LUXR SOLOS ARE BECOMING MAJOR PLAYERS IN CELL-CELL COMMUNICATION IN BACTERIA

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LUXR SOLOS ARE BECOMING MAJOR PLAYERS IN CELL-CELL COMMUNICATION IN BACTERIA

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LuxR solos respond to a variety of signals. In this example, a Salmonella strain carrying an sdiA-dependent srgE-luxCDABE fusion has been struck on the agar within the boundaries of the upper three cartoon bacteria that were drawn on the plastic. Hafnia alvei was struck within the boundaries of the lower bacteria. After nine hours of incubation, luminescence was imaged and pseudocolored to represent intensity (blue and red representing low and high luminescence, respectively). Figure by Dr. Jenee Smith.

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EDITORIAL
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Editorial: LuxR Solos are Becoming Major Players in Cell–Cell Communication in Bacteria

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Keywords: AHL, LuxR solos, quorum sensing, signaling, bacteria

Quorum sensing (QS) is the ability of microbes to sense and respond to their own population density, which typically results in cooperative activity (Fuqua et al., 1994). This form of microbial communication is important to agriculture and human health as they often participate in the regulation of genes important for host interactions.

The classic example of QS in bacteria is performed by the symbiotic bioluminescent bacterium *Vibrio fischeri* (Hastings and Greenberg, 1999). This bacterium colonizes the light organ of a squid and becomes luminescent at high population density. A pheromone of the acylhomoserine lactone class (AHL) is synthesized by the enzyme LuxI, and is a proxy for population density. The AHL is sensed by the transcription factor LuxR, which then activates the transcription of the *luxICDABEG* luciferase operon. Homologous LuxR-LuxI pairs have been found throughout the Proteobacteria (Fuqua et al., 2001); there is divergence among the structures of AHLs produced and detected by LuxI-LuxR pairs, providing some species specificity to the systems.

Several studies and the sequencing of many bacterial genomes has evidenced the presence of many AHL/QS-related *luxR*-type genes, which are unpaired to a cognate *luxI*. These LuxRs possess the typical modular structure having an AHL-binding domain and a DNA-binding HTH domain. These upaired *luxRs*/LuxRs have been called orphans (Fuqua, 2006; Patankar and Gonzalez, 2009) and more recently solos (Subramoni and Venturi, 2009). Several questions arise on the role of LuxR solos in bacteria and recent studies have revealed a number of roles including eavesdropping, intra-species and inter-kingdom signaling. This research topic of *Frontiers in Cellular and Infection Microbiology* is a collection of 10 articles which highlight these different roles as well as the widespread distribution of LuxR solos.

Three articles highlight how widespread LuxR solos are and provide data on their phylogenetic distribution (Gan et al., 2014; Hudaiberdiev et al., 2015; Subramoni et al., 2015). These surveys have shown the presence of one or multiple predicted LuxR solos in many proteobacterial genomes living in different environments, some of them also harboring genes for one or more complete AHL-QS circuits. LuxR solos can be tentatively clustered into meaningful groups or putative orthologs. These LuxR solos subfamilies could respond to different signals and/or having different roles.

The functions of solos can thus far be sub-divided in four categories; as detecting endogenous or exogenous signals, of either the classical AHL type, or of a novel type. The AHL-responsive solos can firstly detect exogenous AHLs, i.e., AHLs synthesized by other organisms, and this category is typified by the LuxR solo, SdiA (Sperandio, 2010; Soares and Ahmer, 2011; Swearingen et al., 2013; Sabag-Daigle et al., 2015). Orthologs of *sdiA* are present in *Escherichia*, *Salmonella*, *Enterobacter*, *Citrobacter*, *Cronobacter*, *Klebsiella*, *Pantoea*, and *Erwinia* (Sabag-Daigle and Ahmer, 2012). The *Pantoea* and *Erwinia* orthologs are part of *luxR-luxI* pairs and represent the ancestral state, and the *luxI* homolog was lost in the remaining genera, giving rise to the *sdiA* solos (Sabag-Daigle and Ahmer, 2012). In this issue, an *sdiA*-regulon study is presented showing a number of genes

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regulated by SdiA in *Enterobacter cloacae* (Sabag-Daigle et al., 2015). Interestingly some target genes were regulated in the complete absence of AHLs and thus AHLs may not be a "folding-switch" for SdiA, in which SdiA only folds correctly in the presence of AHL (Yao et al., 2006; Nguyen et al., 2015), AHL may alter the DNA binding specificity of SdiA so that there are AHL-dependent and AHL-independent promoters. LuxR solos can also be used to detect endogenous AHLs, i.e., AHLs that are made by the species detecting them. This "third wheel" type of LuxR solo is typified by QscR of *Pseudomonas aeruginosa* which harbors two complete AHL QS circuits, namely LasI/R and RhlI/R (Chugani and Greenberg, 2014; Martínez et al., 2015). In this issue, a review regarding the function of QscR is presented (Chugani and Greenberg, 2014; Martínez et al., 2015). QscR is involved in virulence and it responds to LasI generated AHLs, however it has a more relaxed specificity and is more promiscuous than LasR and its regulon overlaps with the one of LasR. The article particularly focuses on its biochemistry since QscR has become a model for understanding QS LuxR homologs.

LuxR solos can also respond to ligands which are not AHLs of either endogenous or exogenous sources. A large sub-family of LuxR solos has been found that specifically recognizes molecules from plants (González and Venturi, 2012; da Silva et al., 2015; Xu et al., 2015). These solos are only found in both pathogenic and beneficial plant-associated bacteria (PAB) and show changes in one or two highly conserved amino acids of the autoinducer binding domain (González and Venturi, 2012). Another member of this subfamily of PAB LuxR solos is reported in this *Frontiers* topic (Xu et al., 2015) as well as studies of protein domain switching between these solos and classic AHL responsive motifs (da Silva et al., 2015). A major step forward will be to identify the class of plant molecules that these solos respond to. Some solos respond to an endogenous, non-AHL, ligand. The LuxR-type receptor PluR of *Photorhabdus luminescens* responds to α-pyrones, while the related organism *Photorhabdus asymbiotica* responds to dialkylresorcinols using the LuxR homolog PauR (Brachmann et al., 2013; Brameyer et al., 2014, 2015; Brameyer and Heermann, 2015; Chen et al., 2015). The synthases for these molecules were determined to be PpyS and DarABC, respectively. In this instance, the LuxR solos turned out not to be solo at all. Instead, these LuxR homologs are paired with previously unrecognized types of synthases. In this topic, a survey of these LuxRs in *Photorhabdus* species is reported (Brameyer et al., 2014).

In summary, LuxR solos are widespread in Proteobacteria hence they are major players in bacterial communication and require more attention. Articles in this topic highlight the different modes of action of LuxR solos which are responding to endogenous and exogenous AHL or non-AHL signals. Further studies could lead to novel ways of controlling bacterial host colonization.

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A bioinformatic survey of distribution, conservation, and probable functions of LuxR solo regulators in bacteria

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LuxR solo transcriptional regulators contain both an autoinducer binding domain (ABD; *N*-terminal) and a DNA binding Helix-Turn-Helix domain (HTH; *C*-terminal), but are not associated with a cognate *N*-acyl homoserine lactone (AHL) synthase coding gene in the same genome. Although a few LuxR solos have been characterized, their distributions as well as their role in bacterial signal perception and other processes are poorly understood. In this study we have carried out a systematic survey of distribution of all ABD containing LuxR transcriptional regulators (QS domain LuxRs) available in the InterPro database (IPR005143), and identified those lacking a cognate AHL synthase. These LuxR solos were then analyzed regarding their taxonomical distribution, predicted functions of neighboring genes and the presence of complete AHL-QS systems in the genomes that carry them. Our analyses reveal the presence of one or multiple predicted LuxR solos in many proteobacterial genomes carrying QS domain LuxRs, some of them harboring genes for one or more AHL-QS circuits. The presence of LuxR solos in bacteria occupying diverse environments suggests potential ecological functions for these proteins beyond AHL and interkingdom signaling. Based on gene context and the conservation levels of invariant amino acids of ABD, we have classified LuxR solos into functionally meaningful groups or putative orthologs. Surprisingly, putative LuxR solos were also found in a few non-proteobacterial genomes which are not known to carry AHL-QS systems. Multiple predicted LuxR solos in the same genome appeared to have different levels of conservation of invariant amino acid residues of ABD questioning their binding to AHLs. In summary, this study provides a detailed overview of distribution of LuxR solos and their probable roles in bacteria with genome sequence information.

Keywords: LuxR solos or orphans, quorum sensing, orthologs, QS domain LuxR proteins, phylogeny

INTRODUCTION

Bacteria sense and respond to changes in external environments through signal transduction systems that include transcriptional regulators for modulating gene expression. A sub-group of LuxR transcriptional regulators with *N*-terminal autoinducer binding domains (ABD) and *C*-terminal Helix-Turn-Helix (HTH) DNA binding domains are known to be involved in quorum sensing (QS) signaling in many proteobacteria (the presence of both domains is referred to as QS domain here on) (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995; Luo and Farrand, 1999). Genes coding for these LuxR regulators usually occur together with a gene coding for the synthesis of *N*-acyl homoserine lactone (AHL) signaling molecules, the LuxI homolog. QS typically involves production of AHLs by a LuxI homolog and their sensing by the LuxR regulator in a cell-density dependent manner to regulate target genes (Fuqua et al., 1994; Zhu and Winans, 2001; Fuqua and Greenberg, 2002). Studies in the last 10 years have uncovered a new group of LuxR regulators that occur without the cognate LuxI homolog and they are referred to as LuxR orphans or solos (Fuqua, 2006; Patankar and Gonzalez, 2009b; Subramoni and Venturi, 2009a). LuxR solos have the same domain organization as canonical LuxR proteins of the QS system, and have been found in different bacteria with important roles in processes such as virulence, plant growth promotion, nodulation, motility, plasmid transfer, antibiotic synthesis, and regulation of QS. They are thought to be important for bacterial signal perception in inter-bacterial and host–bacterial interactions (Soares and Ahmer, 2011; Venturi and Fuqua, 2013).

Several studies have led to the view that LuxR solos may bind to AHLs or to other non-AHL molecules and regulate bacterial traits important for fitness in the environment or in association with their hosts. AHL-binding LuxR solos characterized so far include QscR of *Pseudomonas aeruginosa*, SdiA of *Escherichia coli* and *Salmonella typhimurium*, ExpR of *Sinorhizobium meliloti*, BisR of *Rhizobium leguminosarum* bv. *viciae*, VjbR of *Brucella melitensis*, and PpoR of *Pseudomonas putida* (Ahmer et al., 1998; Chugani et al., 2001; Pellock et al., 2002; Wilkinson et al., 2002; Ahmer, 2004; Delrue et al., 2005; Fuqua, 2006; Subramoni and Venturi, 2009b). Non-AHL binding LuxR solos that recognize yet unknown plant-derived molecules have been studied in several plant-associated bacteria and include OryR of *Xanthomonas oryzae* pv. *oryzae*, XccR of *Xanthomonas campestris* pv. *campestris*,

PsoR of *P. protogenes* and NesR of *S. meliloti* (Ferluga et al., 2007; Zhang et al., 2007; Ferluga and Venturi, 2009; Patankar and Gonzalez, 2009a; Subramoni et al., 2011). A few LuxR solos like CarR of *Serratia marscecens* and CepR2 of *Burkholderia cenocepacia* are also known to regulate target genes in a ligand independent manner (Cox et al., 1998; Malott et al., 2009; Poulter et al., 2011; Ryan et al., 2013). Mostly LuxR solos bind to their ligands and activate expression of their target genes but CarR and CepR2 act as repressors and are known to de-repress target genes in the presence of AHLs.

Similar to QS-associated LuxRs, LuxR solos have been shown to bind to 20-bp palindromic sequences in the promoter regions of genes regulated by them, referred to as "*lux box*" (Devine et al., 1989; Whiteley and Greenberg, 2001; Zhang et al., 2007; Gonzalez et al., 2013). The QS domain LuxR proteins show low sequence similarity (20–25%) but are known to have nine invariant amino acid residues that are critical for ligand and DNA binding properties of these proteins (Whitehead et al., 2001; Zhang et al., 2002). These conserved amino acids are W57, Y61, D70, P71, W85, G113, E178, L182, and G188 with respect to TraR amino acid sequence; the first six amino acids are present in the ABD and the last three amino acids in the HTH domain (Fuqua et al., 1996). The conservation of these key residues is thought to indicate binding of these QS domain LuxRs to AHLs whereas a lack of conservation raises the possibility of binding to other ligands (Patankar and Gonzalez, 2009b).

The availability of an increasing number of bacterial genome sequences has enabled *in silico* analysis for LuxR and LuxI proteins (Sabag-Daigle and Ahmer, 2012). A previous study reported the existence of a much higher number of genes coding for LuxR homologs compared to LuxI homologs in sequenced bacteria suggesting that these genomes might be harboring LuxR solos in addition to canonical LuxRs of QS systems (Case et al., 2008). *In silico* survey of LuxR proteins is complicated by the fact these family of proteins may have different types of domains at the *N*-terminal associated with the *C*-terminal HTH DNA binding domain; one of these domains is the ABD found in QS domain LuxRs. Since only a few LuxR solos have been studied, the distribution, conservation, evolutionary relatedness and functional roles of these interesting group of proteins remains largely unknown.

In this study we have carried out a systematic survey for QS-domain LuxRs in sequenced bacterial genomes included in the Interpro database, and differentiated where possible the putative LuxR solos from LuxR proteins of QS systems. We have also divided several of these LuxR solos into different functionally relevant groups based on their neighboring gene information and determined their relatedness to LuxR solos with known ligands/roles. Our analysis reveals the extent of occurrence of homologs of LuxR solos with known properties and several LuxR solos with probable unknown AHL or non-AHL ligand binding properties. LuxR solos from closely related genomes carrying multiple numbers of these proteins cluster in different sub-groups and have different levels of conservation of amino acids reported to be important for ligand binding. Overall, our analysis has provided a method to classify LuxR solos from sequenced genomes and will enable studies on newly identified members of these type of proteins that are currently being added to the Interpro database.

MATERIALS AND METHODS

IDENTIFICATION OF LuxR SOLOS AND ANALYSIS OF NEIGHBORING LOCI

The complete collection of LuxR proteins with N-terminal ABD, was obtained from the InterPro database (InterPro entry IPR005143), which contains sequences from member databases, PROSITE, Pfam, Prints, ProDom, SMART, and TIGRFAMs (McDowall and Hunter, 2011; Hunter et al., 2012). As of August 31st, 2014, all protein sequences in IPR005143 with the signature "transcriptional factors LuxR-like, autoinducer-binding domain" were analyzed. The combinations of different domain architectures of the proteins analyzed from this Interpro collection include IPR005143 (ABD)–IPR000792 (Transcription regulator LuxR, C-terminal), IPR005143–IPR016032 (Signal transduction response regulator, C-terminal effector), and IPR005143–IPR011991 (Winged HTH DNA-binding domain). Each protein entry was analyzed to determine the following; the sequencing status of the genome to select only the completed ones, the niche or source of the bacterial isolate (animal, environmental, human or plant), the gene products encoded by the flanking genes and the presence of genes coding for complete QS LuxI/LuxR pairs in the same genome.

The protein entries obtained from Interpro were classified as LuxR solos (1) if no gene coding for a LuxI homolog was found in the genome, (2) if no gene was found in the genomic locus near the gene coding for QS domain LuxR protein, or (3) if no unpaired or extra genes coding for LuxI homolog were present in the genome. The genomes carrying these QS domain LuxRs were assigned to three categories: LuxR solos, LuxR solo + QS (if a LuxI/R pair(s) was encoded in the genome in addition to a LuxR solo protein), or QS (if a LuxI/R pair(s) was encoded in the genome but no LuxR solo protein was found). In those cases where LuxR solo proteins were found, the number of genes coding for LuxR solo proteins in each genome was noted. These data were used to generate contingency tables, by using the dynamic table tool available in Microsoft Office 11 and graphs were plotted from the table data using GraphPad Prism.

MULTIPLE SEQUENCE ALIGNMENT AND ANALYSIS FOR INVARIANT AMINO ACIDS

LuxR solos were aligned against TraR amino acid sequence, with Clustal W (Thompson et al., 1994, 2002; Larkin et al., 2007). The presence of all nine key residues previously reported to be invariant in several functionally characterized QS LuxR proteins (W57, Y61, D70, P71, W85, G113, E178, L182, G188 with respect to TraR of *Agrobacterium tumefaciens*) (Whitehead et al., 2001; Zhang et al., 2002) was evaluated by inspection of the alignment.

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES

Evolutionary analyses were conducted using MEGA 6.06 (Tamura et al., 2013). Protein sequences were grouped based on bacterial class, and groups were aligned by MUSCLE (Edgar, 2004). Phylogenetic analyses were performed by using the Maximum Likelihood method based on the JTT matrix-based model

(Jones et al., 1992), which generated trees with the highest log likelihood. In each case significance was estimated by using Bootstrap analysis. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. A group of functionally characterized QS-associated LuxRs and LuxR solo proteins were included in all phylogenetic analyses to determine relatedness (**Table 1**), and transcriptional regulator GerE from *Bacillus subtilis* was included as outgroup, as this sequence is more distantly related to the LuxR solo sequences than they are to each other (Hall, 2013), and has been included previously in similar phylogenetic analyses (Subramoni et al., 2011; Gonzalez and Venturi, 2013).

IDENTIFICATION OF *lux box* AND OPERON PREDICTION

In order to determine the presence of a *lux box* in specific promoters, upstream sequences were retrieved using tools available at RSAT (Thomas-Chollier et al., 2011) and promoter regions identified using BPROM (Solovyev and Salamov, 2011). Twenty base pairs of palindromic sequences in the promoters were then identified using the motif discovery tool of MEME (Bailey et al., 2009). Identified sequences were then aligned with known *lux box* sequences. Operon prediction was carried out using tools available at FGENESB (Tyson et al., 2004).

Table 1 | LuxR solos included as reference in the phylogenetic analyses.

LuxR solo protein	Species and strain	Accession number
RhlR	*P. aeruginosa* PAO1	AAC44036.1
CviR	*Chromobacterium violaceum* ATCC31532	AAP32919.1
CepR CepR2	*B. cenocepacia* J2315	YP 002234479.1 B4EHM0
PfsR	*P. fuscovaginae* UPB0736	CBI67623.1
SinR	*S. meliloti* SM11	AEH78836.1
LuxR	*Vibrio fischeri* ES114	AAA27542.1
TraR	*A. tumefaciens* AAZ50597.1	AAZ50597.1
LasR	*P. aeruginosa* PAO1	AAG04819.1
PmlR	*B. pseudomallei* K96243	YP 110896.1
RpaR	*Rhodopseudomonas palustris* CGA009	NP 945674.1
BjaR	*Bradyrhizobium diazoefficiens* USDA110	NP 767702.1
PluR	*Photorhabdus luminescens subsp. Laumondii* TT01	AGO97061.1
EsaR	*Pantoea stewartii* subsp. *stewartii* DC283	AAA82097.1
SdiA	*S. enterica* subsp. *enterica* serovar *Typhimurium*	AAC08299.1
CinR	*R. leguminosarum 8401*	AF210630.2
QscR	*P. aeruginosa* PAO1	G3XD77
OryR	*X. oryzae* KACC10331	Q5H3E9
ExpR	*S. meliloti* RU11	W0X916
CarR	*S. marcescens* ATCC 39006	AAC38168.1
VjbR	*Brucella melitensis* 16M	Q8YAY5.2
Ger E	*Bacillus subtilis* strain 168	CAA11701.1

CLUSTER ANALYSIS AND IDENTIFICATION OF PUTATIVE ORTHOLOGOUS GROUPS

The entire collection of LuxR solos (almost 5000 proteins) was analyzed by CD-HIT (Huang et al., 2010) to group together all protein sequences that showed sequence identity greater than 90%. This would help to remove very closely related protein sequences from the LuxR solos collection. This reduced subset consisting of representative LuxR solo sequence from each group (657 proteins; data not shown) was used for further analysis. In order to identify closely related members among this reduced collection of LuxR solos, CLANS analysis (Frickey and Lupas, 2004) was carried out. CLANS performs BLAST analysis of each sequence against all other sequences individually based on *P*-values of high-scoring segment pairs and enables two-dimensional visualization of pair-wise sequence similarities. For network-based clustering the *P*-value cut off was set to 10^{-30} and the attraction and repulsion exponents were set to two. In another approach to classify closely related LuxR solos into functionally related groups, the neighboring genes flanking the genes coding for representative LuxR solos were analyzed using SynTax (Oberto, 2013). The conservation of invariant amino acids was also checked. LuxR solos with similar flanking genes and similar amino acid conservation were grouped together.

RESULTS

DISTRIBUTION OF LuxR SOLOS IN SEQUENCED BACTERIAL GENOMES

The complete collection of QS domain LuxR proteins were sourced from Interpro database (IPR005143) and analyzed to identify LuxR solos or orphans according to the criteria outlined in materials and methods. In total, 6030 QS domain LuxR protein sequences from 3540 sequenced genomes were analyzed and an inventory of 4860 LuxR solos and 1170 LuxR proteins that are part of complete QS systems generated (Supplementary Table 1). Majority of LuxR solos were carried by chromosomal loci but some were encoded by plasmids as found in *Oligotropha carboxidovorans*, *Methylobacterium extorquens*, *Agrobacterium* sp., and *R. leguminosarum* bv. *trifolii* CB782. Genes coding for LuxR solos were sometimes located near a gene coding for transposase as found in *M. australicum*, *R. loti*, *R. leguminosarum* bv. *viciae*, *R. tropici*, *S. meliloti*, *Oceanicola batsensis, Octadecabacter arcticus, Paracoccus aminophilus, Roseivivax isoporae, Gluconacetobacter medellinensis, Erythrobacter sp. SD-21, Novosphingobium sp. PP1Y, B. thailandensis, Acidovorax avenae*, and *Alcanivorax pacificus W11-5*. In some cases it was not possible to delineate a LuxR solo or a canonical QS LuxR from multiple QS domain LuxRs present in a genome; for example (1) when two QS domain LuxR proteins occur in tandem near a gene coding for the LuxI homolog (examples include *Nitratireductor aquibiodomus* RA22, *Agrobacterium tumefaciens* 5A, *Rhodobacter sphaeroides* (strain ATCC 17025/ATH 2.4.3), *Sphingobium baderi* LL03 and *S. lactosutens* DS20, (2) when the gene coding for a LuxI homolog was located in a locus genetically unlinked from the locus coding for a

QS domain LuxR homolog or two QS domain LuxR homologs adjacent to each other (as in species belonging to Rhizobiales, Rhodobacteriales, and Burkholderiales), and (3) when truncated LuxR proteins containing only the ABD without the DNA binding domain were present in a genome; genes coding for these proteins were often located near gene(s) coding for a QS domain LuxR protein (Supplementary Table 2). Adjacently located genes coding for two LuxR solos may also occur in genomes without an unpaired LuxI homolog as found in several bacteria belonging to Burkholderiales and Rhizobiales. These are described in more detail later in the Results section.

The taxonomic distribution of LuxR solo proteins in sequenced bacterial genomes was found to be biased due to the availability of a larger number of sequences for some bacterial species with clinical or agricultural importance (**Figure 1A**). For example, a larger number of sequenced genomes are available for Alphaproteobacteria and Gammaproteobacteria species that carry only LuxR solos. However, examination of LuxR solo occurrence at species level was more representative of actual numbers and distribution (**Figure 1B**). QS domain LuxR proteins were found to be mainly restricted to proteobacteria as reported previously (Case et al., 2008); surprisingly a few non-proteobacterial sequenced genomes were also found to carry these proteins (discussed below). Among proteobacteria carrying QS domain LuxRs, 10–15% of sequenced genomes representing 20–25% of bacterial species carried only complete QS systems without any additional QS domain LuxR proteins (**Figures 1A,B**; Supplementary Table 1). On the other hand, majority of bacteria (approximately 75% at species level) harbor one or more LuxR solo or orphan proteins, either with or without complete QS system(s). In Alphaproteobacteria more than 50% of sequenced genomes representing 25% of species carry LuxR solos alone whereas 37% of sequenced genomes constituting 54% species carry both LuxR solos and complete QS systems. In Betaproteobacteria, the numbers of species that contain LuxR solos alone or LuxR solos in addition to complete QS systems are similar although more genomes have been sequenced for the latter. In contrast to this, 80% of Gammaproteobacterial genomes representing 54% species carry only LuxR solos whereas 20% carry both LuxR solos and QS system(s) (**Figures 1A,B**). Very few genomes belonging to Delta-Epsilonproteobacteria carry QS domain LuxR proteins; of those, 50% carry complete QS systems and the rest carry only LuxR solos. Bacteria belonging to *Brucella* sp., *Ochrobactrum* sp., *Acidovorax* sp., *Citrobacter* sp., *Cronobacter* sp., *Escherichia* sp., *Salmonella* sp., *Enterobacter* sp., *Shigella* sp., *Yersinia* sp., *Xanthomonas* sp. as well as several *Pseudomonas* sp. were among those that were found to carry LuxR solos alone without any QS system. Several species that belonged to *Rhizobium* sp., *Mesorhizobium* sp., *Agrobacterium* sp., *Burkholderia* sp., *Aeromonas* sp., *Serratia* sp., and several *Pseudomonas* sp. carry both complete QS systems as well as genes for LuxR solos. A complete list is provided in Supplementary Table 1. The most varied distribution in terms of both presence and numbers of genes coding for LuxR solos and complete QS systems was found in bacteria belonging to *Bradyrhizobium* sp., *Methylobacterium* sp., *Serratia* sp., *Yersinia* sp., and *Pseudomonas* sp.

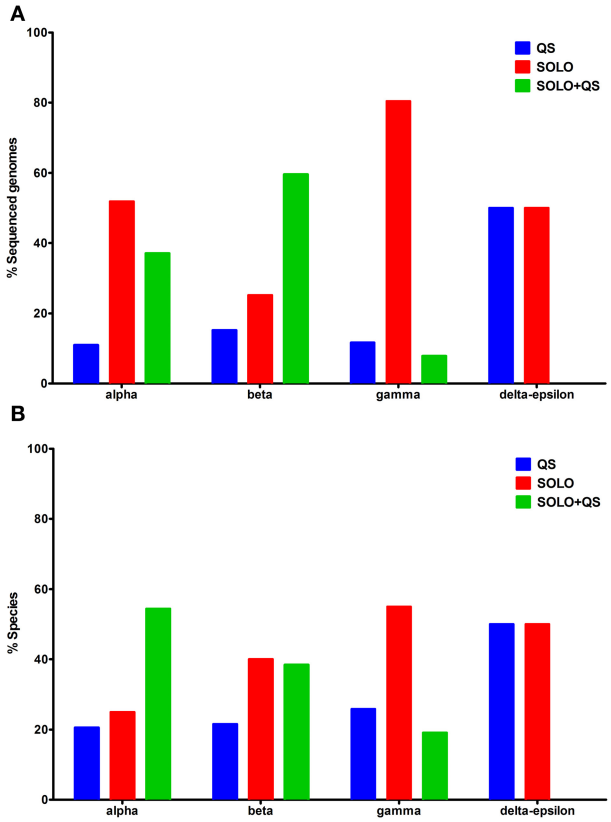

FIGURE 1 | Distribution of QS domain LuxRs in different bacterial classes. (A) Percentage of sequenced genomes carrying QS domain LuxR proteins from each class, having QS LuxRs alone, LuxR solos alone, or both **(B)** Percentage of species carrying QS domain LuxR proteins from each class, having QS LuxRs alone, LuxR solos alone, or both. QS domain LuxR protein sequences were sourced from Interpro database, IPR005143. On the x-axis, alpha-Alphaproteobacteria, beta-Betaproteobacteria, gamma-Gammaproteobacteria, and delta-epsilon-Delta-Epsilonproteobacteria.

BACTERIA CARRYING LuxR SOLOS OCCUR IN DIVERSE ECOLOGICAL NICHE(S)

In order to determine if there was any taxonomic- or niche-specific trend for occurrence of LuxR solos, an analysis of abundance of these proteins with respect to various taxa and ecological niche of bacterial species that harbor them was carried out (**Figure 2**). The number of genes coding LuxR solos in a bacterial genome ranged from one to as high as seven (described below). In Alphaproteobacteria, plant-associated species mostly carried multiple LuxR solos whereas human and animal-associated species typically carried two LuxR solos. A large proportion of environmental isolates belonging to Alphaproteobacteria carried one LuxR solo but a substantial number of species also carried two or more LuxR solos (**Figure 2A**). Among bacteria belonging to Betaproteobacteria, majority of plant-associated and environmental isolates carried one LuxR solo; animal and human associated Betaproteobacteria mostly carried multiple LuxR solos (**Figure 2B**). Exceptions include plant pathogens *B. gladioli* and *B. glumae* harboring multiple solos. Majority of

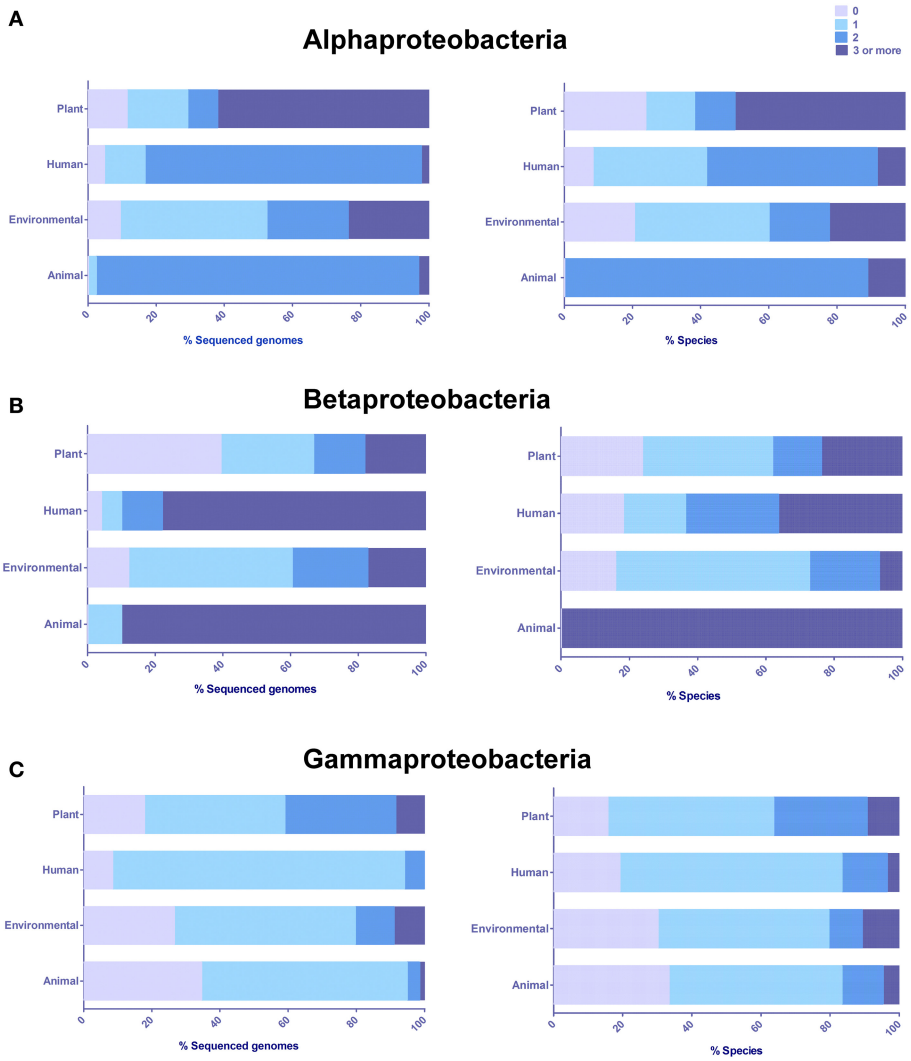

FIGURE 2 | Distribution and abundance of LuxR solos in different classes based on the origin or predominant niche specificity of bacterial species harboring these proteins. (A) Alphaproteobacteria **(B)** Betaproteobacteria, **(C)** Gammaproteobacteria. Plant, Plant-associated bacteria; Human, Human-associated bacteria; Environmental, Environmental isolates; and Animal, Animal-associated bacteria. Some bacterial species were placed in more than one category. The presence of LuxR solos was inferred by analyzing QS domain LuxR proteins of Interpro database, IPR005143, and correlated to niche specificity by generating contingency tables in Microsoft Excel.

bacteria belonging to Gammaproteobacteria carried one LuxR solo irrespective of whether they were environmental or associated with plant, human or animal hosts (**Figure 2C**). In each of the three taxonomic groups a large number of plant-associated and environmental isolates carried multiple LuxR solos; overall, these observations suggest a role for multiple LuxR solos in bacterial species occupying environmental and/or plant-associated niche.

LuxR SOLOS ARE ALSO FOUND IN A FEW SEQUENCED NON-PROTEOBACTERIA

Our analysis of the Interpro database for QS domain LuxR proteins revealed the presence of 11 proteins with this domain architecture in non-proteobacterial genomes. Since a gene coding for a LuxI homolog could not be identified by examination of these non-proteobacterial genome sequences, these proteins were considered as LuxR solos. These were distributed in Actinobacteria (six LuxR solos), Chlamydiae/Verrucomicrobia (two LuxR solos), Nitrospirae (two LuxR solos), and Chrysiogenetes (one LuxR solo), respectively (Supplementary Table 1).

In order to determine the evolutionary relatedness of non-proteobacterial LuxR solos to Gram-negative canonical QS LuxRs and LuxR solos an unrooted phylogenetic tree was generated as described in methods (**Figure 3**). Four major branches could be delineated in the phylogenetic tree; (1) The Actinobacterial (*Mycobacterium* sp., *Streptomyces* sp., and *Rhodococcus* sp.) LuxR solos form a robust clade that branches out from rest of the tree. Three other QS domain LuxRs, namely, RpaR, CinR, and VjbR form another group of this branch. (2) The LuxR solos of *Methylacidiphilum fumariolicum*, *M. infernorum*, *Nitrospira*

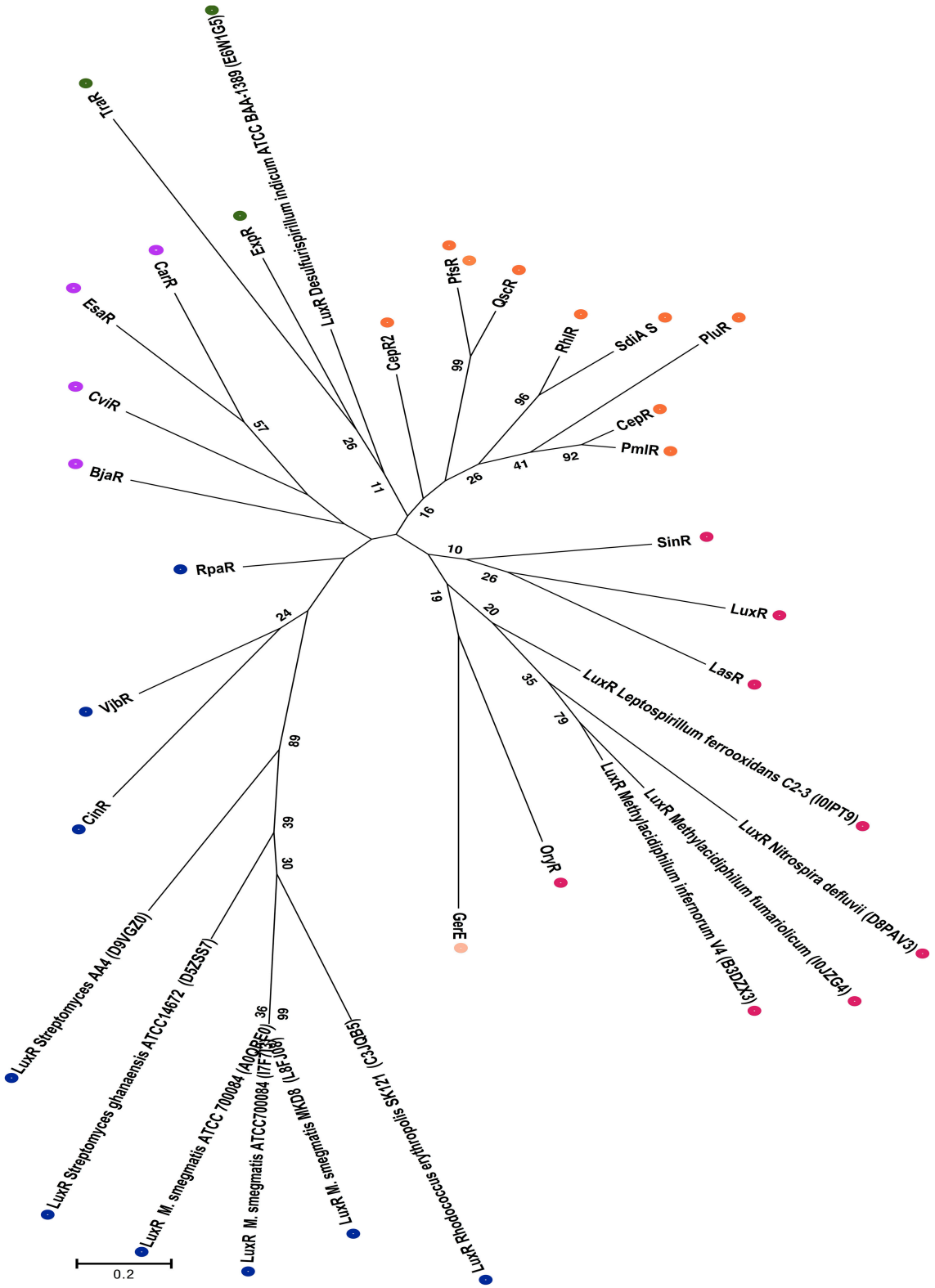

FIGURE 3 | Phylogenetic analyses of non-proteobacterial LuxR solos. This evolutionary history was inferred by using the Maximum Likelihood and the unrooted tree with the highest log likelihood is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, and colored dots indicate different groups as discussed in the results section. The analysis involved 33 amino acid sequences, which included the 11 Non-proteobacterial LuxR solos indicated by their Uniprot identification in the figure. All positions containing gaps and missing data were eliminated. There were a total of 71 positions in the final dataset. Numbers in brackets indicate UNIPROT accession numbers for all proteins analyzed.

defluvii, and *Leptospirillum ferrooxidans* form another separate group clustering together with LasR, LuxR, SinR, and OryR suggesting that they may be evolutionarily closer to these QS domain LuxRs when compared to Actinobacterial LuxR solos. (3) The third branch of the tree is made up of functionally characterized proteobacterial QS domain proteins CarR, EsaR, CviR, and BjaR, indicating high evolutionary relatedness of these proteins. (4) In the fourth major clade of the tree, a LuxR solo of *Desulfurispirillum indicum* was found to cluster with TraR and ExpR; the branching of this LuxR solo also revealed a shared common ancestor with other QS LuxR and LuxR solos like CepR2, PfsR, QscR, RhlR, SdiA, PluR, CepR, and PmlR. In summary, with respect to well-studied proteobacterial QS LuxRs and LuxR solos, the Actinobacterial LuxR solos are evolutionarily distant whereas other non-proteobacterial LuxR solos show a relatively higher degree of evolutionary relatedness.

It was of interest to analyze the sequence features of ligand binding domains of non-proteobacterial LuxR solos as there are no reported studies regarding these proteins. Our analysis revealed lack of conservation in at least two/nine amino acids in all these proteins; importantly the amino acid corresponding to W85 of TraR was changed (**Table 2**). The LuxR solos of *M. fumariolicum* and *M. infernorum* carry W57L and W85R substitutions at the corresponding positions in their proteins. The LuxR solos of *Mycobacterium* sp., *Streptomyces* sp., and *Rhodococcus* sp. carry W57_ and W85D or R substitutions. *D. indicum* and *N. defluvii* solos also carry W85R or W85_ and W57F substitutions. The lack of conservation of these invariant amino acids raises the possibility that these LuxR solos may bind to ligands different from AHLs.

CLUSTER ANALYSIS OF LuxR SOLOS

In order to group related LuxR solos, cluster analysis was carried out using CLANS as detailed in materials and methods. Results using the sub-set of 657 LuxR solo proteins revealed that majority of the LuxR solos remained ungrouped probably due to low sequence similarity. Only a small number (65 out of 657 LuxR solos) clustered together mainly into five groups; these groups are likely a reflection of their taxonomic relationships. Among these, only LuxR solos from closely related species showed conservation of flanking genes (**Figure 4**). These clusters are as follows; cluster 1 consisting of 47 LuxR solos of mixed taxonomy with varying levels of relatedness (as indicated by the differently colored lines), cluster 2 consisting of seven LuxR solos mainly Betaproteobacteria, cluster 3 consisting of five LuxR solos, cluster 4 with three LuxR solos and cluster 5 with two sequences (Supplementary Table 3). Cluster 1 was formed by LuxR solos of Alphaproteobacteria (*Agrobacterium* sp., *Sphingomonas* sp., *Roseivivax* sp., *Sagittula* sp., *Nitratireductor* sp., *Methylobacterium* sp., *Aurantimonas* sp., *Afipia* sp., *Paracoccus* sp., *Sinorhizobium* sp., *Rhodospirillum* sp., *Pelagibaca* sp.), and Betaproteobacteria (*Burkholderia* sp., *Variovorax* sp., *Chromobacterium* sp.), showing high degree of similarity within each group but lesser similarity between them. A few LuxR solos of Gammaproteobacteria represented by *Dickeya* sp., *Klebsiella* sp., *Alcanivorax* sp., *Pseudomonas* sp., as well a LuxR solo from *Streptomyces* were also in cluster 1. *Burkholderia* sp. LuxR solos were distributed in all clusters. A step-wise increase in *P*-value cut off from 10^{-30} to 10^{-1} resulted in merging of these small clusters into a single cluster suggesting that these 65 LuxR solos are closely related. Decreasing *P*-value to 10^{-35} resulted in separation of clusters and at *P*-value of 10^{-200} all proteins separated and remained independent without any cluster formation. These results confirm the fact that LuxR solos from different taxonomic groups have low levels of sequence relatedness making it very difficult to compare across bacterial taxa.

FUNCTIONAL GROUPING OF LuxR SOLOS

In order to group LuxR solos in a biologically relevant manner, an alternate approach was used whereby the subset of representative LuxR solos were analyzed for the genomic context of genes encoding them and the conservation of invariant amino acids of

Table 2 | Conservation analyses for LuxR-solo proteins found in non-proteobacterial genomes.

LuxR proteins found in non-proteobacterial genomes		Key aminoacids in autoinducer binding domain						Key aminoacids in HTH domain		
Accession number	**Species**	**W 57**	**Y 61**	**D 70**	**P 71**	**W 85**	**G 113**	**E 178**	**L 182**	**G 188**
I0JZG4	*Methylacidiphilum fumariolicum*	L	Y	D	P	R	G	E	I	G
B3DZX3	*Methylacidiphilum infernorum* V4	L	Y	D	P	R	G	E	L	G
I7F7I3	*Mycobacterium smegmatis* ATCC700084	gap	Y	K	E	D	G	E	L	G
A0QRE0	*Mycobacterium smegmatis* ATCC700084	gap	Y	K	P	D	G	E	L	G
D9VGZ0	*Streptomyces sp. AA4*	gap	Y	C	P	R	G	E	L	G
D5ZSS7	*Streptomyces ghanaensis* ATCC 14672	gap	Y	D	P	W	G	E	L	G
C3JQB5	*Rhodococcus erythropolis*	gap	Y	D	P	R	G	E	L	G
L8FJ08	*Mycobacterium smegmatis* MKD8	gap	Y	D	P	D	G	E	L	G
E6W1G5	*Desulfurispirillum indicum* strain ATCC BAA-1389	W	Y	D	P	R	G	E	L	G
D8PAV3	*Nitrospira defluvii*	F	Y	D	P	gap	C	E	L	G
I0IPT9	*Leptospirillum ferrooxidans* strain C2-3	W	Y	D	P	gap	G	E	L	G

Each entry was aligned against TraR from Agrobacterium tumefaciens.

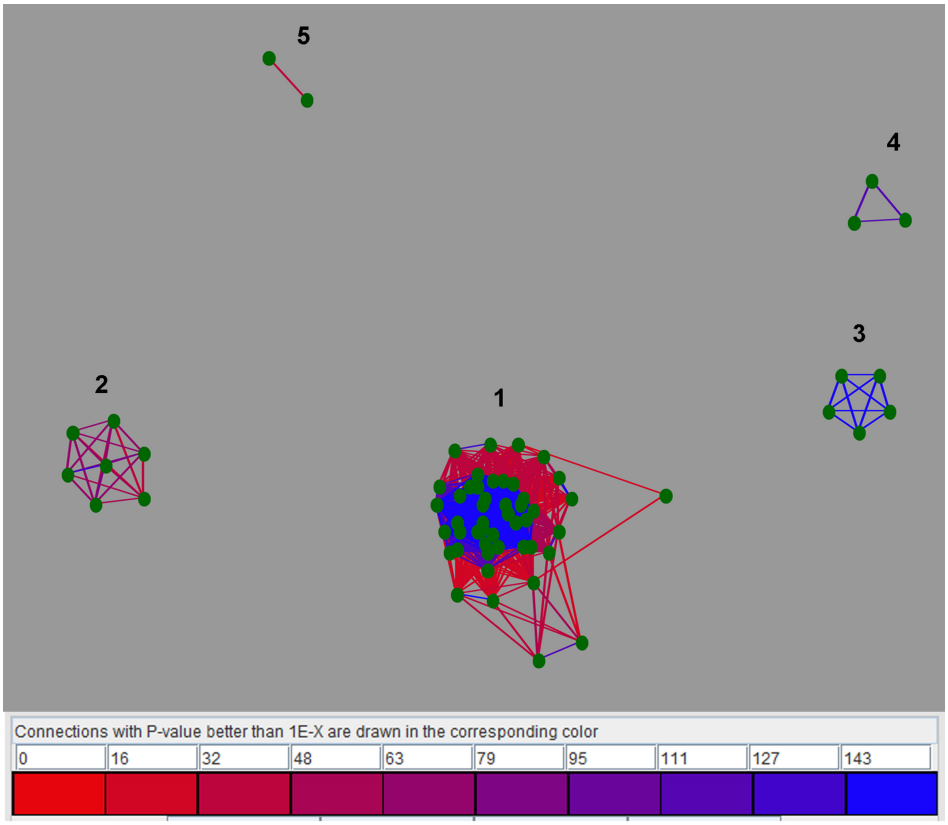

FIGURE 4 | Two-dimensional CLANS clustering of LuxR solos. CLANS analysis was carried out on the representative 657 LuxR solo sequences as detailed in materials and methods. LuxR solos that clustered are represented as green dots and unclustered proteins are not shown. BLAST sequence similarities are indicated by lines shaded from red (*P*-values $< 10^{-35}$), to blue (*P*-values $< 10^{-200}$. Different clusters are indicated by numbers.

ABD. By this approach LuxR solos could be divided into different groups consisting of known and unknown LuxR solos; members of each group are likely to be orthologs (**Tables 3**, **4**). Out of the 657 representative LuxR solos, 272 could be placed into these categories; 385 remained ungrouped using these criteria (Supplementary Tables 4, 5).

Some LuxR solos fell into groups, which had one or more members already well-studied (**Table 3**; Supplementary Table 4). Known groups contained putative orthologs of SdiA, QscR, XccR/OryR, ExpR/AviR, PpoR, CepR2, AvhR, CarR, BlxR, and VjbR. Of these, orthologs of XccR/OryR had the broadest taxonomic distribution as they are present in bacteria belonging to Alphaproteobacteria and Gammaproteobacteria. Orthologs of SdiA (Enterobacteriales), QscR (*P. aeruginosa*) ExpR (Rhizobiales), CepR (Burkholderiales), AvhR (Rhizobiales), CarR (*Serratia* sp., *Yersinia* sp.), VjbR and BlxR (*Brucella* sp. and *Ochrobactrum* sp.), and PpoR (*Pseudomonas* spp) were more restricted in their taxonomical distribution in our analysis based on flanking gene conservation. As expected SdiA, PpoR, BlxR, ExpR group members showed no changes in the invariant amino acids of the ABD whereas XccR/OryR, VjbR, and some members of CepR2 group showed replacement of amino acids at positions W57, Y61, W85, and G113 with respect to TraR amino acid sequence. Since there are several studies describing the representative LuxR solos of groups mentioned above, and their functions, they will not be discussed further here.

Among the representative members of uncharacterized LuxR solos seven different groups belonging to Alphaproteobacteria, one group belonging to Betaproteobacteria and three different groups belonging to Gammaproteobacteria, respectively were identified (**Table 4**; Supplementary Table 5). Of these, only two ortholog groups each belonging to Alphaproteobacteria and Gammaproteobacteria had LuxR solos without changes in the invariant amino acids of the ABD suggesting that these proteins are likely bind to AHLs. In support of this observation it was found that related species carry orthologs of these LuxR solos as part of a complete QS system as seen for groups 2 and 4 (**Table 4**). One ortholog group contained genes coding for LuxR solos always flanked by a gene coding for HchA chaperone protein and occurred in both Alphaproteobacterial and Gammaproteobacterial species. Surprisingly, most sequenced strains of *P. aeruginosa* were found to contain an ortholog of this LuxR solo, in addition to the well-studied QscR, which appears to be restricted to *P. aeruginosa* strains (Supplementary Table 1). Two ortholog groups (groups 6 and 8 representing Rhizobiales and Burkholderiales) had two genes coding for LuxR solos located adjacently; these LuxR solos showed 30–40% homology to each other and varying levels of substitutions in the invariant amino

Table 3 | Groups of known LuxR solos.

Group Known LuxR solo/LuxRI*	Genomic context of LuxR solo	Bacterial species	Homology, Invariant amino acids changed, Reference/(s)
SdiA (*S. typhimurium and E. coli*) ExpRI (*Erwinia*), PhzRI (*Pantoea*)	*yecC, ABC transporter – **sdiA** – yecF, Uncharacterized protein (C) – sirA, Invasion response regulator – uvrC, UvrABC system protein C*	*Enterobacter sp., Klebsiella sp., Cronobacter sp., Escherichia sp., Yokenella sp., Citrobacter sp., Salmonella sp., Shimwellia sp.*	66–82% None Ahmer et al., 1998; Kanamaru et al., 2000; Michael et al., 2001; Sabag-Daigle and Ahmer, 2012
QscR (*P. aeruginosa*)	*Desaturase (C) – **qscR** – phzA, phenazine biosynthesis protein A*	*P. aeruginosa*	97–100% None Chugani et al., 2001; Oinuma and Greenberg, 2011
XccR (*X. campestris*), OryR (*X. oryzae*), PsoR (*P. protegens*), NesR (*S. meliloti*)	*pip, proline imino peptidase/tra, peptide transporter (C) – **xccR/oryR/psoR** – pip, proline imino peptidase/hp, hypothetical protein*	*Pseudomonas sp., Rhizobium sp., Xanthomonas sp., Klebsiella sp., Rhodobacter sp., Agrobacterium sp., Mesorhizobium sp., Citreicella sp., Rahnella sp., Yersinia sp., Brennaria sp., Serratia sp.*	43–90% W57M, Y61W Zhang et al., 2007; Ferluga et al., 2007; Patankar and Gonzalez, 2009a; Subramoni et al., 2011
ExpR (*S. melilotii*), AviR (*A. vitis*)	*chvA, glucan exporter ATP-binding protein (C) – **expR** – pyc, pyruvate carboxylase*	*Agrobacterium sp., Ensifer sp., Rhizobium sp., Shinella sp., Sinorhizobium sp., Hoeflea sp.*	40–93% None Pellock et al., 2002; Zheng et al., 2003; Gao et al., 2014
PpoR (*P. putida*)	*fprB, flavodoxin reductase (C) – **ppoR** – rlmG, 16S RNA, methylase RsmC*	*P. putida, P. fluorescens, P. synxantha, P. moraviensis, P. brassicacearum, P. plecoglossicida, P. entomophila, P. mosselii*	40–79% None Subramoni and Venturi, 2009b; Fernandez-Pinar et al., 2011
CepR2 (*B. cenocepacia*)	*araC, AraC family transcriptional regulator – **cepR2** – geneX, any gene product (C)*	*Burkholderia sp., Variovorax sp., Caulobacter sp.*	30–94% W85A, G113N, E178Q (only some proteins) Malott et al., 2009; Ryan et al., 2013
VjbR (*B. melitensis*)	*hp, hypothetical protein – **vjbR** – tetR, TetR family transcriptional regulator (C)*	*Brucella sp., Ochrobactrum sp., Phyllobacterium sp.*	47–88% W85I/V, G113F Delrue et al., 2005; Uzureau et al., 2010
BlxR (*B. melitensis*)	*ms, Methionine synthase – **blxR** – at, Amino transferase*	*Brucella sp., Ochrobactrum sp.*	78–94% None Rambow-Larsen et al., 2008
AvhR (*A. vitis*)	*Calcium binding protein (C) – **avhR** – nrt, nucleotide binding ABC transporter*	*Agrobacterium sp.*	56–67% W57F, D70S, W85R Hao et al., 2005
BisR (*R. leguminosarum*)	*trbI, conjugal transfer protein TrbI – **bisR** – traR, Conjugal transfer regulator TraR*	*Rhizobium sp.*	59–87% None Wilkinson et al., 2002; Danino et al., 2003
CarR (*S. marcescens*)	*geneX, any gene product – **carR** – carA, carbapenem biosynthesis protein*	*Serratia sp.*	59–62% W57C Cox et al., 1998; Poulter et al., 2011

(C)-Gene in the complementary strand.

**indicates presence of LuxRI in a similar genomic context. Bold values indicate LuxR solos.*

Table 4 | Groups of unknown LuxR solos.

Group	Genomic context of LuxR solo (5′–3′)	Bacterial species	Homology (aa), Invariant amino acids changed
1	*aroQ, 3-dehydroquinate dehydratase II/geneX, any gene product – **luxR solo** – tsf, Translation elongation factor Ts (C) – rpoB, RNA polymerase (C)*	*Roseobacter sp., Phaeobacter sp., Salipiger sp., Jannaschia sp., Roseibacterium sp., Dinoroseobacter sp., Pelagibaca sp., Sagittula sp., Citreicella sp., Roseovarius sp., Ruegeria sp., Oceanibulbus sp., Leisingera sp., Silicibacter sp., Thalassobacter sp., Loktanella sp., Maritimibacter sp., Rhodobacter sp., Defluviimonas sp., Octadecabacter sp., Roseivivax sp., Paracoccus sp., Celeribacter sp., Rubellimicrobium sp., Actibacterium sp.*	58–80% W57Y, Y61F, D70I/G/A, P71V, E178Q
2	*CR, crotonyl-CoA reductase – **luxR solo** – ATPase, helicase, ATP-dependent*	*Roseovarius sp., Oceanibulbus sp., Roseobacter sp., Ruegeria sp.* *raiR/rail homologs of P. gallaeciensis, P. inhibens, L. methylohalidivorans, R. pomeroyi, R. denitrificans, R. litoralis, O. antarcticus and D. shibae are flanked by CR*	44–46% None
3	*acdH, acyl coA dehydrogenase (C) – merR, MerR family transcriptional regulator (C) – **luxR solo** – hp, hypothetical protein – merR, MerR family transcriptional regulator (C)*	*Roseobacter sp., Sulfitobacter sp., Oceanibulbus sp.*	46–58% W57F, D70S (some proteins)
4	*fliG, flagellar motor switch protein (C) – [hp, hypothetical protein (C) – abd, autoinducer binding domain protein (C)] – **luxR solo** – asl, Adenylosuccinate lyase (C)*	*Rubellimicrobium sp., Loktanella sp.* *luxR/luxI homologs of O. antarcticus is flanked by fliG*	53–55% None
5	*tk, thymidine kinase (C) – **luxR solo** – rimO, Ribosomal protein S12p Asp methylthiotransferase (C)*	*Salpiger sp., Roseivivax sp.*	60–62% Y61E/H, D70W, P71S, W85L/V/I, E178Q
6	*fabG2, 3-oxoacyl-[acyl-carrier-protein] reductase (C) – iclR, IclR family transcriptional regulator – **luxR solo** – [**luxR solo**] – mrp, ATP binding Mrp protein*	*Rhizobium sp., Agrobacterium sp., Ensifer sp., Sinorhizobium sp., Shinella sp.*	48–94% 40% (adjacent LuxR solos) W57F/L, D70S, P71T W85Y/V/N/H, G113S/F/C/A
7	*fliF, flagellar M-ring protein – luxR, LuxR superfamily transcriptinal regulator – **luxR solo** – hp, hypothetical protein (C) – flhB, flagellar biosynthetic protein (C)*	*Sinorhizobiumm sp., Mesorhizobium sp., Rhizobium sp., Aquamicrobium sp.*	60–89% W57Y, Y61C/A, D70E, P71E/D
8	*nramp, Natural resistance-associated macrophage protein (C) – **luxR solo** – **luxR solo** (C) – [t3ss, Type 3 secretion genes (C)] – as, asparagine synthase (C)*	*B. cenocepacia, B. multivorans, B. ambifaria, B. thailandensis, B. vietnamiensis, B. dolosa*	66–90% 33% (adjacent LuxR solos) W57Y/F, Y61F, E178Q
9	*geneX, any gene product – **luxR solo** (C) – hchA, HchA chaperone protein (C)*	*Pseudomonas sp., Acinetobacter sp., Vibrio sp., Agrobacterium sp., Beijerinckia sp., Comamonas sp., Stenotrophomonas sp., Serratia sp.*	46–98% Y61F/T/S/I, D70A/R/Q/N, P71T/R/D/K
10	*HAD, HAD-super family hydrolase – **luxR solo** – chp, conserved hypothetical protein (C)*	*P. syringae, P. avellanae, P. viridiflava, P. cichorii*	54–99% None
11	*hadH, 3-hydroxyacyl-CoA dehydrogenase (C) – **luxR solo** – uspG, universal stress protein*	*S. marcescens, S. liquifaciens, S. plymuthica*	77–97% W57Y
12	*geneX, any gene product – **luxR solo** (C) – as II, anthranilate synthase II (C) – as I, anthranilate synthase I (C)*	*P. syringae, P. brassicacearum, P. fluorescens*	45–86% None

(C)-Gene in the complementary strand.

acids of ABD. For the ortholog group of Rhizobiales, a gene coding for a single LuxR solo could be identified in the same genomic context for some species suggesting that genes coding for LuxR solos in tandem probably arose by gene duplication.

In order to determine probable roles of LuxR solos and flanking genes, transcriptional organization and putative functions encoded by these genes was analyzed in representative genomes of each group (**Table 4**). The gene coding for 3-dehydroquinate dehydratase, *aroQ*, was always found in the same transcriptional unit as the gene coding for group 1 LuxR solo suggesting a role for this LuxR solo in aromatic amino acid biosynthesis. Similarly, the gene coding for an ATP-dependent exoDnase/helicase, an enzyme involved in DNA metabolism was found in an operon with the gene coding for group 2 LuxR solo upstream of it. A gene coding for a small hypothetical protein was found downstream of group 3 LuxR solo gene in the same transcriptional unit; function of this hypothetical protein is not known although the genes nearby code for lipid metabolism-related functions. Genes coding for group 4, group 5, and group 6 LuxR solos (both single and two adjacent genes) were in separate transcriptional units. Group 4 LuxR solos occur near genes coding for flagellar motor protein, FliG, which is required for motility. Group 5 LuxR solo gene is flanked on either side by genes coding for thymidine kinase and a ribosomal methyl transferase, both enzymes involved in nucleic acid metabolism. Located upstream of the gene coding for LuxR solo of group 6 is *iclR*, encoding an IclR family of transcriptional regulator; these regulators are known to regulate degradation of QS signals, plant-bacterial interaction and secondary metabolite production (Molina-Henares et al., 2006). Genes coding for Group 7 LuxR solos occur as part of a transcriptional unit with a gene coding for LuxR transcriptional regulator (lacking ABD) whose function is unknown. The two genes coding for group 8 LuxR solos are located adjacent to each other as separate transcriptional units and have either convergent or divergent orientation depending on bacterial species. In some *Burkholderia* sp. they were found adjacent to a gene coding for asparagine synthase whereas in others the type 3 secretion genes were inserted in this locus. Genes coding for group 9 LuxR solos and HchA chaperone protein occur in a single transcriptional unit. Gene coding for group 10 LuxR solo is a single transcriptional unit and present downstream of an operon involved in methionine metabolism. The gene coding for group 11 LuxR solo was located adjacent to an operon of five genes coding for fatty acid metabolism functions. Finally the gene coding for group 12 LuxR solo is part of an operon that codes for enzymes of phenyl alanine/tyrosine metabolism. These observations reveal that genes coding for some of these LuxR solos may be transcriptionally linked with neighboring genes pointing to a role in different metabolic pathways.

FIGURE 5 | Phylogenetic analyses of multiple LuxR solos carried by selected Alphaproteobacterial species. The tree was inferred by using the Maximum Likelihood method. Tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 55 amino acid sequences, from representative species of this class carrying multiple LuxR solos. All positions containing gaps and missing data were eliminated. There were a total of 71 positions in the final dataset. Colors indicate major clusters.

PHYLOGENETIC ANALYSIS OF MULTIPLE LuxR SOLOS IN A BACTERIAL GENOME

According to our results, several proteobacteria species have more than three genes coding for LuxR solos. In order to determine the relatedness between LuxR solos carried by the same genome, phylogenetic analyses was carried out as detailed in materials and methods. Several species were included for the phylogenetic analyses of these proteins in Alphaproteobacteria (**Figure 5**). Several clusters were formed, but in most of the cases, LuxR solos from the same strain clustered together in the same branch of the tree indicating high relatedness among them, most likely associated with duplication events, as it evident for LuxR solos from *L. hongkongensis* DSM17492, *E. adherens* OV14 (green clade) and *H. phototrophica* DHL-43 (yellow clade). Interestingly, one independent clade supported by high bootstrap values stems out

from this tree and this clade includes ExpR and LuxR solos from *Agrobacterium, H. phototrophica*, *E. adherens* and *R. freirei*. In contrast most of the characterized LuxR proteins locate in a distinct branch suggesting lower levels of relatedness among these proteins and Alphaproteobacterial LuxR solos.

LuxR solos from chosen *Burkholderia* species formed three major clades (**Figure 6**). Sequences from the *B. mallei–B. pseudomallei* species group were present in all clusters suggesting that these species harbor LuxR solos of different origins. One cluster was mostly composed of proteins from plant-associated strains belonging to the *B. glumae* and *B. gladioli* species, which formed a clade together with CinR (green group **Figure 6**). Another clade was formed by LuxR solos of *B. mallei*, *B. pseudomallei*, *B. thailandensis* and *B. cenocepacia* that shared an ancestor with TraR, OryR and ExpR (blue group **Figure 6**). The last clade was composed of LuxR solos that clustered with characterized QS domain LuxRs, most of them known to bind AHLs, forming different sub-groups based on relatedness.

Phylogenetic analyses for sequences from the Gammaproteobacteria class, revealed six major clusters consistently, independent of the method (ML, NJ or Parsimony) (**Figure 7**). Importantly, the *Pantoea* LuxR solos included in these analyses grouped together with EsaR, CarR and CviR, whereas *Pseudomonas* sp. LuxR solos where distributed in five clusters. LuxR solos from several *Pseudomonas* sp. grouped together with OryR, TraR and ExpR; other *Pseudomonas* sp. LuxR solos grouped with EsaR and CarR suggesting relatedness to these proteins. Two *Pseudomonas* sp. LuxR solos clustered with CepR2, PfsR, and QscR whereas another two LuxR solos were found with SinR, LasR, and LuxR. The last clade of *Pseudomonas* sp. LuxR solos included characterized LuxR solo SdiA as well as QS-associated LuxRs RhlR, PmlR, CepR, and PluR. Interestingly, LuxR solos found in strains *P. fluorescens* A506, and *Pseudomonas* sp. CFT9 cluster with different characterized LuxR proteins, which indicate that each of them may have evolved from different ancestors.

These observations suggest that multiple LuxR solos carried by a bacterial species may have different evolutionary origins and likely have different ligand binding properties.

DISCUSSION

LuxR proteins with *N*-terminal ABD are well-studied with respect to their role in QS to regulate bacterial community behavior in proteobacteria. QS LuxRs are generally thought to bind to AHLs but recently several studies have identified novel AHL and non-AHL ligands for these proteins raising the possibility of unusual ligands and novel functions for these proteins (Schaefer et al., 2008; Ahlgren et al., 2011; Lindemann et al., 2011; Brachmann et al., 2013). In this context, studies on LuxR solos are gaining importance. Our results the analysis of sequenced genomes reveals that the majority of proteobacterial and a few non-proteobacterial genomes with QS domain LuxRs carry one or more LuxR solos. We have grouped several uncharacterized LuxR solos based on their gene context and conservation of invariant amino acids of their ABD. Several of these LuxR solos had substitutions at the invariant amino acids of the ABD raising the possibility of binding to non-AHL ligands. This approach

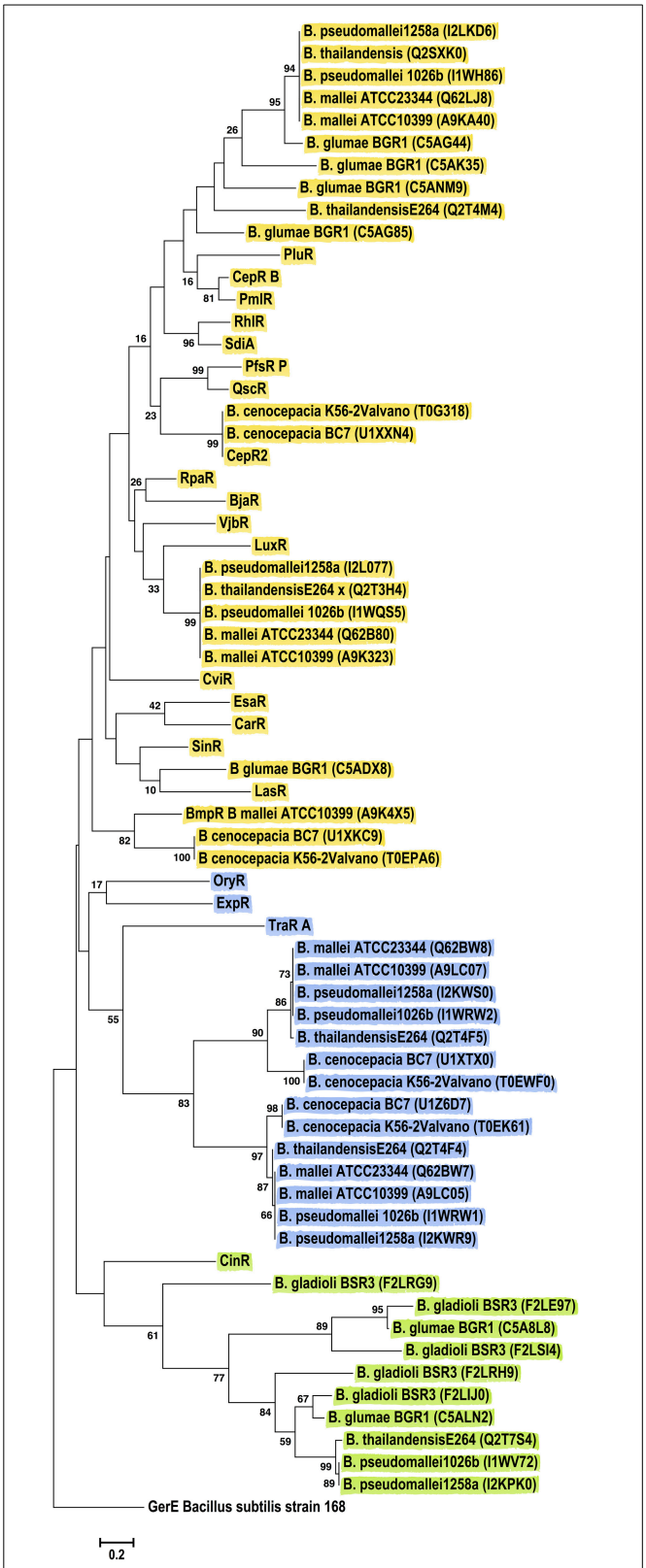

FIGURE 6 | Phylogenetic analyses of multiple LuxR solos carried by the Betaproteobacteria. The tree was inferred by using the Maximum Likelihood method. Tree is drawn to scale, with branch lengths measured in

(Continued)

FIGURE 6 | Continued
the number of substitutions per site. The analysis involved 67 amino acid sequences, representative from the species of the class. This included 46 Betaproteobacterial LuxR solos represented by their Uniprot identification in the figure, in brackets. All positions containing gaps and missing data were eliminated. There were a total of 70 positions in the final dataset. Colors indicate major clusters.

will be of advantage to identify orthologs of LuxR solos that are otherwise difficult to compare across taxonomically distant species due to low sequence homologies.

Our results show that only 40–70% of sequenced bacterial species with QS domain LuxRs carry a complete QS system depending on taxa whereas the remaining carry only LuxR solos. In a previous study where 512 sequenced genomes were analyzed and only 26% of bacteria were reported to contain complete QS system and another 17% only QS domain LuxRs (Case et al., 2008). The differences observed in these two studies is due to the fact that our analyses is based only on genomes that contain QS domain LuxRs and does not consider other completely sequenced bacterial genomes (almost 10 times the numbers of genomes analyzed here) that lack these proteins. Overall, it is clear that among the sequenced genomes containing QS domain LuxR proteins, the majority contain LuxR solos.

Our analysis revealed the presence of genes coding for transposases and pseudogenes adjacent to the genes coding for LuxR solos. Additionally several LuxR solos also occurred near genes coding for proteins having only ABD or even another QS domain LuxR protein (Supplementary Tables 1, 2). These observations suggest a role for horizontal gene transfer and genomic rearrangement events (such as duplication or deletion) associated with occurrence of LuxR solos. LuxR solos with conserved ABD likely evolved by loss of LuxI homolog as related species carrying both *luxR*/*luxI* at the same genomic context were identified in a previous study for SdiA similar to our observations for other LuxR solos (Sabag-Daigle and Ahmer, 2012). The presence of proteins having only autoinducer domain needs to be looked into as several species harbor these proteins; although these proteins have only the ABD, some of these proteins are longer than QS domain LuxRs (Supplementary Table 2). TrlR of *A. tumefaciens* has only ABD and it is known to bind to TraR forming dimers and block its function (Chai et al., 2001). It is possible that ABD proteins function to sequester their cognate ligands or interact with QS domain LuxRs in other bacteria.

The very small numbers of LuxR solos that were detected in non-proteobacterial genomes showed only a limited level of relatedness to proteobacterial QS domain LuxR proteins. Although it is not known if these proteins are functional, it will be interesting to find out if these LuxR solos bind to AHLs or non-AHL ligands. In our analyses we also identified two QS domain LuxR proteins and a gene coding for LuxI homolog in a genetically unlinked locus of *Enterococcus gallinarum*, a Gram-positive bacterium (Supplementary Table 2). This raises the possibility that AHLs may be produced by Gram-positive bacteria as well. In fact, recently, a Gram-positive bacterium belonging to *Exuigobacterium* genera has been reported to produce 3-oxo-C8 HSLs (Biswa and Doble, 2013). Promoter regions of the non-proteobacterial LuxR solos showed the presence of 20-bp palindromic sequences (data not shown); however their role in LuxR solo mediated regulation needs to be determined.

Functionally characterized LuxR solos with known ligands are very few in number (Patankar and Gonzalez, 2009b; Subramoni and Venturi, 2009a). We have sorted LuxR solos from sequenced genomes into functionally relevant groups to understand their relatedness to well-studied LuxR solos and their probable biological roles. However, LuxR solos show only 18–25% homology to each other and it is difficult to group them into clusters based on sequence similarity as revealed in our analysis of these proteins using CLANS method. Using an alternate approach of conservation of flanking genes and invariant amino acids of ABD, we grouped a larger number of LuxR solos in to putative orthologs (Supplementary Tables 4, 5). The ortholog proteins of each group identified here are not comprehensive as it is likely that the genomic context of LuxR solo orthologs may have diverged during evolution; this might account for a number of LuxR solos that could not be grouped.

Genes coding for characterized LuxR solos are known to occur along with functionally linked genes and often regulate their expression. For example, QscR is known to regulate expression of the adjacent phenazine biosynthetic operon and XccR/OryR regulates adjacent proline imino peptidase (*pip*) (Chugani et al., 2001; Mavrodi et al., 2001; Ledgham et al., 2003; Ferluga et al., 2007; Zhang et al., 2007). Our analysis of genomic context of LuxR solos in each group revealed that several LuxR solos occur in a transcriptional unit with neighboring genes; it is possible that these genes are functionally associated with LuxR solos. The presence of 20-bp palindromic sequences were detected upstream of these transcriptional units (Supplementary Table 5); however further analyses and experimental verification will be required to confirm whether they are *lux box* motifs actually bound by LuxR solos. Interestingly, the sequence of several of these palindromic motifs appeared to be conserved within the promoters of LuxR solos of a particular group. Palindromic sequences were also detected upstream of −35 box of promoters of several Alphaproteobacterial LuxR solo containing transcriptional units of environmental/marine strains but they lacked the $CT(N_{12})AG$ motif typical of known *lux box* sequences (Supplementary Table 5). Further analysis of promoter regions of flanking genes and experimental validation has to be carried out to understand regulation of neighboring genes by these unknown LuxR solos.

Multiple LuxR solos present in the same genome showed different levels of relatedness to characterized QS domain LuxRs suggesting different ligand binding properties and different origins. In particular, multiple LuxR solos were found in several plant-associated Alphaproteobacteria, *Pseudomonas* sp., and *Burkholderia* sp. suggesting a role for these proteins in adaptation of these bacteria to diverse habitats. It is possible that LuxR solos of different ligand specificity were acquired by these bacteria from different sources by horizontal gene transfer as it is known to be highly prevalent in many of these species, especially those belonging to *Pseudomonas* sp. (Qiu et al., 2009;

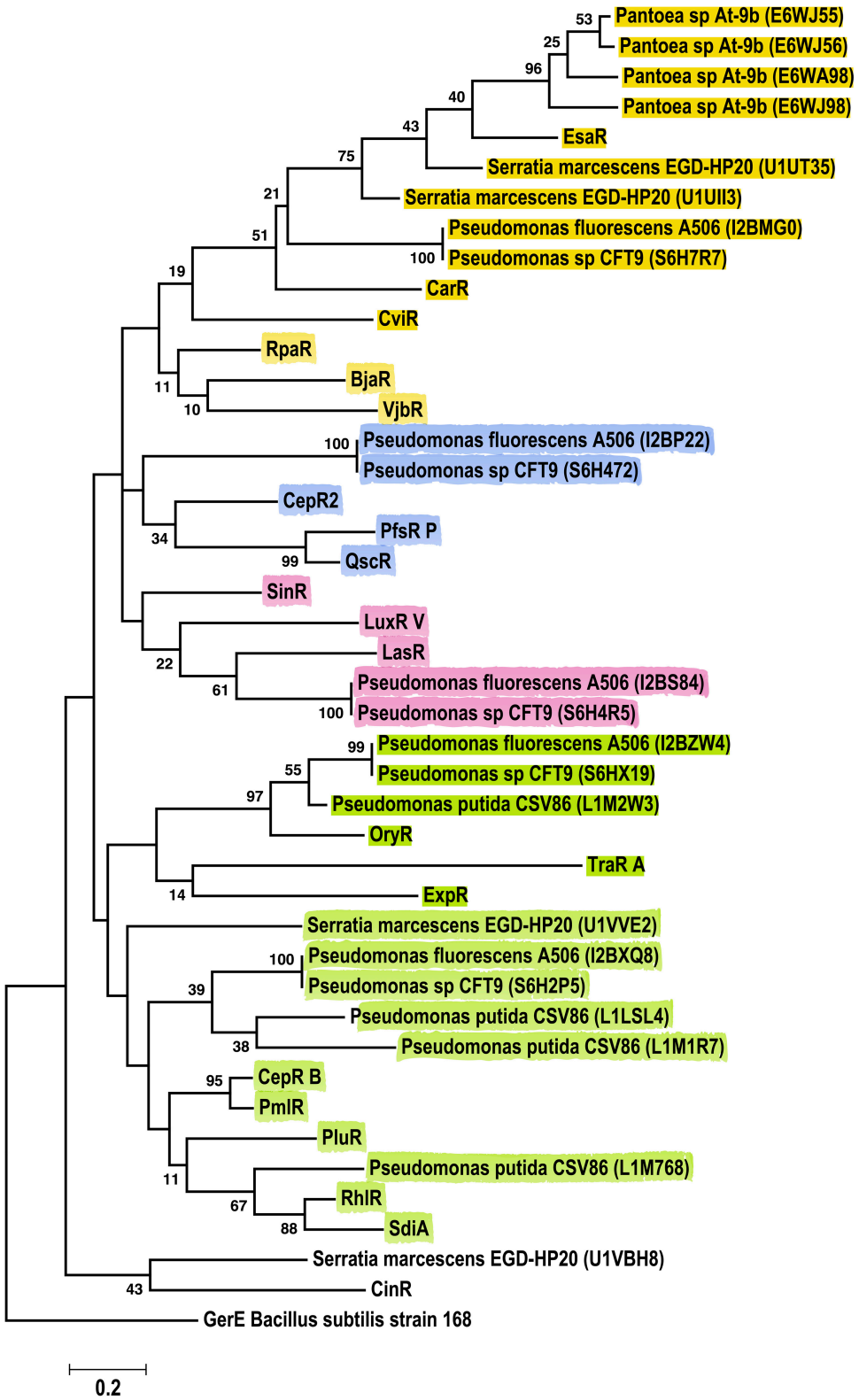

FIGURE 7 | Phylogenetic analyses of multiple LuxR solos carried by the Gammaproteobacteria. The tree was inferred by using the Maximum Likelihood method. Tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 44 amino acid sequences, representative from the species of the class. This included 23 Gammaproteobacterial LuxR solos represented by their Uniprot identification in the figure. All positions containing gaps and missing data were eliminated. There were a total of 64 positions in the final dataset. Colors indicate major clusters.

Subramoni et al., 2011). This seems to be the case for the multiple LuxR solos from *Pseudomonas fluorescens* A506, which branch out with different characterized LuxR solos in the phylogenetic analyses (**Figure 7**); bootstrap values also support this grouping. Further analysis of the conservation of invariant amino acids of ABD and ligand binding experiments are required to understand the different roles of multiple LuxR solos in the same genome.

In summary, the systematic analysis and functional grouping of LuxR solos carried out in this study provides new information regarding the taxonomic and niche specific distribution, evolutionary origins, variation in ligand binding domain and probable roles of these proteins in bacteria which could be studied in the future.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2015.00016/abstract

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Census of solo LuxR genes in prokaryotic genomes

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luxR genes encode transcriptional regulators that control acyl homoserine lactone-based quorum sensing (AHL QS) in Gram negative bacteria. On the bacterial chromosome, *luxR* genes are usually found next or near to a *luxI* gene encoding the AHL signal synthase. Recently, a number of *luxR* genes were described that have no *luxI* genes in their vicinity on the chromosome. These so-called solo *luxR* genes may either respond to internal AHL signals produced by a non-adjacent *luxI* in the chromosome, or can respond to exogenous signals. Here we present a survey of solo *luxR* genes found in complete and draft bacterial genomes in the NCBI databases using HMMs. We found that 2698 of the 3550 *luxR* genes found are solos, which is an unexpectedly high number even if some of the hits may be false positives. We also found that solo LuxR sequences form distinct clusters that are different from the clusters of LuxR sequences that are part of the known *luxR-luxI* topological arrangements. We also found a number of cases that we termed twin *luxR* topologies, in which two adjacent *luxR* genes were in tandem or divergent orientation. Many of the *luxR* solo clusters were devoid of the sequence motifs characteristic of AHL binding LuxR proteins so there is room to speculate that the solos may be involved in sensing hitherto unknown signals. It was noted that only some of the LuxR clades are rich in conserved cysteine residues. Molecular modeling suggests that some of the cysteines may be involved in disulfide formation, which makes us speculate that some LuxR proteins, including some of the solos may be involved in redox regulation.

Keywords: N-acyl homoserine lactone, quorum sensing, LuxR, solo, orphan

Introduction

Quorum sensing (QS) is a general intercellular signaling mechanism that allows bacterial populations to synchronize their behavior in a cell-density dependent manner (Fuqua et al., 1994; Miller and Bassler, 2001). Density dependent responses enable populations to solve problems that single bacterial cells cannot, such as the colonization of new habitats, infection of host organisms, etc. Originally studied in a few species only, a variety of QS mechanisms are now recognized throughout the entire bacterial world (Whitehead et al., 2001; Waters and Bassler, 2005; Case et al., 2008; Schaefer et al., 2008; Lindemann et al., 2011; Brachmann et al., 2013).

One of the simplest and the best studied among the QS mechanisms is *N-acyl Homoserine Lactone* (AHL) based signaling (briefly AHL QS) which is present in many Gram negative bacteria, including important human, animal and plant pathogens that occur in a wide variety of environments. In the AHL QS system (**Figure 1A**), AHL

production is carried out by an AHL synthase that belongs to the LuxI protein family. The AHL molecules produced by *luxI* accumulate both inside and outside of cell membrane in equilibrium between the external and internal signal levels. The AHL molecules inside the cells bind to the signal receptor/regulator protein LuxR which will regulate transcription of both the *luxI* gene as well as other, downstream regulated genes. The *luxI* and *luxR* genes form a typical positive feedback loop usually referred to as an autoinduction circle, which is coupled to external signal concentration via the diffusible AHL molecules.

The regular arrangement of *luxI* and *luxR* genes was observed already in early studies. A review of Goryachev describes two canonical arrangements for *luxI* and *luxR* genes, a tandem arrangement (both genes on the same strand) and a convergent arrangement (with the two genes on opposite strands) (Goryachev, 2009). However, as more genome sequences became available, a number of further topological arrangements were found (Gelencsér et al., 2012a,b; Choudhary et al., 2013). Currently there are about 17 topologies known and it was also shown that the chromosomal neighborhood of AHL circuits contain a few recurrent elements, such as negative regulators of QS and genes involved in DNA mobilization.

Importantly it was also found that QS genes in a given local arrangement (topology) are apparent orthologs with respect to each other while they are paralogs with respect to *luxR* genes in different topological arrangements. For instance, the sequence of a LuxR protein within a tandem topology of *Burkholderia cepacia* is more similar to a LuxR protein of *P. aeruginosa* with the same topology than to another LuxR protein within its own genome which is part of a different type of chromosomal arrangement (such as RMI consisting of *luxR*, *rsaM* and *luxI*). In other words, AHL QS genes cluster according to topology which can be easily recognized in similarity cladograms.

An interesting subgroup of *luxR* genes are those which have no *luxI* gene in their chromosomal neighborhoods. The *qscR* gene of *P. aeruginosa LESB83* is a typical example, and C. Fuqua introduced the term "*orphan luxR*" for this gene in a seminal paper (Fuqua, 2006). As a large number of other genes were found subsequently in a variety of other regulatory and genomic contexts, the generic term solo was introduced for this larger group of genes (Subramoni and Venturi, 2009).

Current views suggest two kinds of regulatory scenarios for solo LuxR proteins (**Figures 1B,C**). In one of them (**Figure 1B**), the solo LuxR responds to the signal produced by an AHL QS circuit within the same cell. The *P.aeruginosa qscR* gene is an example of this scenario. In the other scenario (**Figure 1C**) the solo LuxR protein responds to an external signal which is not necessarily an AHL type molecule. Sequence conservation studies identified a number of conserved residues that are responsible for AHL binding (for a review see, Covaceuszach et al., 2013). Lamba and associates noticed that the AHL binding residues are conspicuously absent in a few solo LuxR proteins (Covaceuszach et al., 2013; Gonzalez and Venturi, 2013; Patel et al., 2014). On this basis, AHL-binding and non-AHL binding LuxR sequences can be tentatively distinguished. It was hypothesized that the identified proteins respond to external signals.

Identifying solo *luxR* genes in genomes is a delicate task, because the LuxR protein is structurally related to other, abundant protein families. Namely, LuxR is composed of two domains, the DNA-binding domain GerE (PFAM id: PF00196) and the autoinducer binding domain (PFAM id: PF03472). Both domains can be found in a variety of other proteins, for instance the GerE domain is part of 273 different types of protein architectures reviewed in the PFAM database (Finn et al., 2008). An ORF can be predicted as a genuine LuxR protein if it bears similarities to both domains, and in addition, the two domains should be in the right serial order, and the total length of the ORF must be in the range of known LuxR proteins (Gelencsér et al., 2012a,b; Choudhary et al., 2013). When looking for canonical QS circuits, false positives can be filtered out by requiring that *luxR* and *luxI* genes be within a certain distance on the chromosome (less than 3000 bp for simple topologies like RI, RMI, RLI and less than 3400 bp for RXMI topologies). When looking for solo *luxR* genes, we do not have such filtering criteria so there is a danger of accepting more false positives. In addition, a *luxR* gene may erroneously appear as a solo because one fails to detect the *luxI* gene in the vicinity, or because it is associated with a novel kind of signal synthase previously not recognized as a QS gene. Sequencing problems can easily cause such mistakes.

This article is concerned with the identification of solo *luxR* genes in the presently available bacterial genomes. We used rigorous criteria to screen complete and draft genomes, both at the proteome and at the DNA sequence level, and found that solo *luxR* genes are more frequent than previously thought. A large number of the solos are not likely to bind AHLs, so there is room for looking for new molecules binding to solo LuxR proteins. We also noted that a few groups of LuxR sequences contain a relatively large number of conserved cysteine residues and raised the hypothesis that they might be involved in sensing oxidative stress.

Data and Methods

For the purposes of the present survey we term a *luxR* gene a solo if it has no *luxI* gene in its vicinity (within 3000 bp up and downstream), and its chromosomal neighborhood is not obviously similar to any of the known AHL QS gene neighborhoods.

The genomic data used in this study were obtained from NCBI's publicly available repository of genomes. For mapping and identifying LuxR solo proteins, Hidden Markov Model (HMM) recognizers were used using Hidden Markov Model recognisers built using the HMMER program, HMMER 3.0 http://hmmer.janelia.org/, as described previously previously (Gelencsér et al., 2012a,b; Choudhary et al., 2013). We scanned 2771 complete and 6970 draft genomes, which in total contained around 25 million proteins.

Results and Discussion

LuxR Solos Form Separate Clades

Of 3550 *LuxR* genes (106 hypothetical) detected in total, 884 (21 hypothetical) were found to be member of AHL circuits and 2698 (85 hypothetical) were solo *luxR* genes, which makes 75% of all *LuxR* genes. The accession numbers of genes are given in Supplementary Material Table 1. LuxR protein sequences were

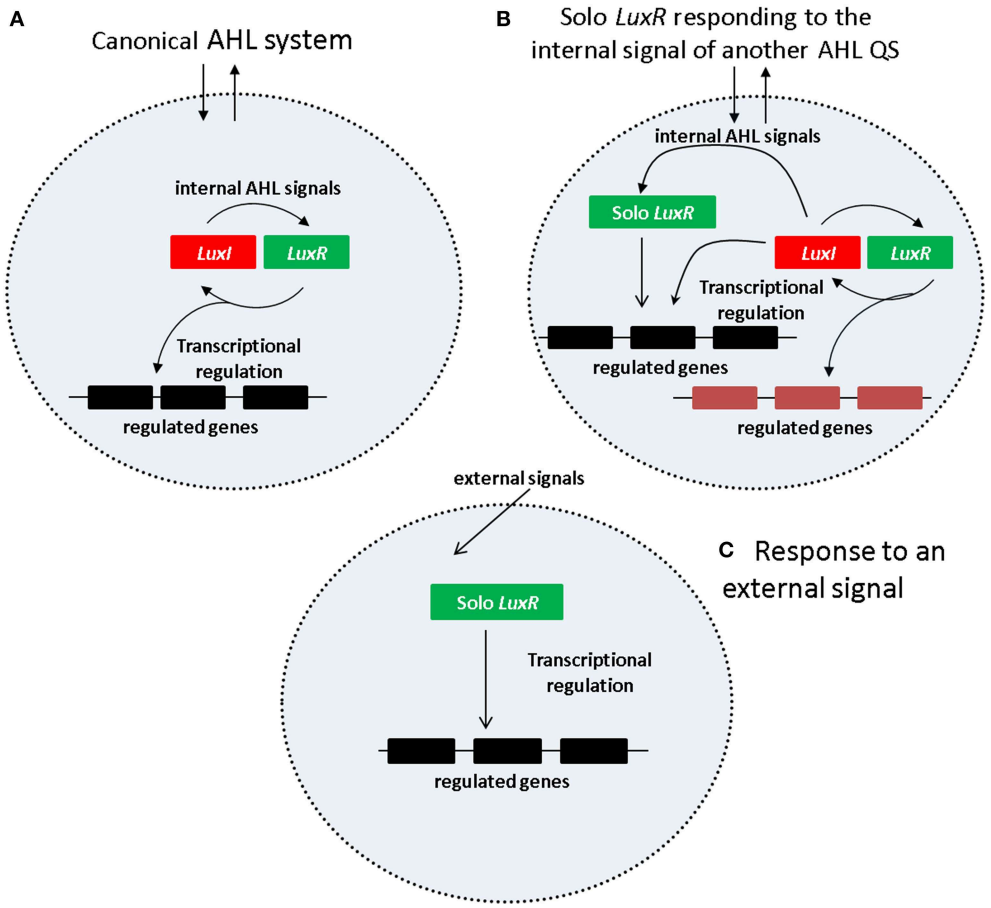

FIGURE 1 | Regulatory mechanism of AHL QS system. (A) Canonical AHL system. **(B)** Solo LuxR responding to the signal of a nonadjacent LuxI synthase. **(C)** Solo LuxR responding to an external signal.

previously shown to cluster according to the topological arrangement of the QS system genes. A sequence similarity clustering of all LuxR sequences showed that LuxR solos form separate clusters that are distinct from the LuxR sequences of complete QS systems. The entire cladogram is deposited in Supplementary Materials Data Sheet 1, a tree representing the *Burkholderia* genus is shown in **Figure 2**. It is apparent that solo LuxRs cluster separately and also that there are distinct types of LuxR solo sequences. This suggests that LuxR solos may be involved in distinct functions.

Novel Topological Arrangements for LuxR Solos

While checking the local topologies in the clades of the *Burkholderia* tree (**Figure 2**) we discovered a novel topology type for solo *LuxR* genes, it was found that two solo *LuxR* genes are sometimes found adjacent to each other. We termed this new arrangement as the "twin *LuxR*" topology. We found two types of this arrangement, one of them is found in *Burkholderia*, the other one is in various other species (**Figure 3**).

These distributions of topologies are difficult to present for a large dataset, therefore for illustration purposes we show a cladogram for the *Burk holderia* genus (**Figure 2**). It is conspicuous that the solo *luxR* genes form separate clades and, same as for other topologies, so they are orthologous with respect to sequences within the clade and paralogous with respect to *luxR* genes present in different topologies, including those within the same genome. In other words we can conclude that at least some solo *luxR*s carry an independent function, so they evolve independently from other *luxR*s within the same genome.

AHL Binding Motifs

In addition to local gene arrangements, we also found that LuxR sequences differ in terms of their characteristic sequence motifs. Previously, Venturi and associates observed a number of sequence motifs that characterize AHL-binding and non-AHL binding LuxR proteins, respectively (Covaceuszach et al., 2013; Gonzalez and Venturi, 2013; Patel et al., 2014). We tested these motifs and found that a sequence contains either an AHL-binding motif (one or more of 8 motifs), or a non-AHL binding motif (one or more of 3 motifs). We then added the respective labels to the sequences and found that if an AHL binding consensus motif is present in a sequence, it is conserved in the entire clade of the tree presented in **Figure 2**. On the contrary, if the AHL binding consensus motifs are absent, non-AHL binding motifs are inevitably present in the entire clade. On the one hand, this conveys confidence to the specificity of the motifs, on

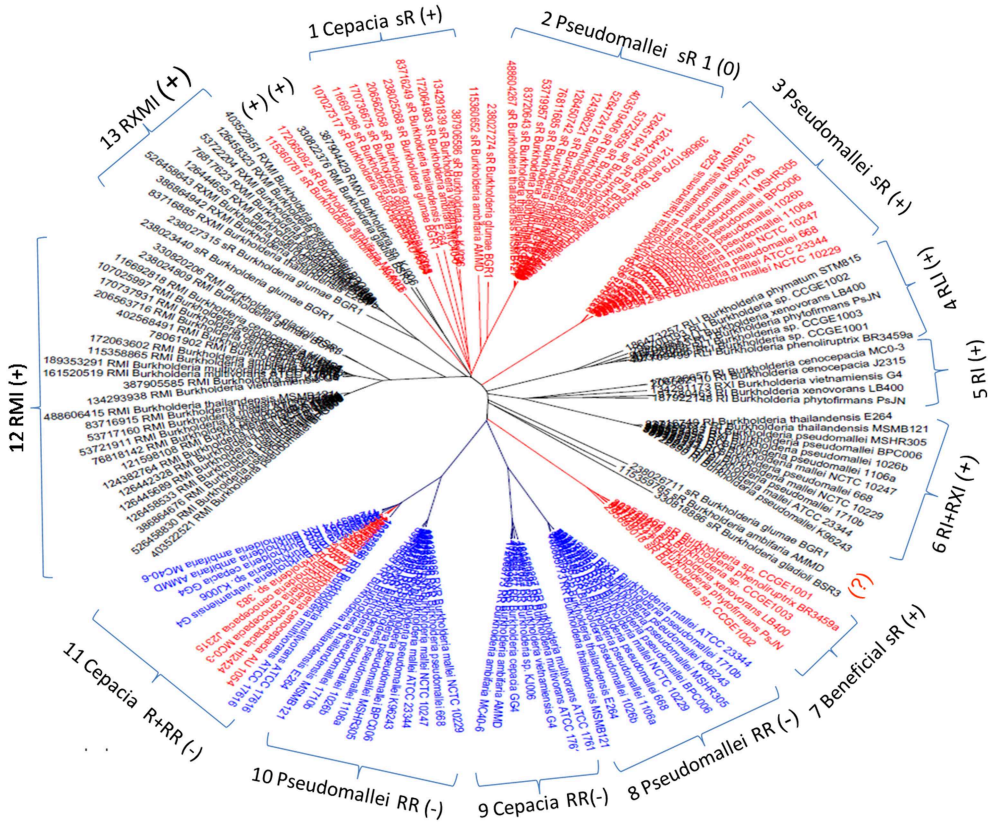

FIGURE 2 | Cladogram of *luxR* genes in the *Burkholderia* genus. Red, solo *luxR*s; Blue, solo *luxR*s in twin topology; Black, *luxR* genes in known QS circuits. (+) and (–) indicates clades where the AHL binding motives are present and absent respectively. In the tags, R stands for *luxR*, I for *luxI*, M is *rsaM*, L is *rsaL* and X stands for any other gene between *luxR* and *luxI* (*X* genes are frequently hypothetical genes of unknown function). As a topological symbol of the clades, R stands for soloR, RR is twin topology, and RI is a canonical QS arrangement consisting of *luxR* and *luxI* genes. A detailed description of topological arrangements is given in (Gelencsér et al., 2012a,b; Choudhary et al., 2013).

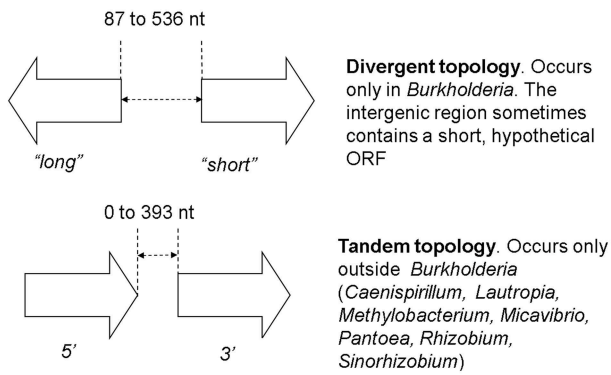

FIGURE 3 | Twin *luxR* arrangements and the suggested notation. The genes occurring in these topologies are listed in Supplementary Materials Table 2. It is important to note that the **two** arrangements are taxonomically distinct, i.e., **one** taxon contains either **one** or the other.

the other hand, the fact that only solo LuxR clades contain the non-AHL binding motifs supports the fact that some of the solo clades are involved in signaling other than AHL. There were a few sequences (outside the *Burkholderia* genus) that contained neither the AHL-binding, nor the non-AHL binding motif (Data Sheet 2). On the one hand, this fact points to the tentative nature of this kind of analysis. On the other hand, it can also point to novel signal types that are not similar to the ones originally included in the analysis of Venturi and associates (Covaceuszach et al., 2013; Gonzalez and Venturi, 2013; Patel et al., 2014).

Cysteine Residues—LuxR Proteins as Redox Sensors?

We also observed that the LuxR proteins of the *Burkholderia* genus differ in terms of the number of cysteine residues (**Table 1**, column 4). Some of the clades shown in **Figure 2** have 6 or 7 conserved cysteine residues while others have one or none. Characteristically, the numbers are conserved within the clades, so again we are tempted to believe that these differences may have a functional role. For instance, the one clade of solo LuxR proteins in *B. pseudomallei* has 7 cysteine residues (see multiple alignment in Supplementary Material Image 1), while another solo LuxR clade from *B. pseudomallei* has only 1. It is worth noting that the cysteine residues are mostly located within the autoinducer domain, not in the DNA binding domain.

TABLE 1 | Presence of AHL-binding and non-AHL-binding sequence motifs in the LuxR proteins in the Burkholderia genus.

LuxR clade (in Figure 2)	AHL binding motif	Non-AHL binding motif	Cysteine content (min-max, average)
RLI	+		4–4, 4
RI	+		2–3, 2.08
RI_RXI	+		3–7, 5.4
Beneficial	+		2–3, 2.17
Pseudomallei RR		+	4–6, 4.18
Cepacia-RR2		+	1–2, 1.71
Pseudomallei RR 2		+	4–6, 4.92
Cepacia_RR		+	1–2, 1.71
Cepacia_soloR		+	4–5, 4.2
RMI	+		2–6, 4.37
RXMI	+		0
Cepacia_soloR	+		2–5, 3.56
Pseudomallei_soloR 1			1–3, 1.23
Pseudomallei_soloR 2	+		7–14, 8

TABLE 2 | Potential disulfide bridges predicted for various clades of Burkholderia LuxR proteins.

Clade	Cys1[a]	Cys2	Cα-Cα distance [Angstrom]
Cepacia solo	111	79	4.3
Pseudomallei long	107	91	4.1
Pseudomallei solo	116	78	4.4
	174	47	4.3

[a] *Positions given according to the numbering of the 1H0M PDB structure.*

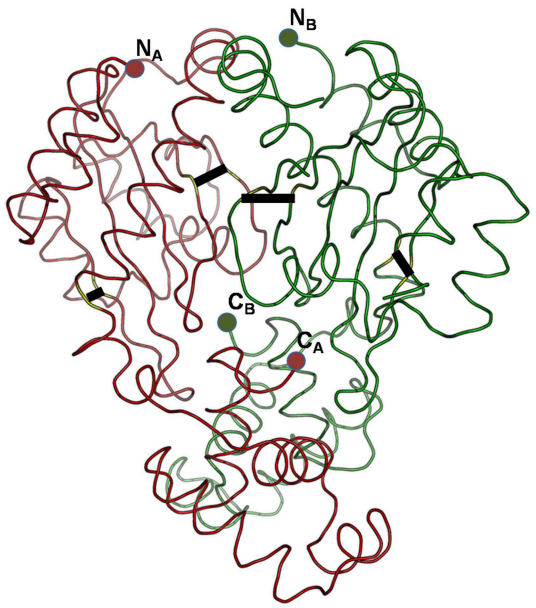

FIGURE 4 | Potential disulfide bridges in the *Burkholderia pseudomallei* solo LuxR proteins which form one clade on phylogenetic tree (Figure 2). Four potential disulfide bridges are mapped on the X-ray structure of the active conformation of the TraR dimer, which was crystallized along with the autoinducer (not shown) and target DNA (not shown) (PDB code: 1H0M). The four bridges are (from left to right): A48–A169, A82–A116, B82–B116, and B48–B169.

The asymmetric distribution of cysteines between clades and between protein domains makes us speculate about the potential functional role of the cysteine residues. One of the plausible ideas is disulfide-based redox regulation mediated by cysteines which is a well-known mechanism in bacterial transcription factors—for a review see (Ilbert et al., 2009). In theory, disulfide formation in a regulatory protein can reinforce active, folded conformations (up-regulation) or conversely, it can lock the protein into inactive, unfolded aggregates (down-regulation). A well-known example is the OxyR repressor, a LysR-type transcription factor that is responsible for the regulation of the antioxidant defense in a large variety of bacteria (Christman et al., 1989). Same as LuxR, OxyR consists of a helix-turn-helix type DNA-binding domain and another domain that mediates dimerization. Oxidative stress results in the formation of an intramonomeric disulfide bridge in the LysR domain, which activates OxyR ptoein by changing the DNA binding specificity. As a result, OxyR becomes rapidly activated and induces the transcription of its target genes (Storz and Tartaglia, 1992; Hausladen et al., 1996; Aslund and Beckwith, 1999). Interestingly, OxyR protein has 6 cysteines, out of which only 2 are involved in disulfide formation.

Can the cysteines conserved in LuxR proteins make disulfile bonds that reinforce the active structure? This cannot be answered on a theoretical basis, but preliminary insights can be gained from the experimentally determined 3D structures of LuxR proteins. We used the crystal structure of the TraR protein of *Agrobacterium tumefaciens* (PDB code: 1H0M) as a template. In this structure the LuxR dimer is bound to AHL and to cognate DNA so this is an active conformation of a LuxR protein (Vannini et al., 2002). Consequently, if a conserved cystein pair in a LuxR homolog can be aligned with positions that are within the distance range of disulfide formation, the resulting disulfide bond can by definition reinforce the active conformation of the protein. We aligned all *Burkholderia* LuxR protein sequences to this template and determined whether or not the positions of conserved cysteine residues are within Cα-Cα distance range of disulfide formation (**Table 2**; the procedure is described in Supplementary Material Data Sheet 3). Interestingly, such disulfide bridge possibilities were found only among the solo LuxR proteins, and all of them were predicted within the autoinducer domain (). In the example shown in **Figure 4**, potential disulfide bridges found in *Burkholderia pseudomallei* solo LuxR proteins that form one clade on phylogenetic tree (**Figure 2**) were mapped on the X-ray structure of the active conformation of the TraR dimer. The highlighted disulfide bridges may thus stabilize the active conformation.

In silico prediction of disulfide bridges *per se* cannot be regarded as a proof for LuxR proteins participating in redox responses. Nevertheless, the facts that, on the one hand, such predicted bridges were found only in solo LuxR proteins and only in their autoinducer domain and the analogy with the OxyR protein, on the other hand, makes us suggest that the role of

some of the solo LuxRs in oxidative stress responses be further investigated in wet lab experiments.

Conclusions

We present a large scale survey of *luxR* genes, trying to understand the mechanisms and phylogenic patterns of solo *luxR*s. We found that out of 3550 LuxR proteins found in the NCBI sequence repository, 2698 are solos, which is a surprisingly large number even if we suppose that some of these *luxR* solos may be associated with unknown or unidentified synthase genes. Transcriptional regulatory circuits can co-evolve independently from the target genes (Cases and De Lorenzo, 2005). Phylogenetic analysis of LuxRs (**Figure 2**) suggests that the evolution of solo LuxRs may be independent from the evolution of QS operons. The fact that taxonomically conserved solo LuxR proteins often contain non-AHL binding consensus motifs while QS-bound LuxRs tend to contain AHL-binding motifs, supports this hypothesis. Furthermore, we observed novel chromosomal arrangement pattern (topology) types, which we name *twin solo LuxRs* which is an addition to the arrangement types described previously (Gelencsér et al., 2012a,b; Choudhary et al., 2013). Last but not least, we hypothesize that some solo *luxR* genes might participate in redox regulation.

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2015.00020/abstract

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Whole genome sequencing and analysis reveal insights into the genetic structure, diversity and evolutionary relatedness of *luxI* and *luxR* homologs in bacteria belonging to the *Sphingomonadaceae* family

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Here we report the draft genomes and annotation of four *N*-acyl homoserine lactone (AHL)-producing members from the family *Sphingomonadaceae*. Comparative genomic analyses of 62 *Sphingomonadaceae* genomes were performed to gain insights into the distribution of the canonical *luxI/R*-type quorum sensing (QS) network within this family. Forty genomes contained at least one *luxR* homolog while the genome of *Sphingobium yanoikuyae* B1 contained seven Open Reading Frames (ORFs) that have significant homology to that of *luxR*. Thirty-three genomes contained at least one *luxI* homolog while the genomes of *Sphingobium* sp. SYK6, *Sphingobium japonicum,* and *Sphingobium lactosutens* contained four *luxI*. Using phylogenetic analysis, the sphingomonad LuxR homologs formed five distinct clades with two minor clades located near the plant associated bacteria (PAB) LuxR solo clade. This work for the first time shows that 13 *Sphingobium* and one *Sphingomonas* genome(s) contain three convergently oriented genes composed of two tandem *luxR* genes proximal to one *luxI* (*luxR-luxR-luxI*). Interestingly, *luxI* solos were identified in two *Sphingobium* species and may represent species that contribute to AHL-based QS system by contributing AHL molecules but are unable to perceive AHLs as signals. This work provides the most comprehensive description of the *luxI/R* circuitry and genome-based taxonomical description of the available sphingomonad genomes to date indicating that the presence of *luxR* solos and *luxI* solos are not an uncommon feature in members of the *Sphingomonadaceae* family.

Keywords: *luxI/R*, *luxR* solos, *Novosphingobium*, quorum-sensing, *Sphingomonadaceae*, phylogenetic, whole genome sequencing

INTRODUCTION

Members of the *Sphingomonadaceae* family are Gram-negative glycosphingolipid-containing bacteria that belong to the α-4 subclass of proteobacteria (Yabuuchi et al., 1990). This family possesses a variety of metabolic capabilities that are potentially advantageous pertaining to a variety of bioremediation capabilities (White et al., 1996). Based on phylogenetic, chemotaxonomic and phenotypic observations, the *Sphingomonas* genus has been expanded to include three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Yabuuchi et al., 1990). Recently, a fifth genus was added to include, *Sphingosinicella* (Maruyama et al., 2006; Geueke et al., 2007; Yoon et al., 2008; Yasir et al., 2010).

Regarding niche, sphingomonads have been isolated from a variety of terrestrial and aquatic environments, including; water supplies, respirators, blood, wounds, dialysis equipment, patients with septicemia, peritonitis, meningitis, and wound infections, soils, deep subsurface sediments, corroding copper pipes and in plants (White et al., 1996; Gan et al., 2009).

Members of the *Sphingomonas* genus are able to catabolize a wide range of natural recalcitrant and anthropogenic compounds including; biphenyl, naphthalenes, pyrene, furans, oestradiol, polyethylenglycols, chlorinated phenols, and various biocides such as carbofuran, 2,4-D and mecoprop (Ogramab et al., 2000; Basta et al., 2004; Stolz, 2009). It was shown that the biphenyl- and naphthalene-degrading *Sphingomonas aromaticivorans* F199 strain and other sphingomonads that degrade additional xenobiotic compounds contain large plasmids encoding the catabolic pathways (Romine et al., 1999; Ogramab et al., 2000; Basta et al., 2004, 2005). Evidence also supports that these replicons can only be transferred among sphingomonads (Ogramab et al., 2000; Basta et al., 2004) by conjugal transfer and that gene and gene cluster rearrangements in the plasmids occur post conjugation (Tiirola et al., 2002). The presence of multiple insertion

elements in sphingomonads suggests a role in the establishment of degradative pathways and in plasmid rearrangements and differences in gene cluster localization in members of the *Sphingomonadaceae* family (Dogra et al., 2004; Muller et al., 2004; Thiel et al., 2005). A recent study comparing the genomes of 26 sphingomonads suggests diverse adaptations and biodegradative capabilities in this group within the phylum *Alphaproteobacteria* (Aylward et al., 2013). Given this complexity in niche environments, biodegradation capabilities and genome rearrangements, the whole genome sequencing of additional sphingomonads has the potential to enhance our understanding of the diversity within this group and may contribute to important biotechnological applications such as bioremediation in the future.

Quorum sensing (QS) is a system commonly employed by bacteria to monitor its cell density prior to regulating gene expression (Fuqua et al., 1994; Miller and Bassler, 2001; Waters and Bassler, 2005; Schuster et al., 2013). In one type of QS system from Gram-negative bacteria, the bacteria produce and detect chemical signals called *N*-acyl-homoserine lactones (AHL). These signals are produced by the enzyme AHL synthase, a member of the LuxI-type protein family. The AHL compounds are detected by a transcriptional regulator belonging to the LuxR-type family. A typical AHL-QS system contains a LuxI and a LuxR protein that are usually in a genomic context regarding proximity of the genes on the chromosome (Choudhary et al., 2013). Upon reaching a concentration threshold measured by the cell density, the AHL signal is detected by the cognate LuxR and can activate population-wide-responses leading to the coordination of gene activation or repression. In Gram-negative bacteria, AHL dependent QS regulation is used to regulate the production of diverse responses such as; the activation of virulence factors, conjugation, the production of antimicrobial metabolites, the regulation of enzyme secretion, the production of bioluminescence and the anabolism of polysaccharide production which is correlated to biofilm formation (Miller and Bassler, 2001; Fuqua and Greenberg, 2002; Waters and Bassler, 2005).

Besides the presence of the canonical *luxI/luxR* pairs, many bacteria contain additional *luxR* transcriptional regulators that are not in a genomic context regarding proximity to a *luxI* gene. These unpaired *luxR* genes have been termed solos and orphans and are homologous to QS LuxR-type transcriptional regulators in that LuxR solos contain the AHL-binding domain at the N terminus and a DNA-binding helix-turn-helix (HTH) domain at the C terminus (Fuqua, 2006; Case et al., 2008; Subramoni and Venturi, 2009; Tsai and Winans, 2010; Cude and Buchan, 2013; Gonzalez and Venturi, 2013). The solo LuxR-type transcriptional activators increase the regulatory range by responding to endogenously produced AHLs and by "listening-in" on exogenous signals produced by other bacteria. Recently, a subfamily of LuxR solos have been found that respond to plant-produced compounds and were subsequently named the plant associated bacteria (PAB) luxR solos (Ferluga et al., 2007; Zhang et al., 2007). In addition, LuxI solos were identified first by Zan et al. (2012) and have also been subsequently identified in *Sulfitobacter, Ruesgeria,* and *Phaeobacter* genera all within the *Roseobacter* clade (Cude and Buchan, 2013).

Members of the sphingomonads have been shown to synthesize AHL signals (D'angelo-Picard et al., 2005; Gan et al., 2009; Huang et al., 2013; Schaefer et al., 2013). Previous work by our group have, isolated, identified, sequenced and annotated the genome of an AHL-producing *Novosphingobium* sp. Rr 2-17 isolated from a grapevine tumor (Gan et al., 2009, 2012). Comparative genomic analysis of Rr 2-17 and five additional members from the genus *Novosphingobium* validated the presence of canonical *luxI/luxR* pairs. Furthermore, a putative *luxR* solo in strain PP1Y of the *Novosphingobium* genus was identified (Gan et al., 2013). Our initial and continuing work with a group of sphingomonads documented to degrade natural and anthropogenic compounds identified a subset of four sphingomonads capable of producing AHL QS signals. We decided to sequence their whole genomes to corroborate AHL-producing phenotype with the presence of *luxI* and *luxR* homologs in the whole genomes and more importantly to contribute molecular resources for future genetic work pertaining to microbial-based bioremediation.

Leveraging on the expansion of microbial genomics data, the additional objectives of this study are to (1) provide an updated genomic distribution of *luxI/R* homologs in the *Sphingomonadaceae* family, (2) update and validate sphingomonad taxonomy using genome-based approach, (3) provide a comprehensive LuxR phylogeny and (4) identify putative LuxR solos and LuxI solos in the currently sequenced sphingomonads.

MATERIALS AND METHODS

STRAINS, CULTURE CONDITIONS AND EXTRACT PREPARATION

The bacterial strains (kindly provided by Andreas Stolz, Institut fur Mikrobiologie, Universitat Stuttgart, Stuttgart, Germany) used in this work were cultured on R2A minimal agar media. To prepare extracts for the detection of AHL compounds, the four sphingomonads were grown on potato dextrose agar medium for 4 days and were resuspended in 10 mls sterile purified water. Equal volume of acidified ethyl acetate (aEtOAc) was added to the resuspended bacteria and the mixture was agitated for 3 h at 25°C with shaking at 150 rpm followed by centrifugation to separate the aqueous phase from the aEtOAc phase. Under these conditions, AHLs partition into the non-polar aEtOAC phase. The aEtOAc was aspirated off, dried in a Savant speed-vac and resuspended in aEtOAc to produce a 20-fold concentrated aEtOAc extracts. These extracts were used in AHL detection assays.

E. coli JM109, *Agrobacterium tumefaciens* A136 and *Chromobacterium violaceum* CV026 were grown in Luria-Bertani (LB). Each bacterial biosensor reporter strain used in this work is listed in Supplemental Table 1 along with its AHL receptor protein and cognate AHL signal. All media and growth conditions are as previously described by our group (Scott et al., 2006; Gan et al., 2009; Lowe et al., 2009; Savka et al., 2011).

BIOASSAYS FOR AHL QS SIGNAL DETECTION USING AHL-DEPENDENT BIOSENSOR STRAINS

An overnight culture of these four biosensors were grown in LB with the appropriate antibiotic and diluted 1:10 in LB and 200 µl of the diluted cell suspension was added to the round bottom tubes (12 × 50 mm) containing dried aEtOAc samples or pure AHL signals as controls. Cognate AHL signal for *E. coli*

biosensors JM109 (pSB401) was 3-oxo-C6-HSL at 50 nM; for JM109 (pSB536) was C4-HSL at 1 μM; for JM109 (pSB1075) was 3-oxo-C12-HSL at 1 nM, unless otherwise noted. For the *A. tumefaciens* A136 biosensor pure C8-HSL was used at 50 nM. Tubes were incubated at 30°C with shaking for 5 to 6 h before bioluminescence was measured using a Turner Designs TD 20/20 luminometer. The TD 20/20 luminometer was adjusted to different sensitivities due to the varying responses of the JM109 series of biosensors to their cognate AHL signal. Unless noted, relative light units (RLU) measurements were made at 30.0, 39.9, 50.1, and 30.0% sensitivity for LuxR-, AhyR, LasR, and TraR-based biosensors, respectively. Luminescence is measured and given in RLU per triplicate sample. RLUs were determined with a 20-s integration period. Mean values of the RLUs were obtained with three independent biological samples.

For "T"-streak assays, the *Chromobacterium violaceum* colorless mutant, CV026 was used. In the presence of exogenous QS signals CVO26 produces the purple pigment violacein, indicating the presence of AHL in the sample. *C. violaceum* wild type strain was used as a positive control. *E. coli* DH5α was the negative control in the T-streak plate assays. The biosensor, controls, and samples were grown on tryptone—yeast extract medium mixed with PDA medium (1:1, v/v). Each isolate was tested at least two times using the "T"-streak bioassay. The whole cell AHL-dependent biosensor assays were performed as previously described by our group (Scott et al., 2006; Gan et al., 2009; Lowe et al., 2009; Savka et al., 2011).

WHOLE GENOME SEQUENCING, ASSEMBLY AND ANNOTATION

Genomic DNA was extracted using the GenElute™ (Sigma-Aldrich, St. Louis, MO) and converted into next generation sequencing library using Nextera XT (Illumina, San Diego, CA) according to the manufacturer's instructions. Whole genome sequencing was performed using the MiSeq (Illumina, San Diego, CA) at the Monash University Malaysia Genomics Facility. The raw data for each bacterium were error-corrected and assembled using Spades v2.5 (default setting) (Bankevich et al., 2012). The generated contigs were scaffolded and gap-closed using SSPACE and GAPFiller respectively (Boetzer et al., 2011; Boetzer and Pirovano, 2012). Genome annotation was performed using Prokka and InterProScan5 (Jones et al., 2014; Seemann, 2014).

WHOLE GENOME-BASED PHYLOGENY ASSIGNMENT

Publicly available complete and draft genome sequences (<250 contigs) from the genus *Novosphingobium, Sphingomonas, Sphingopyxis,* and *Sphingobium* were downloaded. Subsequently, gene/protein prediction was performed using Prodigal2.60 (default setting) (Hyatt et al., 2010). PhyloPhlAn was used to construct phylogenetic tree from the resulting predicted proteins based on 400 highly conserved microbial proteins (Segata et al., 2013).

SYSTEMATIC BIOINFORMATICS IDENTIFICATION OF LuxI, LuxR AND LuxR SOLO HOMOLOGS

A systematic methodology for the accurate and stringent identification of LuxI, LuxR, and LuxR solo homologs is presented in **Figure 1**. Briefly, the predicted proteomes were scanned for protein family domain (PFAM) specifically the autoinducer synthase domain (PFAM signature: PF00765) and the autoinducer binding domain (PFAM signature: PF03472) that are universally present in reported LuxR and LuxI homologs, respectively, using profile hidden Markov models-based similarity search (*E*-value <1e-5). The short listed candidates were further annotated using the more time consuming but comprehensive InterProScan5. To qualify as an authentic LuxR homolog, the shortlisted protein must contain four signature LuxR homolog Interproscan identifiers e.g., IPR005143 (autoinducer binding), IPR016032 (Signal transduction response regulator, C-terminal effector), IPR011991 (Winged helix-turn-helix DNA-binding domain), and IPR000792 (Transcription regulator LuxR, C-terminal) that are universally present in functionally validated LuxR homologs. An authentic LuxI homolog on the other hand, must contain both IPR001690 (Autoinducer synthesis protein) and IPR018311 (Autoinducer synthesis, conserved site). Cognate LuxI and LuxR homologs were then manually identified based on the coordinate and close proximity of their respective protein-coding genes.

MAXIMUM LIKELIHOOD APPROXIMATION OF THE LuxR PHYLOGENY

Functionally validated LuxR homologs, PAB LuxR solos and the putative sphingomonad LuxR homologs were combined and aligned with MAFFT-LINSI using the default setting (Katoh and Standley, 2013). The resulting protein alignment was then used as the input for maximum likelihood phylogenetic analysis using FastTree2 (Price et al., 2010). The constructed tree was visualized and graphically edited using FigTree (Rambaut, 2014).

VISUALIZATION OF *LuxR* AND *LuxI* SOLOS GENE NEIGHBORHOOD, LuxR HOMOLOGS ALIGNMENT AND PAIRWISE IDENTITY MATRIX CONSTRUCTION

Contigs containing the identified *luxR* solo genes were extracted from the genome, annotated with Prokka (default setting) and subsequently visualized in EasyFig (Sullivan et al., 2011). Additionally, sphingomonad LuxR homologs clustered with the PAB LuxR solos were aligned with MAFFT-LINSI (Katoh and Standley, 2013) and visualized using ALINE (Bond and Schuttelkopf, 2009). Pairwise identity matrix for selected LuxR homologs was constructed using SDT (Muhire et al., 2014).

RESULTS

GENOME STATISTICS OF THE FOUR NEWLY SEQUENCED SPHINGOMONADS AND THEIR ABILITY TO PRODUCE AHL SIGNALS

The genome assembly and annotation statistics of four genomes of *Sphingomonas* known for their biodegradation ability in addition to their isolation source and notable features are presented in **Table 1**. Culture extracts prepared from each of the four sphingomonads strains in this study chosen for whole genome sequencing activated at least two AHL-dependent whole cell bacterial biosensors (Supplemental Table 1). *Sphingomonas paucimobilis* EPA505 activated light production in the TraR-based *Agrobacterium* A136 and in the LasR-based *E. coli* JM109 (pSB1075) biosensors and activated pigment synthesis in the CviR-based *Chromobacterium* biosensor. The *Sphingobium herbicidovorans* NBRC16415, *Sphingobium yanoikuyae* B1 and *Novosphingobium resinovorum* KF1 activated light production in

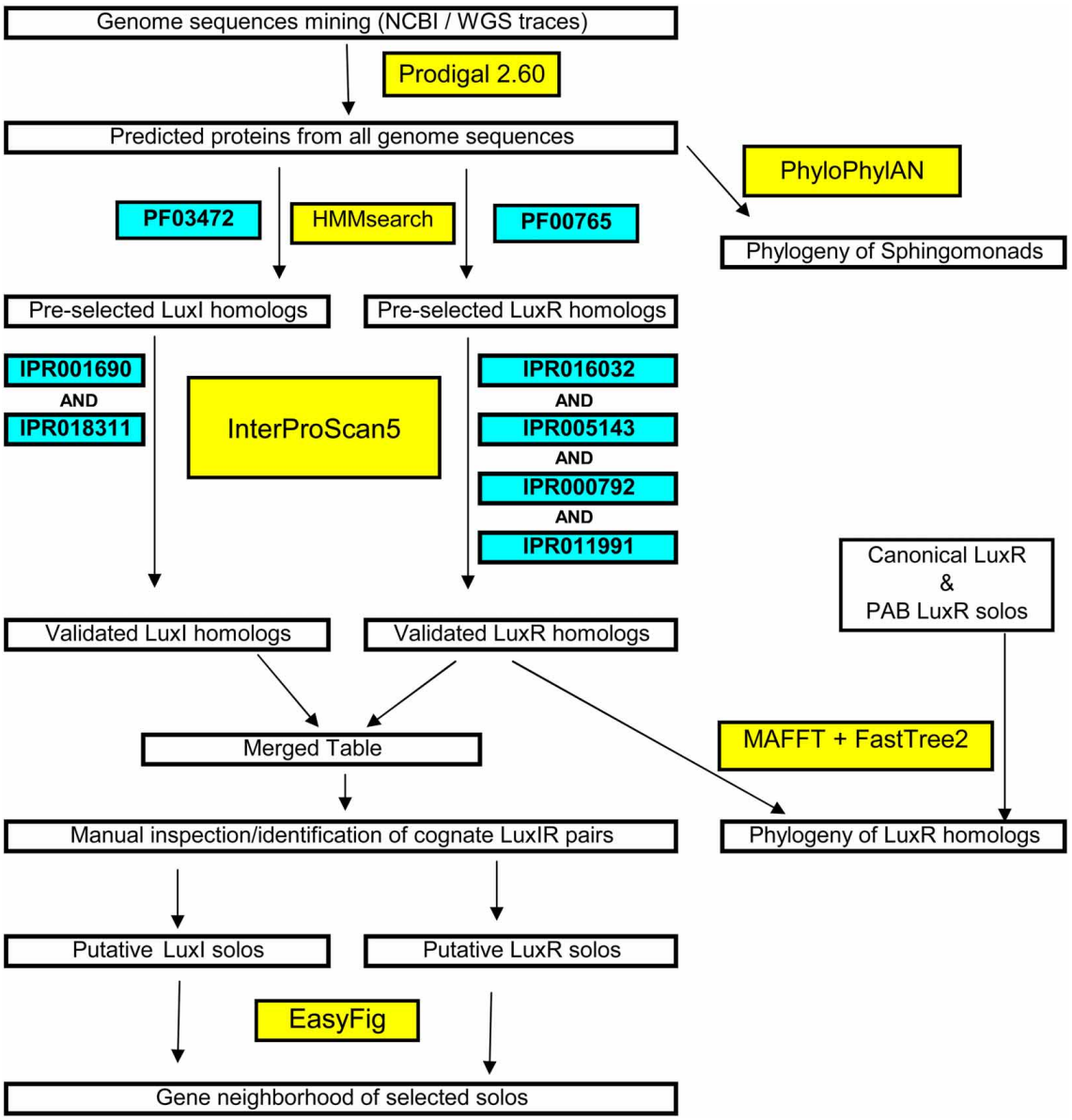

FIGURE 1 | Flowchart of the systematic and stringent bioinformatics methodology used in this study for the identification of *luxI/R* in sphingomonads and large-scale phylogenetic/phylogenomic tree construction.

the TraR- and activated pigment synthesis in the CviR-based biosensors (**Table 2**). These results are consistent with findings by others that AHL QS signal production in members of the sphingomonad group is not uncommon (D'angelo-Picard et al., 2005; Gan et al., 2009; Huang et al., 2013; Schaefer et al., 2013).

PHYLOGENOMIC ANALYSIS OF CURRENTLY SEQUENCED SPHINGOMONAD

Analysis of the currently sequenced sphingomonads (**Figure 2** and see Supplemental Table 4 for accession number) indicates that there is a sequencing bias toward the genera *Sphingobium, Novosphingobium,* and *Sphingomonas*. Very recently, our group sequenced and annotated the genomes of two additional cave *Sphingopyxis* genomes that enabled the expansion of the taxon sampling size (Gan et al., 2014). Species from the genera *Sphingobium* and *Novosphingobium* form robust monophyletic lineages with extremely high (>90%) nodal support. Based on phylogenomic analysis, the *Sphingobium* clade is the sister group to the clade of *Sphingopyxis* and *Novosphingobium*. Notably, species from the genus *Sphingomonas* display considerable paraphyletic distributions, indicating incongruence between molecular and biochemical-based taxonomic assignment. Phylogenomic analysis also suggests that *Sphingomonas* sp. SKA85 and the classic *Sphingomonas paucimobilis* EPA505 (Nohynek et al., 1996) may have been misclassified at the genus level as evidenced by its tight clustering within the *Sphingobium* group.

Table 1 | Strain information, genome assembly and annotation statistics of the sequenced sphingomonads used in this study.

Strain	A. N.*	Size (bp)	No. of contigs	N50	GC%	Some compounds degraded	Isolation source	References
Novosphingobium resinovorum KF1	JFYZ01	6,304,486	115	171,782	65.06	2, 3, 4,6-tetrachlorophenol	Fluidized-bed reactor	Takeuchi et al., 2001
Sphingobium herbicidovorans NBRC16415	JFZA01	4,032,326	62	178,990	62.44	2, 4-dichlorophenoxyacetate	Soil	Zipper et al., 1996
Sphingobium yanoikuyae B1	JGVR01	5,683,787	116	158,314	63.94	Toluene, biphenyl	Polluted stream	Yabuuchi et al., 1990
Sphingomonas paucimobilis EPA505	JFYY01	4,874,185	81	285,203	63.93	Fluoranthene, naphthalene(s)	Creosote waste site	Muller and Wittmann-Liebold, 1997

**A.N., Accession Number.*

Table 2 | Production of *N*-acyl-homoserine lactones by four newly sequenced strains of the *Sphingomonas, Sphingobium* and *Novosphingobium* group assayed by five AHL-dependent biosensor strains*.

Genus and species/strain	AhyR‡	LuxR‡	TraR‡	LasR‡	CviR#
Sphingomonas paucimobilis EPA505	−	−	+++	+	+++
Sphingobium herbicidovorans NBRC16415	−	−	++	−	+++
Sphingobium yanoikuyae B1	−	−	++	−	+
Novosphingobium resinovorum KF1	−	−	+++	−	+

**Abbreviations include: AhyR, AHL receptor from Aeromonas hydrophilia; LuxR, from Vibrio fisheri; TraR, from Agrobacterium tumefaciens; LasR, from Pseudomonas aeruginosa; CviR, from Chromobacterium violaceum.*

‡Scores for biosensor detection of AHL in strain extracts are based on the following criteria: -, < 2-fold higher than background levels of relative light units (RLU) bioluminescence; +, > 2-fold higher than background RLUs; ++, 50 to 75-fold higher than background RLUs; +++, > 75-fold higher than background in RLUs.

 # CviR, AHL-dependent receptor of biosensor strain CV026. Scores were relative violacein pigment production in T-streak bioassays on PDA/TYE (1:1) agar media.

THE PRESENCE AND COMPOSITION OF SPHINGOMONAD *LuxR* AND *LuxI* HOMOLOGS ARE DIVERSE

The analysis of 62 sphingomonads genomes provides genetic evidence that QS is a common trait within the family. 40 of the 62 genomes analyzed contain at least one putative *luxI* or *luxR* homolog with 33 of them containing at least 1 putative canonical *luxI/R* homolog pair (**Table 3** and See Supplemental Tables 2, 3 for a complete information of the identified *luxR* and *luxI* homologs). The non-universal presence of QS genes in members of the same species e.g., *Sphingobium yanoikuyae* and *Sphingobium xenophagum* may imply that QS is a trait that is subject to purifying selection. It is also worth noting that members of the currently sequenced *Sphingomonas* have a relatively incomplete *lux*-based QS capacity as evidenced by the sparse presence of *luxI* and *luxR* homologs in this genus.

LuxR PHYLOGENY REVEALS DIVERSE ORIGIN OF SPHINGOMONAD LuxR AND SUPPORTS THE MONOPHYLETIC CLUSTERING OF LuxR SOLOS FROM PLANT ASSOCIATED BACTERIA

A majority of the sphingomonad LuxR homologs form a big clade that is a sister group to the clade containing the functionally validated BjaR and RhlR (**Figure 3**) (Cubo et al., 1992; Lindemann et al., 2011). Consistent with previous reports, the PAB LuxR solos e.g., NesR, XagR, XccR, OryR, and PsoR (Ferluga et al., 2007; Zhang et al., 2007; Ferluga and Venturi, 2009; Chatnaparat et al., 2012; Gonzalez et al., 2013) formed a robust and well-defined monophyletic group. Based on phylogenetic clustering, six sphingomonad LuxR homologs may share a common (but distant) ancestry with the PAB LuxR solos clade. Alignment of these six putative LuxR homologs shows substitution in the highly conserved amino acid in the regulatory domain e.g., Y61W that is similarly reported in PAB LuxR solos. With the exception of a LuxR homolog from *Sphingobium herbicidovorans* NRBC 16415 (JFYZ01∼contig3_10) that has a W57V substitution, the W57 residue was conserved in the remaining five sphingomonad LuxR homologs. Furthermore, other substitutions were observed in the conserved D70 and W85 residues for four out of the six sphingomonad LuxR homologs (**Figure 4**). In general, the three conserved residues in the DNA-binding domain (E178, L182, and G188) are conserved across the LuxR homologs alignment with the exception of L182I substitution in a *Sphingomonas* sp. S17 LuxR homolog (AFGG01∼contig50_9).

THE GENE NEIGHBORHOOD OF SPHINGOMONAD *LuxR* SOLO AND *LuxR* DOUBLE IS NOT CONSERVED

Investigation of the genes flanking the putative *luxR* solos in our sequenced genomes reveals some intriguing findings (**Figure 5A**). In *Sphingobium herbicidovorans* NBRC 16415, its putative *luxR* solo is convergently oriented with respect to a *luxI/R* pair and while in *N. resinovorum* [contig 2], it is located four genes downstream of a *luxI/R* pair. Furthermore, the gene coding for a possibly truncated LuxR-like protein is located immediately downstream of the *luxR* solos in *S. yanoikuyae* and *N. resinovorum* (contig2) (**Figure 5A**), suggesting the occurrence of *luxR* gene duplication and/or recombination in that region. In addition to the tandem *luxR* duplication (*luxR* double) in strain NBRC16415, further analysis of the sphingomonad genomes led to the identification of additional tandem *luxR* duplication

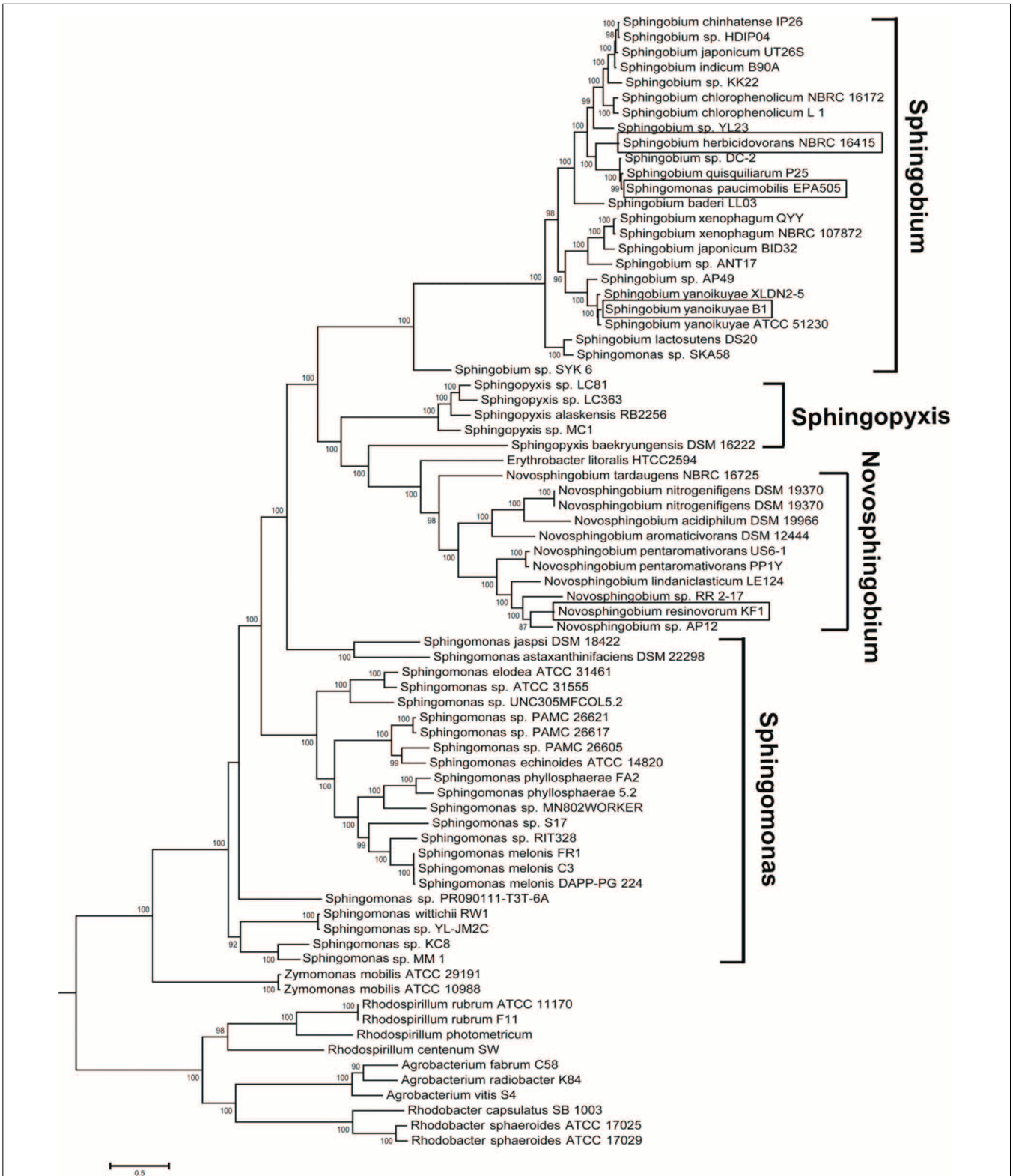

FIGURE 2 | Phylogenomic tree depicting the evolutionary relationship of currently sequenced sphingomonads based on approximately 400 conserved single-copy genes. The four whole genomes sequenced in study were shown in rectangle boxes. Selected members from the genera *Rhodospirillum, Agrobacterium and Rhodobacter* were designated as outgroup. Bootstrap support of less than 50% was not shown.

Table 3 | Distribution and organization of the *luxI* and *luxR* homologs and the presence of direct double *luxR-luxI* topology identified in the whole genome sequences of 40 sphingomonads.

Organism	Canonical *luxR, luxI*	*luxR-luxR-luxI* and neighborhood variation[#]	Unpaired *luxI* (solos)	Unpaired *luxR* (solos)
NOVOSPHINGOBIUM (7/11) [‡] [6,0,0,3] *				
Novosphingobium lindaniclasticum LE124	1	0	0	0
Novosphingobium pentaromativorans PP1Y	2	0	0	1
Novosphingobium pentaromativorans US6-1	1	0	0	0
Novosphingobium resinovorum KF1[†]	2	0	0	2
Novosphingobium sp. AP12	1	0	0	0
Novosphingobium sp. RR 2-17	1	0	0	0
Novosphingobium tardaugens NBRC 16725	0	0	0	2
SPHINGOBIUM (18/24)[‡] [11,11,8,12] *				
Sphingobium baderi LL03	0	2 with T3 and T5	1	3
Sphingobium chinhatense IP26	1	0	1	0
Sphingobium chlorophenolicum NBRC 16172	2	0	1	1
Sphingobium chlorophenolicum L1	0	1 with T1	1	1
Sphingobium herbicidovorans NBRC 16415[†]	1	1 with T3	0	1
Sphingobium indicum B90A	0	1 with T1	1	1
Sphingobium japonicum UT26S	2	1 with T1	0	1
Sphingobium lactosutens DS20	2	1 with T1	0	0
Sphingobium sp. ANT17	1	0	0	0
Sphingobium sp. AP49	1	0	0	0
Sphingobium sp. DC-2	0	0	0	1
Sphingobium sp. HDIP04	0	1 with T4	1	1
Sphingobium sp. KK22	1	1 with T3	2	0
Sphingobium sp. SYK6	2	1 with T1	0	1
Sphingobium sp. YL23	0	1 with T3	0	0
Sphingobium xenophagum QYY	0	1 with T2	0	1
Sphingobium yanoikuyae ATCC 51230	2	0	1	5
Sphingobium yanoikuyae B1[†]	2	0	0	1
SPHINGOMONAS (11/22)[‡] [5,2,0,7] *				
Sphingomonas elodea ATCC 31461	0	0	0	1
Sphingomonas paucimobilis EPA505[†]	2	0	0	1
Sphingomonas sp. KC8	1	0	0	0
Sphingomonas sp. MM1	0	1 with T6	0	0
Sphingomonas sp. PAMC 26617	0	0	0	1
Sphingomonas sp. PAMC 26621	0	0	0	1
Sphingomonas sp. S17	0	0	0	1
Sphingomonas sp. SKA58	1	1 with T1	0	0
Sphingomonas sp. UNC305MFCOL5.2	1	0	0	0
Sphingomonas sp. YL-JM2C	2	0	0	2
Sphingomonas wittichii RW1	0	0	0	3
SPHINGOPYXIS (4/5)[‡] [4,0,0,0] *				
Sphingopyxis alaskensis RB2256	2	0	0	0
Sphingopyxis sp. LC363	1	0	0	0
Sphingopyxis sp. LC81	2	0	0	0
Sphingopyxis sp. MC1	1	0	0	0

[‡] *(Number of genomes luxI and/or luxR)/(Total genome).*

* *Number of genomes with luxI and luxR of four described category (separated by comma).*

[†] *Strains sequenced in this study.*

[#] *Topology variation and conservation of phyH in convergent double luxR, luxI gene neighborhoods (See **Figure 5B** for detailed topology variation).*

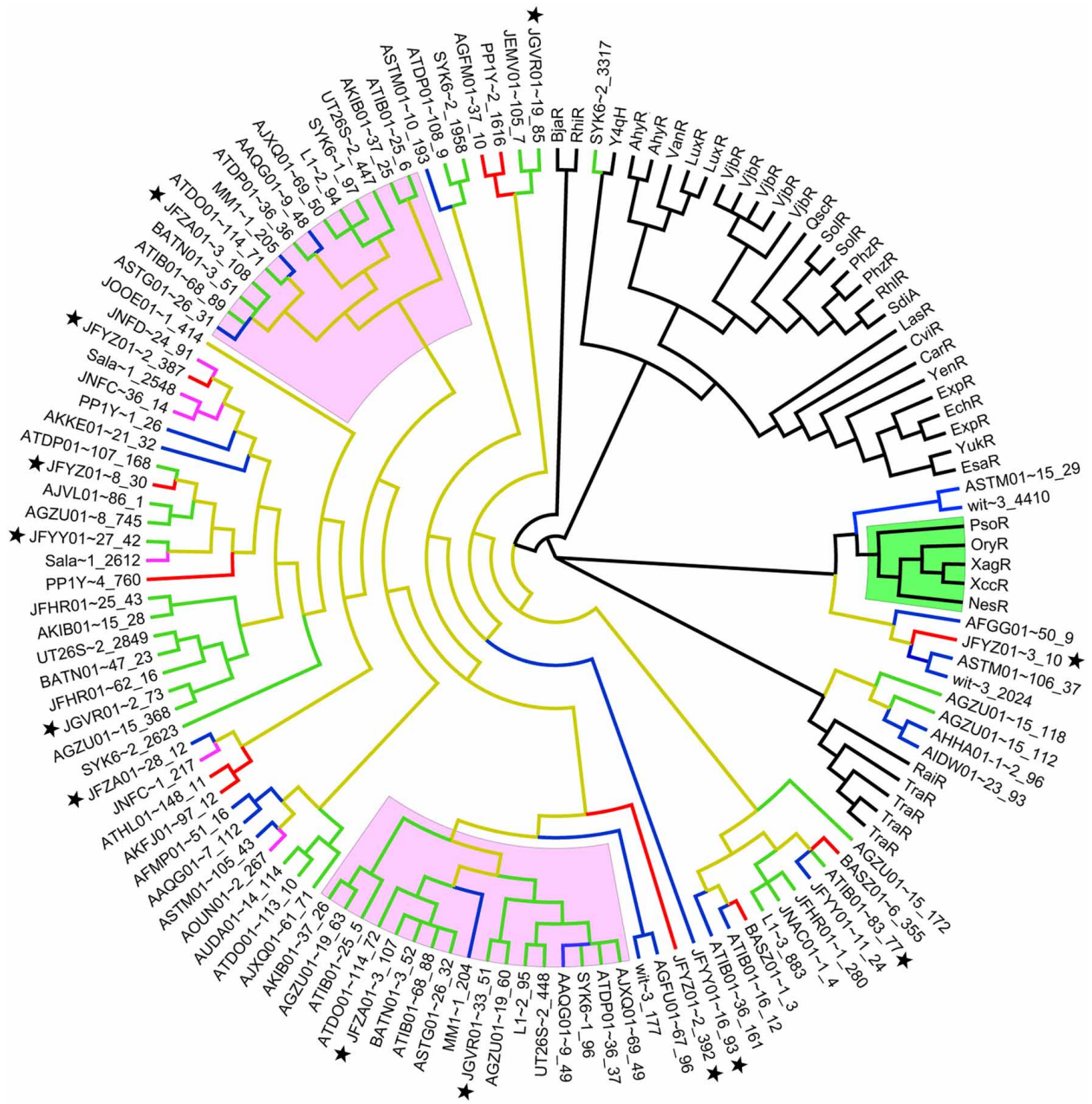

FIGURE 3 | Unrooted phylogenetic tree of functionally validated LuxR homologs, Plant associated bacteria (PAB) LuxR Solos (Gonzalez and Venturi, 2013) and Identified sphingomonad LuxR Homologs. Clades highlighted in green and pink represent PAB LuxR solos and sphingomonad double LuxR, LuxI, respectively. Branches colored in brown, blue, red, green, and purple represent the *Sphingomonadaceae*, *Sphingomonas*, *Novosphingobium*, *Sphingobium*, and *Sphingopyxis* lineages, respectively. Black star next to taxa name indicates LuxR homologs from strains sequenced in this study. Accession numbers and aligned sequences are available in Supplemental Data 1.

(**Table 3** and **Figure 3**) with variable gene neighborhood at the 5′ end (**Figure 5B**).

PAIRWISE COMPARISON BETWEEN MEMBERS OF THE SAME CONVERGENT DOUBLE *LuxR* GROUP SHOWS CONSIDERABLE SEQUENCE DIVERGENCE

The amino acid pairwise identity between members of the same LuxR double group is in the range of 50%. On the contrary, up to 94% pairwise identity could be obtained for members from different LuxR double group (**Figure 6**). This is consistent with the LuxR phylogenetic tree with whereby LuxR double members from the same group do not form a tight cluster with one another (**Figure 3**). Given that *luxR* double is almost exclusively observed in the genus *Sphingobium*, *luxR* double may originate from an ancient tandem gene duplication in the common ancestor of the genus *Sphingobium* followed by a neofunctionalization-oriented

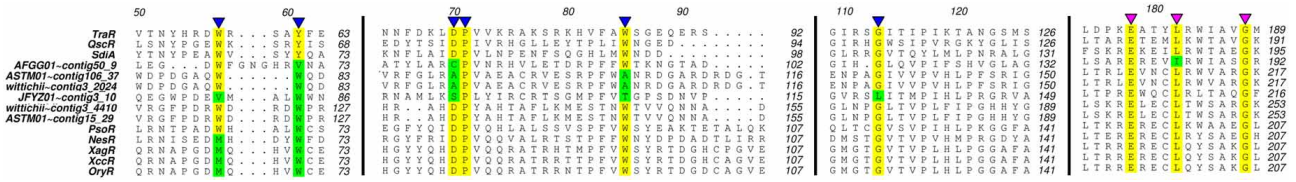

FIGURE 4 | Alignment of plant associated bacteria solos. LuxR solos and selected sphingomonad LuxR homologs. Number above the alignment corresponds to the residue number of the TraR protein. Regions highlighted in yellow indicate the invariant sites of canonical LuxR homologs (Fuqua and Greenberg, 2002) while variation from the conserved site was highlighted in green. The conserved sites corresponding to autoinducer binding and DNA binding were indicated by blue and purple triangles, respectively.

functional divergence of the *luxR* duplicate that was subsequently retained in several strains of the genus *Sphingobium*. The presence of a *luxR* double in a non-*Sphingobium* strain e.g., *Sphingomonas sp.* MM1 may then be attributed to horizontal gene transfer.

IDENTIFICATION OF LuxI SOLOS

Two putative *luxI* solos were identified in *Sphingobium* sp. KK2 strain that reside on different contigs and one in *Sphingobium chinhatense* IP26 strains (**Figure 7A**). A gene coding for N-terminal truncated/mutated LuxR-like protein is located immediately upstream and convergently oriented to the putative *luxI* solo in *Sphingobium chinhatense* IP26 and *Sphingobium* sp. KK2. Multiple sequence alignment of the three putative LuxI solos in sphingomonads with LuxI-type family proteins showed all 10 amino acid residues required for AHL synthase activity are conserved and supports that these three *luxI* solos encode enzymes involved in AHL synthesis (**Figure 7B**). Additionally, *phyH* gene coding for phytanoyl dioxygenase is located immediately downstream of and convergently oriented with respect to one of the *luxI* solos in strain KK2 which is frequently observed in several well-described *luxI/R* pairs (Gan et al., 2013).

DISCUSSION

The biochemical and genetic characterization of *Novosphingobium* sp. Rr 2-17 isolated from grapevine tumor provided the first glimpse of QS ability in the genus *Novosphingobium* (Gan et al., 2009). The genome sequencing of strain Rr 2-17 and subsequent comparative genomic analysis with five additional members from the genus *Novosphingobium* validates the presence of *luxI/R* homolog(s) (Gan et al., 2012) and even more intriguingly, a *luxR* solo in this genus (Gan et al., 2013). Expanding from our previous study, we present four new whole genome sequences of AHL QS signal producing strains in the sphingomonad group and to our knowledge presents the most comprehensive genomic surveillance of sphingomonads for the distribution of *luxI/R* homologs to date. In addition, the work presents the most updated and accurate genome-based taxonomy validation of the currently sequenced sphingomonads. Although previous works provided convincing biochemical test results to support the reclassification of *Sphingomonas*, the constructed phylogeny based on the 16S rRNA gene failed to provide satisfactory bootstrap support particularly in the splits that separated the major genus in *Sphingomonadaceae* (Takeuchi et al., 2001). Our phylogenomic approach dramatically improves the bootstrap support at these major splits that highlights the presence of strong phylogenetic signal afforded by the utilization of nearly 400 universal proteins. Further, the paraphyletic clustering of the genus *Sphingomonas* underscores the overlooked diversity of *Sphingomonas* that may benefit from further sub-classification in addition to its current classification into three well-known genera e.g., *Novosphingobium, Sphingopyxis,* and *Sphingobium* and the recently proposed genus, *Sphingosinicella* (Takeuchi et al., 2001).

The phylogenetic clustering of sphingomonad LuxR homologs shows no evidence of phylogeny congruence i.e., inconsistent clustering of LuxR homologs from members of the same genus. Given that a majority of the sphingomonad LuxR homologs form a large clade among themselves, the incongruence with the newly constructed species phylogeny (**Figure 2**) can be explained by a combination of horizontal gene transfer and gene duplication within the *Sphingomonadaceae* family followed by speciation as proposed previously (Lerat and Moran, 2004). Interestingly, four sphingomonad LuxR homologs formed a monophyletic clade that is sister group to TraR and RaiR. The distant relationship between the this sphingomonad LuxR clade and the major sphingomonad LuxR homologs clade coupled with the localization of both *traR* and *raiR* genes on the plasmid e.g., Ti plasmid and non-symbiotic plasmid respectively (Piper et al., 1993; Gray et al., 1996; Oger and Farrand, 2002) suggest the acquisition of these four *luxR* homologs via plasmid-mediated horizontal gene transfer. This warrants future work focusing on the identification of plasmid-coded *luxR* homolog through plasmid isolation and sequencing to confirm the origin of the distant sphingomonad *luxR* homologs.

Five out of six of the sphingomonad LuxR homologs that are more closely related to PAB LuxR solos than the rest of the LuxR homologs (**Figure 3**) appear to share one of the two major signature e.g., Y61W in PAB solos (**Figure 4**). Recent cartography analysis of the ligand-binding sites of the LuxR homologs has demonstrated that Y61 residue is directly involved in ligand binding (in addition to W57, D70, and W85) (Covaceuszach et al., 2013). Therefore, substitution at Y61 in these specific sphingomonad LuxR homologs is a strong indicator of their inability to bind to AHL. Three dimensional structure modeling of these proteins followed by comparison of binding/active sites regarding substrate preference(s) will shed lights into the protein characteristic of these atypical sphingomonad LuxR homologs. Recently, a LuxR-homolog from *Photorhabdus* that has some substitutions in the conserved 9 aa residues in LuxR homologs was shown to bind to a bacterial-produced pyrone instead of AHLs or plant exudates (Brachmann et al., 2013; Brameyer et al., 2014). It should be noted

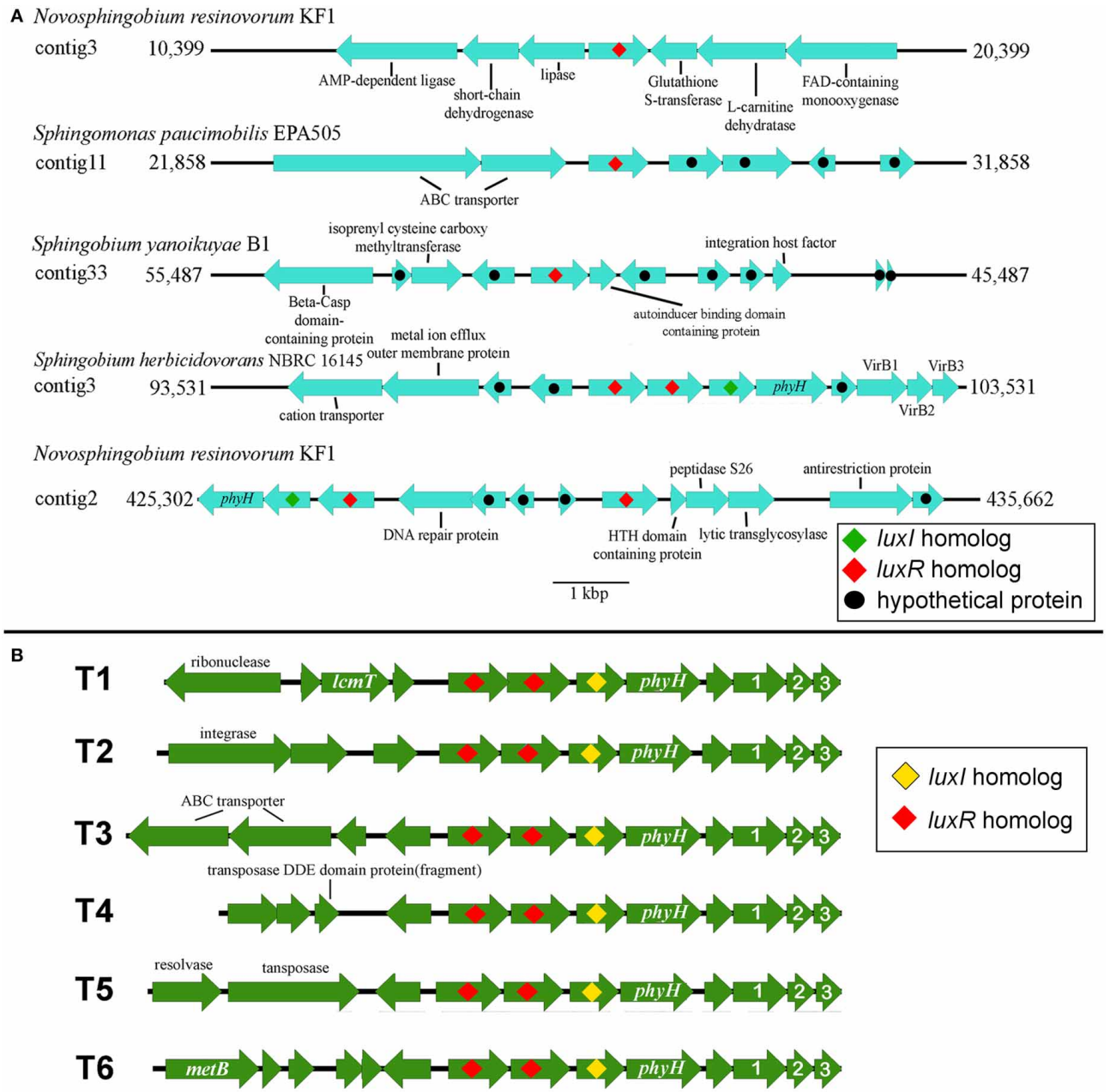

FIGURE 5 | Gene organization of *luxR* Solos and *luxR-luxR-luxI* in the four whole genome sequenced sphingomonads. (A) EasyFig generated linear comparison of *luxR* solos in the genomic region of selected sphingomonads. Approximately 5000 bp of genomic region flanking the *luxR* solos is shown. **(B)** Gene orientation of the identified convergent double *luxR, luxI* group and its gene neighborhood variation. T1 to T6 denotes different gene neighborhoods identified in the convergent double *luxR, luxI*. Arrows without label represent gene coding for hypothetical protein. Please see **Table 2** for topology variation present in sphingomonad genomes. The numbers "1," "2," and "3" represent *virB1*, *virB2*, and *virB3* genes respectively. Additional abbreviations include: *lcmT*, Isoprenylcysteine carboxyl methyltransferase; *metB*, Cystathionine gamma-synthase; *phyH*, phytanoly dioxygenase.

that the structural-activity relationship(s) of LuxR solos is beyond the scope of this study.

The occurrence of two *luxR* homologs in tandem is not novel in the realm of alpha-bacteria and has been previously reported in the genus *Roseobacter*, noted as topology N (Cude and Buchan, 2013). However, the gene neighborhood of the double *luxR* in various sphingomonads is significantly different from topology N to justify the proposal of a new topology that we will coin as topology T. Topology T represents the convergently oriented *luxR-luxR-luxI-phyH*-X-*virB1-virB2-virB3* topology whereby X denotes gene coding for hypothetical protein. It is also worth noting that one or more mobile elements are present upstream of the

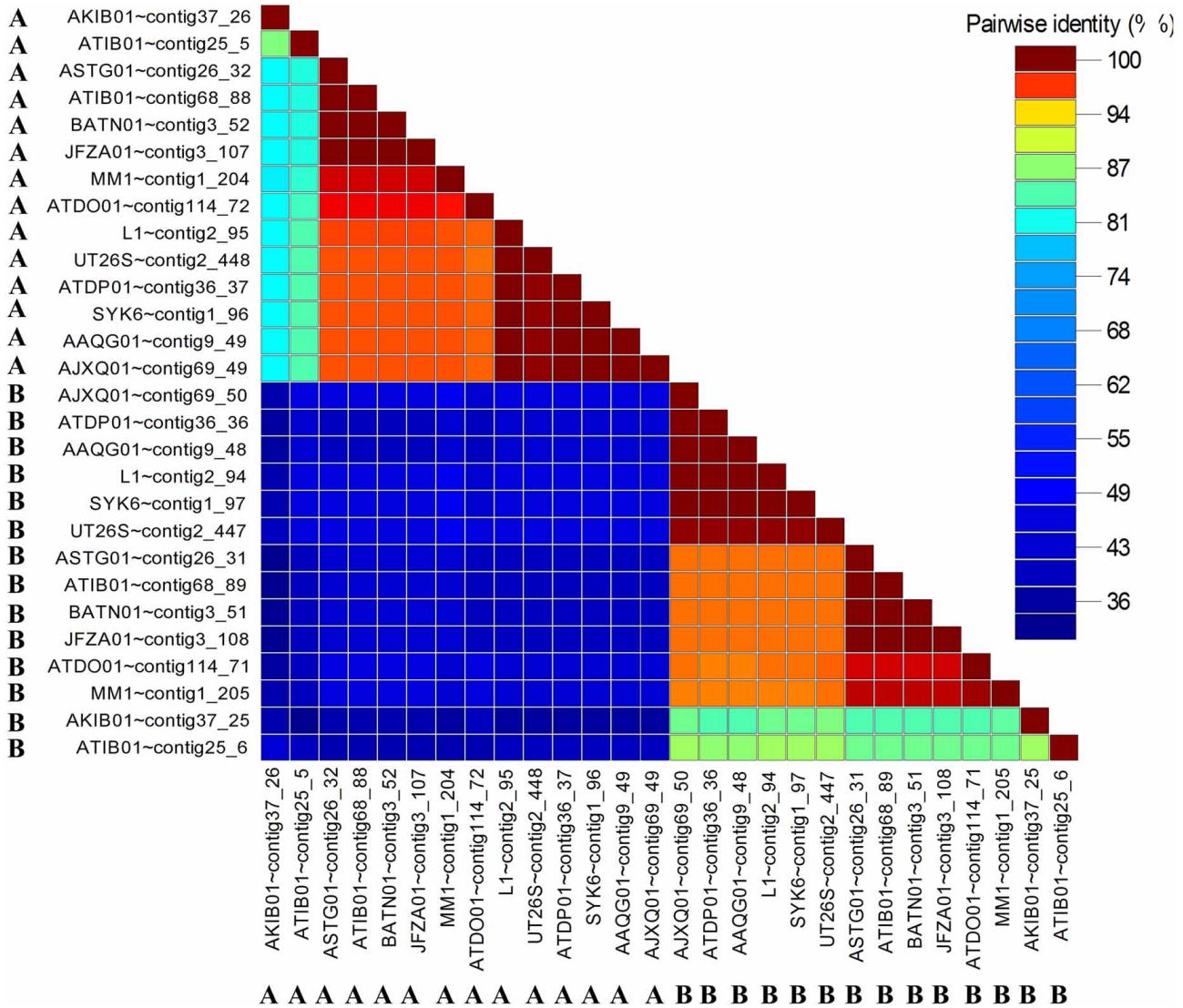

FIGURE 6 | Pairwise identity matrix of identified convergent luxR-luxR-luxI (LuxR-A and LuxR-B) in sphingomonads. The letters A and B correspond to different partner *luxRs* in the *luxR-luxR-luxI*. The genes coding for LuxR homologs with the same symbol and color were convergently oriented with respect to each other.

double *luxR* in three out of the six topology variants, indicating past transposition event(s) and/or transposition potential of the gene cluster.

The low pairwise identity between members of the same LuxR double group (**Figure 6**) support the distantly shared ancestry as observed in the LuxR phylogenetic tree (**Figure 3**, shaded in pink). Furthermore, the low pairwise identity between members of the same LuxR double group and retention of convergent double *luxR* in the genomes of several *Sphingobium* strains suggests that the sphingomonad LuxR duplicate has undergone sufficient functional divergence which may correlate to the evolution of the organism to be more competitive regarding niche adaptation. The presence of the complete LuxR signature domains in both members of the same convergent double LuxR group suggests the retention of the core LuxR function with perhaps dissimilar substrate range and/or DNA-binding region that warrants future protein characterization and transcription study.

In addition to harboring the newly described QS gene circuit arrangement, some members of the currently sequenced genus *Sphingobium* exhibit another interesting feature of QS signaling, e.g., presence of *luxI* solos. The assignment of LuxI solos based on the presence of signature amino acid residues in canonical LuxI homologs and the absence of unassociated *luxR* in the vicinity of its protein coding gene (**Figure 7** and **Table 3**) provide strong evidence that the LuxI solos identified in both *Sphingobium* sp. KK2 and *Sphingobium chinhatense* IP26 are authentic. The presence of a gene coding for a putative N-terminal truncated LuxR-like protein immediately upstream of the *luxI* solo gene in strain IP26 is suggestive of the *luxI* solo previously being part of a functional *luxI/R* pair instead of having been acquired independently.

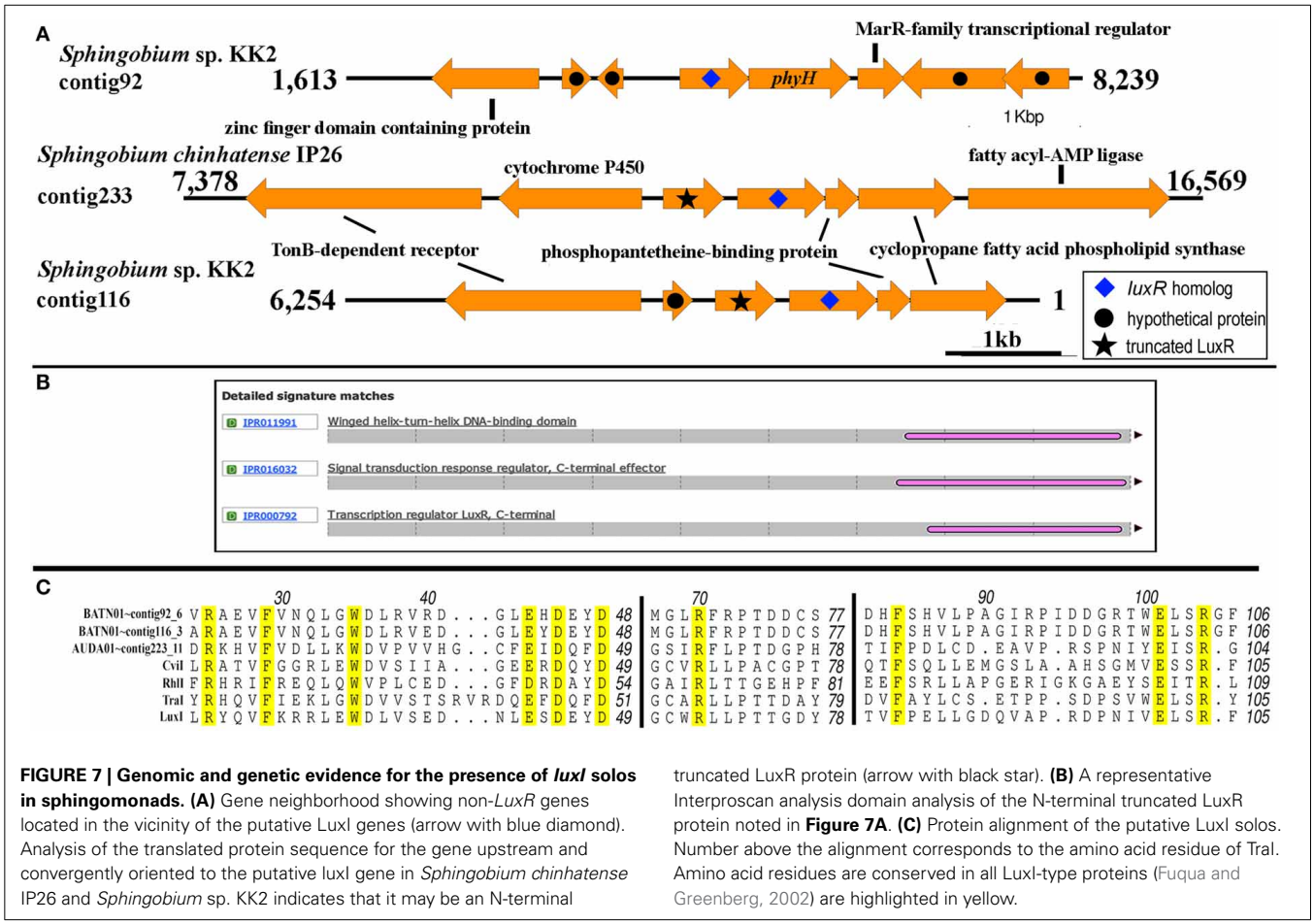

FIGURE 7 | Genomic and genetic evidence for the presence of *luxI* solos in sphingomonads. (A) Gene neighborhood showing non-*LuxR* genes located in the vicinity of the putative LuxI genes (arrow with blue diamond). Analysis of the translated protein sequence for the gene upstream and convergently oriented to the putative luxI gene in *Sphingobium chinhatense* IP26 and *Sphingobium* sp. KK2 indicates that it may be an N-terminal truncated LuxR protein (arrow with black star). **(B)** A representative Interproscan analysis domain analysis of the N-terminal truncated LuxR protein noted in **Figure 7A**. **(C)** Protein alignment of the putative LuxI solos. Number above the alignment corresponds to the amino acid residue of TraI. Amino acid residues are conserved in all LuxI-type proteins (Fuqua and Greenberg, 2002) are highlighted in yellow.

However, this may not be the case for another *luxI* solo located in contig92 of *Sphingobium* sp. KK2 with more than 800 bp of an upstream non-protein coding region. Recently a detailed study of LuxR-LuxI type QS network in *Ruegeria* sp. KLH11 (Zan et al., 2012) confirmed the presence of a functional LuxI solo, SscI and demonstrated that SscI and a paired-LuxI homolog, SsbI, produced the same AHLs e.g., 3-OH-C14:1-HSL and 3-OH-C14-HSL that indirectly affect QS-dependent gene regulation by another LuxI/R pair homologs, SsaI/R. Given the presence of one or more *luxI/R* pairs in the strain KK2 and IP26 genomes, it is tempting to speculate that a similar level of QS network complexity may operate in both *Sphingobium* strains.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00188/abstract

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Identification of *sdiA*-regulated genes in a mouse commensal strain of *Enterobacter cloacae*

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Many bacteria determine their population density using quorum sensing. The most intensively studied mechanism of quorum sensing utilizes proteins of the LuxI family to synthesize a signaling molecule of the acylhomoserine lactone (AHL) type, and a protein of the LuxR family to bind AHL and regulate transcription. Genes regulated by quorum sensing often encode functions that are most effective when a group of bacteria are working cooperatively (e.g., luminescence, biofilm formation, host interactions). Bacteria in the *Escherichia, Salmonella, Klebsiella,* and *Enterobacter* genera do not encode an AHL synthase but they do encode an AHL receptor of the LuxR family, SdiA. Instead of detecting their own AHL synthesis, these organisms use SdiA to detect the AHLs synthesized by other bacterial species. In this study, we used a genetic screen to identify AHL-responsive genes in a commensal *Enterobacter cloacae* strain that was isolated from a laboratory mouse. The genes include a putative type VI secretion system, *copA* (a copper transporter), and *fepE* (extends O-antigen chain length). A new transposon mutagenesis strategy and suicide vectors were used to construct an *sdiA* mutant of *E. cloacae*. The AHL-responsiveness of all fusions was entirely *sdiA*-dependent, although some genes were regulated by *sdiA* in the absence of AHL.

Keywords: SdiA, LuxR solo, *Enterobacter*, regulon, acylhomoserine lactone, transposon mutagenesis, suicide vector, quorum sensing

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Introduction

Many bacteria monitor their population density (often called quorum sensing) as one of many inputs to gene regulation. The genes regulated by quorum sensing are often those that provide maximal benefit when expressed simultaneously throughout a population (Schuster et al., 2013). The classic example is the expression of luciferase by *Vibrio fischeri*, in which luminescence is most effective when the entire population participates (Hastings and Greenberg, 1999). Other examples include the expression of conjugation functions, biofilm formation, or various aspects of host interaction (Rutherford and Bassler, 2012).

The most intensively studied type of quorum sensing utilizes a protein of the LuxI family to synthesize a signaling molecule of the acylhomoserine lactone (AHL) type (Schaefer et al., 1996). Accumulation of the AHL results in its detection by a transcription factor of the LuxR family. In the

case of *V. fischeri*, the LuxR-AHL complex binds upstream of the *luxICDABEG* operon to activate the expression of luciferase (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995). Thus, the population of bacteria cooperate to create light and illuminate their host, the squid *Euprymna scolopes* (Chun et al., 2008; Miyashiro and Ruby, 2012).

Homologous LuxI/LuxR regulatory systems have been identified in numerous Proteobacteria (Case et al., 2008). Some bacteria that live in mammalian intestinal tracts encode AHL synthases, although AHLs themselves have not yet been demonstrated to be present in this environment (Swearingen et al., 2012). Interestingly, a LuxR homolog, SdiA, has been

TABLE 1 | Strains and plasmids.

Strain or plasmid	Genotype	Source or references
STRAINS		
14028	Wild-type *Salmonella enterica* subspecies *enterica* serovar Typhimurium	American type culture collection
AL4001	*E. coli* BA4000 *gadW*4001::mTn5*luxkan2*	Dyszel et al., 2010b
BA4000	Nal^R resistant mutant of *E. coli* BW25113	Dyszel et al., 2010b
BW20767	*E. coli leu*-63::IS10 *recA*1 *creC*510 *hsdR*17 *endA*1 *zbf*-5 *uidA*(Δ*MluI*)::*pir*+ *thi* RP4-2-*tet*::Mu-1*kan*::Tn7	Metcalf et al., 1996
JLD400	*Enterobacter cloacae* mouse isolate	Ali et al., 2014
JLD401	*Enterobacter cloacae* mouse isolate, Nal^R	This study
JLD500	JLD401 ENC_40870::mTn5*luxkan2*	This study
JLD501	JLD401 ENC_10940::mTn5*luxkan2*	This study
JLD502	JLD401 ENC_30820::mTn5*luxkan2*	This study
JLD504	JLD401 ENC_11220IG::mTn5*luxkan2*	This study
JLD505	JLD401 ENC_07270::mTn5*luxkan2*	This study
JLD506	JLD401 ENC_02820::mTn5*luxkan2*	This study
JLD507	JLD401 ENC_22440::mTn5*luxkan2*	This study
JLD508	JLD401 ENC_22440::mTn5*luxkan2*. ENC_22440 is a *copA* homolog	This study
JLD509	JLD401 ENC_30820::mTn5*luxkan2*	This study
JLD511	JLD401 ENC_10940::mTn5*luxkan2*	This study
JLD513	JLD401 ENC_40870::mTn5*luxkan2*	This study
JLD514	JLD401 ENC_40870::mTn5*luxkan2*	This study
JLD515	JLD401 ENC_14970IG::mTn5*luxkan2*	This study
JLD516	JLD401 ENC_14970IG::mTn5*luxkan2*	This study
JLD517	JLD401 ENC_40870::mTn5*luxkan2*	This study
JLD518	JLD401 ENC_40870::mTn5*luxkan2*	This study
JLD519	JLD401 ENC_40870::mTn5*luxkan2*	This study
JLD800	AL4001 *sdiA*271::*cam*	Dyszel et al., 2010b
ASD401	JLD401 *sdiA32*::mTn5-FC	This study
ASD500	JLD401 ENC_40870::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD501	JLD401 ENC_10940::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD502	JLD401 ENC_30820::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD504	JLD401 ENC_11220IG::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD505	JLD401 ENC_07270::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD506	JLD401 ENC_02820::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD508	JLD401 ENC_22440::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD515	JLD401 ENC_14970IG::mTn5*luxkan2 sdiA32*::mTn5-FC	This study
ASD708	BW20767 + pASD708	This study
PLASMIDS		
pUT mTn5lux kan2	Suicide vector, ori R6K, mini-Tn5 Km2 *luxCDABE* transposon, mob+ (RP4) Amp^R Kan^R	Winson et al., 1998
pMO197	Suicide vector, *oriT oriV sacB* TcR, *ccdB*, Tet^R	This study
pMO704	Suicide vector, *oriT oriV sacB* TcR, *ccdB*, Amp^R	This study
pASD704	pCR8/GW/TOPO, *E. cloacae sdiA*	This study
pASD706	pCR8/GW/TOPO, *E. cloacae sdiA32*::mTn5-FC	This study
pASD708	pMO197, *E. cloacae sdiA32*::mTn5-FC	This study
pCR8/TOPO/GW	Cloning vector, $Spec^R$	Invitrogen
pJNS25	P_{srgE}-*luxCDABE* p15A ori Tet^R	Smith and Ahmer, 2003

TABLE 2 | Oligonucleotides.

Name	Sequence	Description
BA247	GAGTCATTCAATATTGGCAGGTAAACAC	Binds within *luxC* coding region, used for sequencing insertion site of mTn*5luxkan2* transposon
BA1090	GAATGTATGTCCTGCGTCTTGAGTA	Binds within *luxC* coding region; used for sequencing insertion site of mTn*5luxkan2* transposon
BA2276	CAGTAAGTATGAGGGATATAGACTTTTTCACCTG	Binds upstream of *E. cloacae sdiA* gene
BA2277	GAGCACACCTGAATTTGCCACTGCCGAGAATAAC	Binds downstream of *E. cloacae sdiA* gene
BA2219	CTGTCTCTTATACACATCTGTGTAGGCTGGAGCTGCTTC	Binds the P1 region of pCLF4.pCLF3, pKD3, pKD4 for amplification of the FRT-cam/kan-FRT cassette with ME sequences
BA2220	CTGTCTCTTATACACATCTCATATGAATATCCTCCTTAG	Binds the P2 region of pCLF4.pCLF3, pKD3, pKD4 for amplification of the FRT-cam/kan-FRT cassette with ME sequences
BA1598	GATCTTCCGTCACAGGTAGG	Binds within the chloramphenicol resistance marker (C2)
BA2343	GCGTTCAATTTGCTCCAGATGCCGCTTCTGG	Binds upstream of *E. cloace sdiA* gene
IPCRF	TTTTGGTGATAATAGTGTTTACCTGCC	Forward primer for inverse PCR with miniTn5
IPCRR	TTTTTTTAGTCATACGTATCCTCCAAGCC	Reverse primer for inverse PCR with miniTn5
BA2447	GAAAAGGATAGCACAGGATCTGAGAAAGG	Primer binds within ENC_14960; used with BA1090 for identification of insertion site
BA2448	GCCACAGCGTGAATTGCAGGTGCTGGATGCGC	Primer binds within ENC_40870; used with BA1090 for identification of insertion site

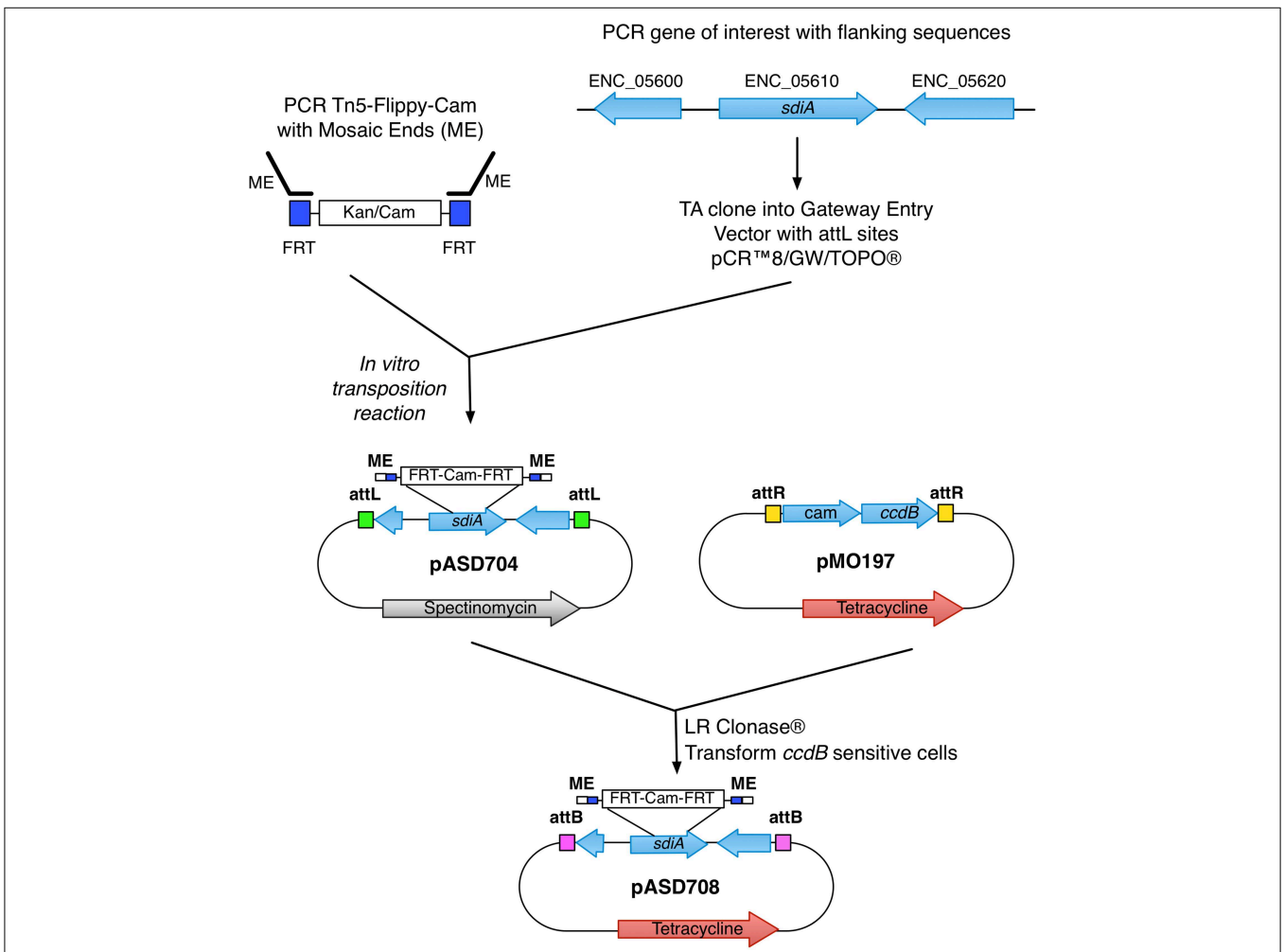

FIGURE 1 | Generation of the *sdiA* mutant in *E. cloacae*. Schematic of the construction and generation of the *sdiA* mutant in *E. cloacae* using the mTn5-FC and novel suicide vector pMO197.

identified in the *Enterobacteriaceae*, including the genera *Escherichia*, *Salmonella*, *Enterobacter, Klebsiella,* and *Citrobacter*. However, these organisms do not encode a cognate AHL synthase (Smith and Ahmer, 2003; Sabag-Daigle and Ahmer, 2012). Instead, it has been shown that SdiA of *E. coli* and *Salmonella enterica* detect the AHLs produced by other species of bacteria (Michael et al., 2001; Smith and Ahmer, 2003; Dyszel et al., 2010a,b; Sperandio, 2010a; Soares and Ahmer, 2011; Sheng et al., 2013). In *Salmonella enterica* serovar Typhimurium, SdiA positively regulates two loci, (1) the *rck* (resistance to complement killing) operon located on the virulence plasmid, pSLT (Ahmer et al., 1998; Michael et al., 2001; Smith and Ahmer, 2003; Abed et al., 2014); and (2) *srgE* (*sdiA*-regulated gene), a single gene horizontal acquisition that encodes an effector

TABLE 3 | AHL-responsive, *sdiA*-dependent fusions identified in *E. cloacae.*

			***sdiA*-dependent, AHL-dependent fold change in reporter expression[a,b]**					
Gene Hits	**Insertion Site**	**Fusions**	**37°C shaking**	**30°C shaking**	**37°C standing**	**30°C standing**	**37°C motility agar**	**30°C motility agar**
ENC_40870	Hypothetical protein	JLD500						
		JLD513	67.7	**317.4**	32.2	49.9	128.0	236.2
		JLD514						
		JLD517	5.7	2.7	9.3	3.4	**9.9**	2.2
		JLD518						
		JLD519						
ENC_10940	Hypothetical protein	JLD501	45.0	28.6	48.8	13.4	41.9	**50.9**
		JLD511	**27.4**	9.2	19.3	8.6	9.1	8.1
ENC_30820	*fepE*	JLD502	46.9	**58.2**	41.6	25.9	40.6	50.7
		JLD509	**24.0**	7.4	13.0	8.2	5.7	4.3
ENC_11220	Intergenic region	JLD504	72.4	82.5	223.8	45.7	**15975.8**	6912.2
			22.4	33.6	41.9	18.2	**178.6**	37.3
ENC_07270	Hypothetical protein in a putative type VI	JLD505	44.6	82.3	90.4	79.5	**233.1**	155.3
	secretion system operon		1.9	2.0	**3.4**	1.3	2.5	2.0
ENC_02820	Phage integrase	JLD506	13.1	8.6	**14.7**	12.0	5.7	6.6
ENC_22440	*copA*	JLD507 JLD508	8.6	5.1	**9.3**	5.9	2.1	8.6

[a] *Top number is the largest fold change in sdiA-dependent expression throughout the time course for each fusion. The bottom number is the largest fold change in AHL-dependent expression throughout the time course for each fusion. The highest sdiA-dependent or AHL-dependent fold change is indicated for each fusion in bold.*

[b] *For fusions ENC_02820 and ENC_22440 only the sdiA-dependent fold change is displayed (calculated from the cultures that included AHL). Neither fusion exhibited statistically significant AHL-dependent changes in expression.*

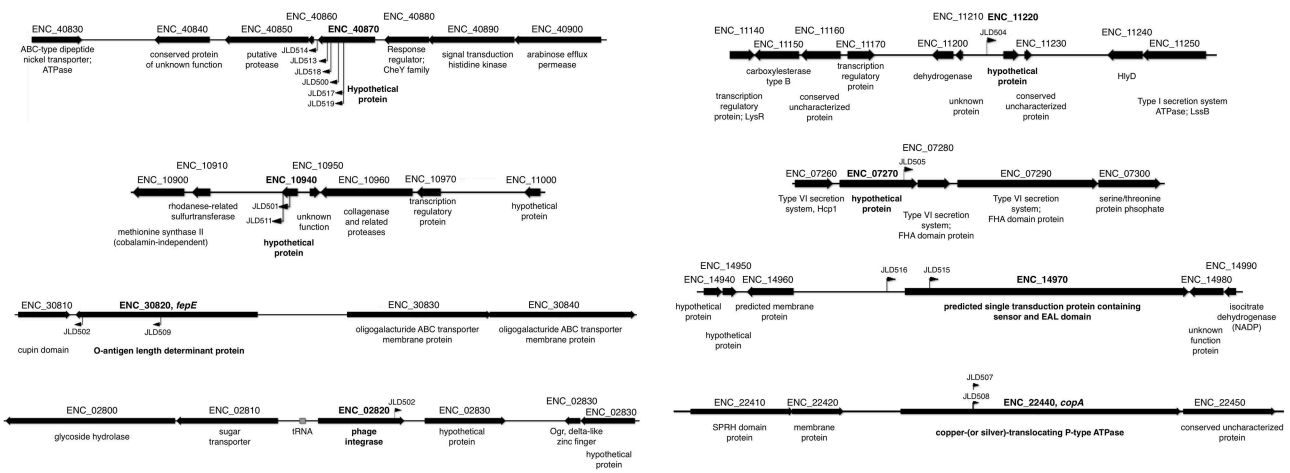

FIGURE 2 | Insertion points of AHL-regulated genes in *E. cloacae*. Diagram of the location of AHL-regulated mTn5-*luxCDABE* insertions. The mTn5-*luxCDABE* insertion points and orientation are indicated by flags.

protein that is secreted by type III secretion system 2 (T3SS2) (Smith and Ahmer, 2003; Habyarimana et al., 2014). SdiA in EHEC functions to activate expression of genes involved in the glutamate-dependent acid resistance system (*gad)* and has also been found to repress the expression of flagella genes and the enterocyte effacement (LEE) locus (Van Houdt et al., 2006; Lee et al., 2008; Nikaido et al., 2008; Dyszel et al., 2010b; Hughes et al., 2010; Nguyen and Sperandio, 2012; Nguyen et al., 2013; Sheng et al., 2013). Competition assays in cattle of wild-type EHEC and an isogenic *sdiA* mutant indicate a defect of the *sdiA* mutant in colonization of rumen and the recto-anal junction (RAJ) (Hughes et al., 2010; Sheng et al., 2013). This phenotype was shown to correlate with lack of *gad* activation in the rumen and a failure to repress the LEE locus in the RAJ in the absence of *sdiA* (Hughes et al., 2010; Nguyen et al., 2013). In a plant-associated isolate of *Enterobacter*, an *sdiA* mutation derepresses the *csgBAC* operon leading to an overproduction of curli fimbrae (Shankar et al., 2012). The *sdiA* mutant has increased root colonization and biofilm formation correlating with the increased expression of curli adhesion molecules (Shankar et al., 2012).

We wanted to study the role of *sdiA* in a commensal member of the murine microbiota. Laboratory strains of *E. coli* K-12 and EHEC do not colonize mice well. Commensal strains of *E. coli* recovered from mice are very rare in the literature, and during microbiome studies *E. coli* has been found to be rare or non-existent in mice depending on strain and vendor. In this study, we performed a genetic screen to identify AHL-responsive genes of an *Enterobacter cloacae* strain that was isolated from laboratory mice (Ali et al., 2014). We utilized a transposon to create chromosomal *luxCDABE* fusions in a wild-type background, with *sdiA* at its natural position in the chromosome. We screened these fusions to identify those that are AHL-responsive. A new suicide vector and novel mutagenesis strategy were then used to mutate *sdiA* in each fusion strain. The AHL-responsiveness of all of the fusions was entirely *sdiA*-dependent, but a few genes were regulated by *sdiA*, including one gene repressed by *sdiA*, largely in the absence of AHLs. This ligand-independent activity of SdiA has important implications for our understanding of the role of this LuxR solo (Patankar and Gonzalez, 2009; Subramoni and Venturi, 2009).

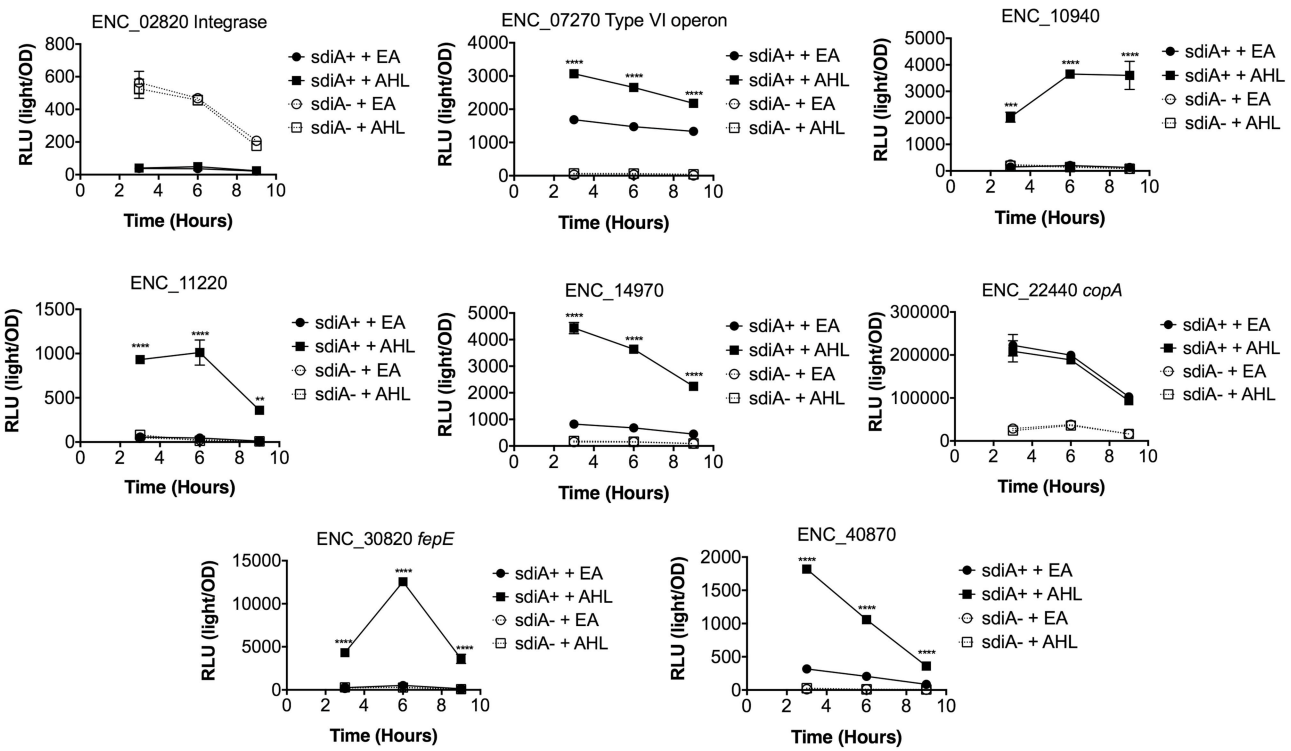

FIGURE 3 | Regulation of AHL-regulated genes in *E. cloacae* in LB broth at 37°C shaking. Expression of mTn5*luxCDABE* fusion strains in either the wild-type (closed symbols) or *sdiA* mutant backgrounds (open symbols) with either 1 μM oxoC6 (squares) or 0.1% ethyl acetate (EA) solvent control (circles) in LB broth. Luminescence is reported in relative light units (light/OD_{590}). Data was collected at 3, 6, and 9 h time points. All data points are the average of three technical replicates and error bars indicate SEM. This is a representative graph of three independent biological replicates. The statistical significance of AHL-dependent changes in gene expression are designated with *p*-values (≤0.05 = *, ≤0.005 = **, ≤0.0005 = ***, ≤0.00005 = ****). The *sdiA*-dependent changes in gene expression were larger than the AHL-dependent changes and were statistically significant (p=0.00005) for all fusions in all growth conditions for at least one time point. The $sdiA^+$ and *sdiA* mutant strains for each fusion are indicated in parentheses: ENC_02820 (JLD506/ASD506), ENC_07270 (JLD505/ASD505), ENC_10940 (JLD501/ASD501), ENC_11220 (JLD504/ASD504), ENC_14970 (JLD515/ASD515), ENC_22440 (JLD508/ASD508), ENC_30820 (JLD502/ASD502), and ENC_40870 (JLD500/ASD500).

Materials and Methods

Bacterial Strains and Media

Bacterial strains are listed in **Table 1**. Bacteria were routinely grown in Luria-Bertani (LB) broth or on LB agar unless otherwise stated. LB motility agar was also used (LB broth + 0.3% agar). Chloramphenicol (cam), kanamycin (kan), tetracycline (tet), ampicillin (amp), and nalidixic acid (nal) were used at 20, 50, 10, 200, and 50 μg/ml, respectively. *N*-(3-oxo-hexanoyl)-L-homoserine lactone (oxoC6) was obtained from Sigma-Aldrich and dissolved in ethyl acetate that had been acidified by the addition of 0.1 ml glacial acetic acid per liter (EA) (Pearson et al., 1994). The stock concentration of oxoC6 was 1 mM and it was used at a final concentration of 1 μM. Solvent controls were performed by using EA alone at 0.1%.

Constructing Transposon Based Luciferase Fusions and Screening for AHL Responsiveness in *E. cloacae*

Transposon mutagenesis was performed by mating BW20767+pUTmTn*5luxkan2* (Winson et al., 1998) and JLD401, a spontaneous nalidixic acid resistant mutant of *Enterobacter cloacae* strain JLD400. The two strains were plated on LB plates at 37°C overnight. Cells were then scraped with sterile PBS and plated on LB kan nal. 10,000 single colonies were patched into 96-well plates with 0.3% motility agar in the presence of oxoC6 or the solvent control, EA, at 37°C for 9 h. Plates were read with a Wallac Victor3 (Perkin Elmer) plate reader. Those wells that had greater than 3-fold difference after 9 h were streaked for isolation on LB kan nal plates at 37°C overnight. For confirmation, one colony from each plate was inoculated into LB kan nal broth or 0.3% motility agar in 96-well format in the presence of oxoC6, or the solvent control, EA. Plates were read on the Victor plate reader every 3 h. Those fusions that demonstrated greater than 2.5-fold AHL-dependent induction after 9 h were saved for future studies.

Identification of Transposon Insertion Sites

Genomic DNA was isolated from overnight cultures of the transposon insertion mutants using the GenElute™ Bacterial Genomic DNA Isolation kit (Sigma Aldrich, St. Louis, MO). The transposon insertion site in the genomic DNA was sequenced

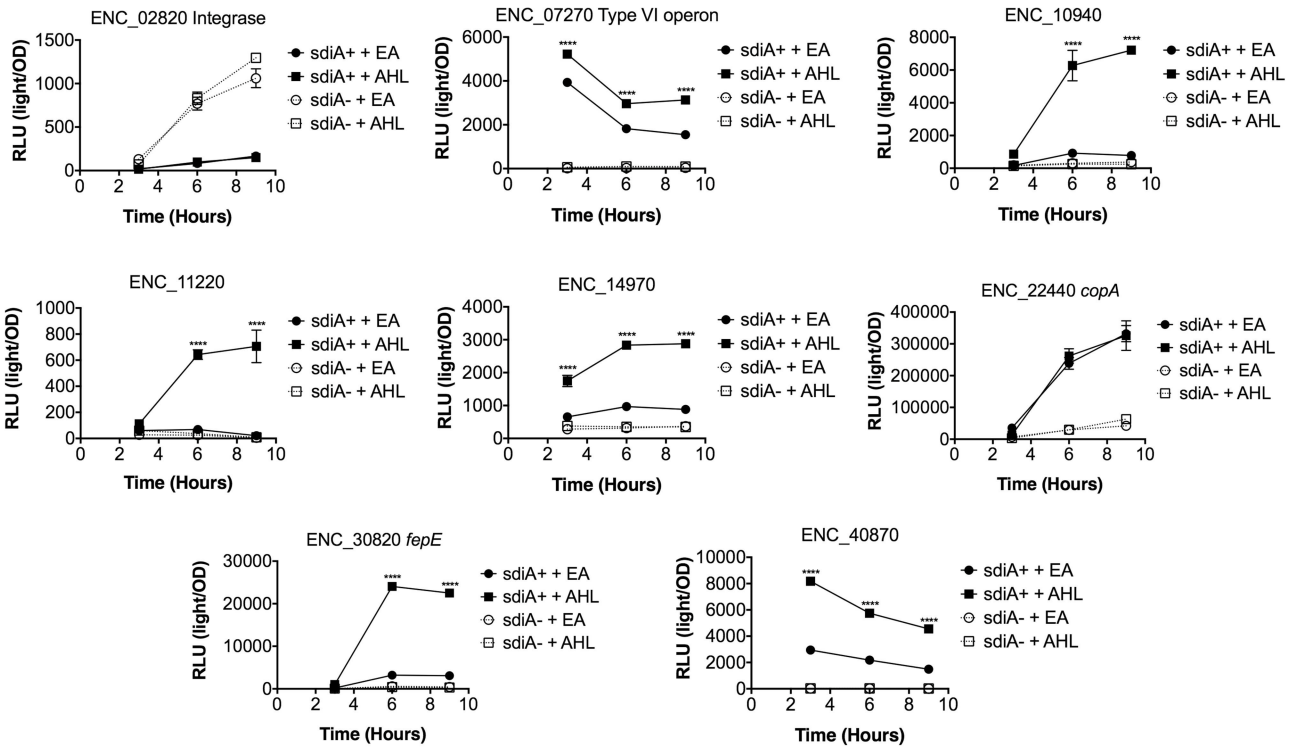

FIGURE 4 | Regulation of AHL-regulated genes in *E. cloacae* in LB broth at 30°C shaking. Expression of mTn*5luxCDABE* fusion strains in either the wild-type (closed symbols) or *sdiA* mutant backgrounds (open symbols) with either 1 μM oxoC6 (squares) or 0.1% ethyl acetate (EA) solvent control (circles) in LB broth. Luminescence is reported in relative light units (light/OD_{590}). Data was collected at 3, 6, and 9 h time points. All data points are the average of three technical replicates and error bars indicate SEM. This is a representative graph of three independent biological replicates. The statistical significance of AHL-dependent changes in gene expression are designated with *p*-values (≤0.05 = *, ≤0.005 = **, ≤0.0005 = ***, ≤0.00005 = ****). The *sdiA*-dependent changes in gene expression were larger than the AHL-dependent changes and were statistically significant (*p*=0.00005) for all fusions in all growth conditions for at least one time point. The *sdiA*$^+$ and *sdiA* mutant strains for each fusion are indicated in parentheses: ENC_02820 (JLD506/ASD506), ENC_07270 (JLD505/ASD505), ENC_10940 (JLD501/ASD501), ENC_11220 (JLD504/ASD504), ENC_14970 (JLD515/ASD515), ENC_22440 (JLD508/ASD508), ENC_30820 (JLD502/ASD502), and ENC_40870 (JLD500/ASD500).

using Sanger sequencing with two different primers, BA247 and BA1090 (**Table 2**). Both sequencing primers bind within the *luxC* coding region oriented out of the transposon. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). DNA sequencing was performed by the Plant Microbe Genomics Facility at The Ohio State University. The sequence adjacent to the transposon insertion site was used for BLASTN searches using the BLAST program at the National Center for Biotechnology Information (NCBI).

Not all insertion points were identified using Sanger genomic DNA sequencing. The rest were identified using inverse PCR. Genomic DNA was digested with *Nla*III (NEB) for 3 h at 37°C. The enzyme was inactivated for 20 min at 65°C, then T4 DNA ligase (NEB) was added to the digestion reaction in a total reaction volume of 200 μL at 16°C overnight. The ligation reaction was purified using the QiaQuick PCR purification kit (Qiagen), digested with *Xmn*I for 3 h at 37°C, then heat inactivated at 65°C for 20 min. This digest was used in a PCR reaction using the primers IPCRF and IPCRR (**Table 2**) using *Taq* DNA polymerase (NEB). The PCR product was sequenced using the IPCRF primer at the Plant Microbe Genomics Facility at The Ohio State University.

Liquid and Motility Agar Assays for *Lux* Fusions

Strains were grown in LB kan for *sdiA*+ strains or LB kan cam for *sdiA* mutants, and grown at 37°C shaking overnight. They were then subcultured 1:100 in triplicate into either LB broth or LB motility agar containing the appropriate antibiotics and either 1 μM oxoC6 or 0.1% EA as the solvent control and then placed in the well of a black clear bottom 96-well plate. The plate was grown with shaking at 37°C and time points were taken by placing the 96-well plate in the Wallac Victor plate reader. For broth cultures, both OD_{590} and luminescence were measured. For the motility agar assays, only the luminescence was measured. For the AHL concentration sensitivity assay, an *sdiA*-regulated fusion was assayed in LB broth or motility agar with 10-fold dilutions of either oxoC6 or oxoC8 starting at a concentration of 1 μM. AHL-dependent changes in gene expression were analyzed using a Two-Way ANOVA over the time course. *P*-values were marked accordingly (≤0.05 = *, ≤0.005 = **, ≤0.0005 = ***, ≤0.00005 = ****). The *sdiA*-dependent changes in gene expression were larger than the AHL-dependent changes and were statistically significant ($p \leq 0.00005$) for all fusions in all growth conditions for at least one time point.

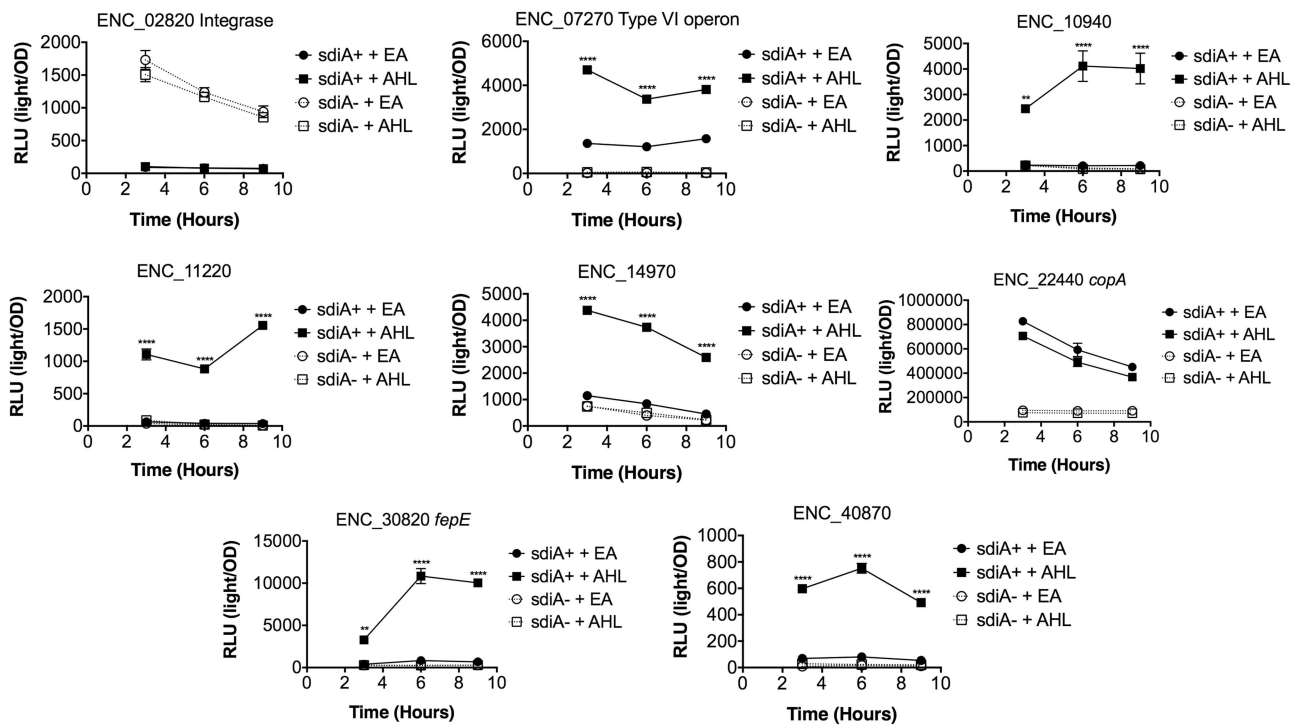

FIGURE 5 | Regulation of AHL-regulated genes in *E. cloacae* in LB broth at 37°C standing. Expression of mTn*5luxCDABE* fusion strains in either the wild-type (closed symbols) or *sdiA* mutant backgrounds (open symbols) with either 1 μM oxoC6 (squares) or 0.1% ethyl acetate (EA) solvent control (circles) in LB broth. Luminescence is reported in relative light units (light/OD_{590}). Data was collected at 3, 6, and 9 h time points. All data points are the average of three technical replicates and error bars indicate SEM. This is a representative graph of three independent biological replicates. The statistical significance of AHL-dependent changes in gene expression are designated with *p*-values (≤0.05 = *, ≤0.005 = **, ≤0.0005 = ***, ≤0.00005 = ****). The *sdiA*-dependent changes in gene expression were larger than the AHL-dependent changes and were statistically significant ($p \leq 0.00005$) for all fusions in all growth conditions for at least one time point. The *sdiA*$^+$ and *sdiA* mutant strains for each fusion are indicated in parentheses: ENC_02820 (JLD506/ASD506), ENC_07270 (JLD505/ASD505), ENC_10940 (JLD501/ASD501), ENC_11220 (JLD504/ASD504), ENC_14970 (JLD515/ASD515), ENC_22440 (JLD508/ASD508), ENC_30820 (JLD502/ASD502), and ENC_40870 (JLD500/ASD500).

Construction of an *sdiA* Mutant of *E. Cloacae*, Using a New Suicide Vector and Transposon Mutagenesis Strategy

The new suicide vectors are derivatives of pDMS197 (Edwards et al., 1998) and pGP704 (Miller and Mekalanos, 1988), respectively, that have been modified for the Gateway cloning system of Invitrogen, which uses phage attachment sites for *in vitro* recombination reactions. To modify these vectors, a blunt-ended DNA fragment (Reading Frame Cassette C) obtained from Invitrogen containing *attR*-cam^r-*ccdB*-*attR* was ligated into the *Sma*I site of pGP704 and pDMS197, resulting in pMO704 and pMO197, respectively (**Figure 1**).

With the suicide vectors completed, the *sdiA* gene of *E. cloacae* was amplified by PCR using primers BA2276 and BA2277 (**Table 2**) and cloned into the Gateway entry vector pCR8/GW/TOPO creating pASD704. This vector has *attL* sites flanking the inserted PCR product. Mutations were created in this cloned *E. cloacae sdiA* gene using *in vitro* transposon mutagenesis. We utilized a new mTn*5* derivative that we named mTn5-FC where FC stands for flippy-cam (**Figure 1**). Essentially, the DNA sequences of the optimized mosaic ends of Tn*5* (Goryshin and Reznikoff, 1998) are appended to PCR primers (BA2219 and BA2220) that are used to amplify the FRT-cam^r-FRT cassette from pCLF3 (Santiviago et al., 2009). The resulting PCR product is the transposon. To mutagenize the *sdiA* gene on pASD704, plasmid DNA and transposon DNA were mixed in the presence of transposase enzyme in an *in vitro* transposition reaction. This reaction mix was then transformed into DH5αλpir and plated on LB spec cam. The resulting colonies were screened for transposon insertions in the *sdiA* gene using PCR with primers BA2276 and BA1598. One insertion of interest, named *sdiA32*::mTn5-FC (pASD706), was then used in an "LR" cloning reaction to recombine the mutated *sdiA* gene into the new pMO197 suicide vector by transforming BW20767, creating pASD708. This vector was then mobilized into each of the *E. cloacae* strains, selecting for replacement of the wild-type *sdiA* allele with the mutant allele, which was verified by PCR using primers BA2343 and BA1598.

Results

Identification of AHL-Responsive Transcriptional Fusions in *E. cloacae*

A murine isolate of *Enterobacter cloacae* strain, JLD400, was found to be genetically tractable and sensitive to all antibiotics tested except ampicillin (Ali et al., 2014). It is easily

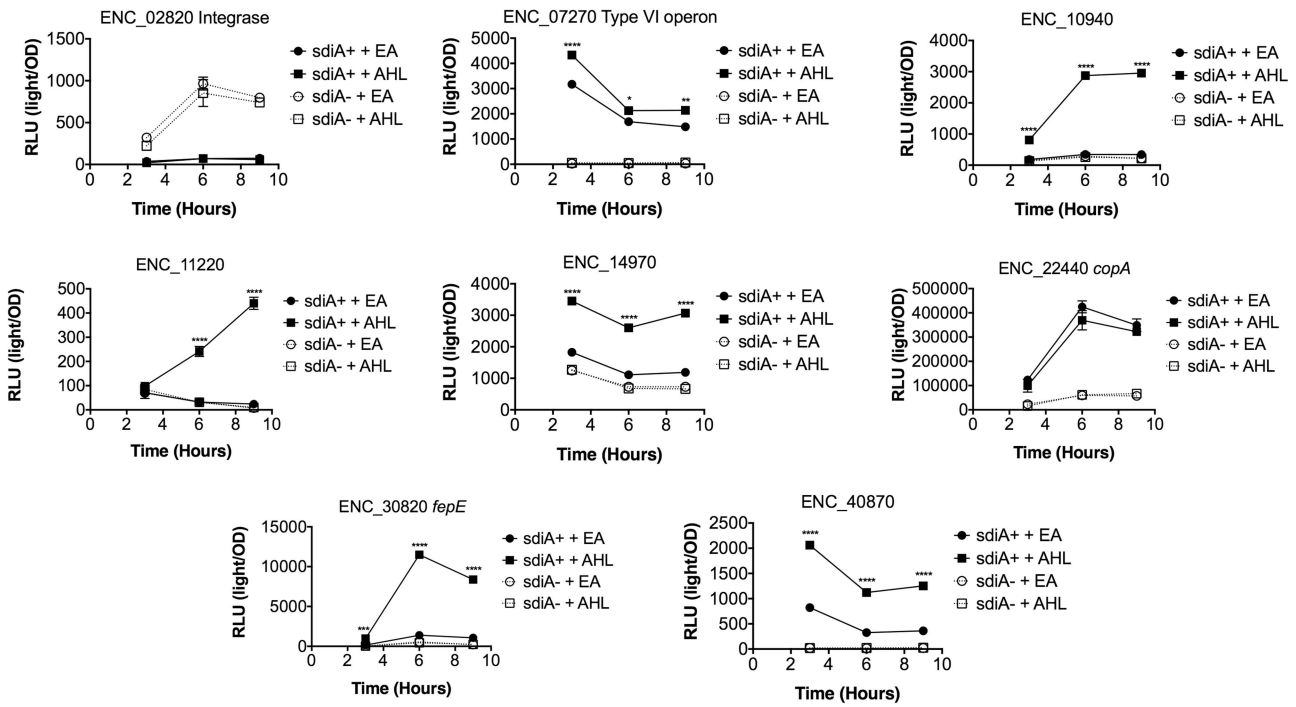

FIGURE 6 | Regulation of AHL-regulated genes in *E. cloacae* in LB broth at 30°C standing. Expression of mTn*5luxCDABE* fusion strains in either the wild-type (closed symbols) or *sdiA* mutant backgrounds (open symbols) with either 1 μM oxoC6 (squares) or 0.1% ethyl acetate (EA) solvent control (circles) in LB broth. Luminescence is reported in relative light units (light/OD_{590}). Data was collected at 3, 6, and 9 h time points. All data points are the average of three technical replicates and error bars indicate SEM. This is a representative graph of three independent biological replicates. The statistical significance of AHL-dependent changes in gene expression are designated with *p*-values (≤0.05 = *, ≤0.005 = **, ≤0.0005 = ***, ≤0.00005 = ****). The *sdiA*-dependent changes in gene expression were larger than the AHL-dependent changes and were statistically significant ($p = 0.00005$) for all fusions in all growth conditions for at least one time point. The *sdiA*$^+$ and *sdiA* mutant strains for each fusion are indicated in parentheses: ENC_02820 (JLD506/ASD506), ENC_07270 (JLD505/ASD505), ENC_10940 (JLD501/ASD501), ENC_11220 (JLD504/ASD504), ENC_14970 (JLD515/ASD515), ENC_22440 (JLD508/ASD508), ENC_30820 (JLD502/ASD502), and ENC_40870 (JLD500/ASD500).

electroporated and readily serves as a recipient in RP4-mediated conjugation. A mutant resistant to nalidixic acid was isolated after passage on LB with nalidixic acid (JLD401, **Table 1**).

In order to identify genes that are regulated in response to AHLs in *E. cloacae*, we constructed random transcriptional fusions to the luciferase genes of *Photorhabdus luminescens* (*luxCDABE)* using the transposon mTn*5-luxCDABE* (Winson et al., 1998). Previous screens for *sdiA*-regulated targets in *Salmonella* have utilized plasmid-borne *sdiA,* which bypasses the AHL requirement, but this approach has been shown to have pleotropic effects in *E. coli* (Ahmer, 2004; Dyszel et al., 2010b). Therefore, we took an alternate strategy in which we mutagenized the wild-type strain with the mTn5-*luxCDABE* transposon while *sdiA* remained in its native position in the chromosome and screened 10,000 mutants for responsiveness to synthetic AHL (oxoC6, which is detected by SdiA of *E. coli* and *Salmonella*). Seventeen insertions were identified that demonstrated an increase in luminescence greater than 2.5-fold.

The transposon insertion point was identified for all 17 insertions using either of two methods: (1) sequencing genomic DNA using two different sequencing primers that bind within the transposon sequence and are oriented outward, or (2) inverse PCR and subsequent sequencing of the product. After identification of the transposon insertion sites, a confirmatory PCR was performed using a primer within the transposon and another within the putative AHL-responsive gene. A positive PCR reaction confirms that the transposon insertion is in the correct location, but we also sequenced the resulting PCR product to further define the transposon insertion site. All 17 AHL-responsive fusions were located within 8 unique genes (**Table 3**, **Figure 2**). The genome sequence of our *Enterobacter cloacae* isolate is not known, but BLAST searches revealed that the majority of these sequences were most similar to the genome sequence of *Enterobacter cloacae* subspecies *cloacae* NCTC 9394 (FP929040). The transposon insertions of six strains (JLD500, JLD513, JLD514, JLD517, JLD518, and JLD519) were within ENC_40870, which encodes a hypothetical protein only present in *Enterobacter cloacae*. JLD501 and JLD511 each contained an insertion in ENC_10940, which encodes a hypothetical protein with a secretion signal predicted by SignalP within the first 20 amino acids (Petersen et al., 2011). JLD502 and JLD509 each contained an insertion in ENC_30820, which encodes a homolog of FepE, a protein that increases the length of O antigen chains (Murray et al., 2003; Crawford et al., 2012, 2013). JLD504 has an insertion within the intergenic region of ENC_11220, which encodes a hypothetical protein. JLD505 contained an insertion

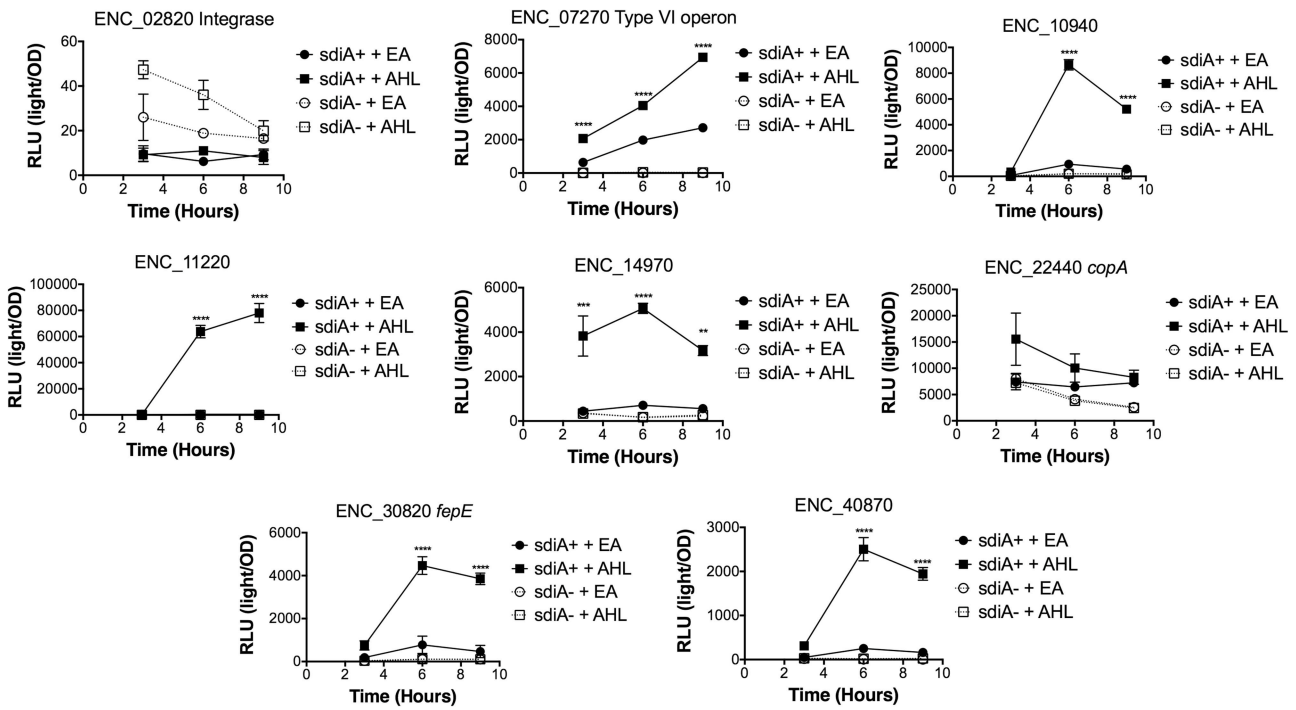

FIGURE 7 | Regulation of AHL-regulated genes in *E. cloacae* in motility agar at 37°C. Expression of mTn*5*/*luxCDABE* fusion strains in either the wild-type (closed symbols) or *sdiA* mutant backgrounds (open symbols) with either 1 μM oxoC6 (squares) or 0.1% ethyl acetate (EA) solvent control (circles) in LB broth. Luminescence is reported in relative light units (light/OD_{590}). Data was collected at 3, 6, and 9 h time points. All data points are the average of three technical replicates and error bars indicate SEM. This is a representative graph of three independent biological replicates. The statistical significance of AHL-dependent changes in gene expression are designated with *p*-values (≤0.05 = *, ≤0.005 = **, ≤0.0005 = ***, ≤0.00005 ≤ ****). The *sdiA*-dependent changes in gene expression were larger than the AHL-dependent changes and were statistically significant (*p*=0.00005) for all fusions in all growth conditions for at least one time point. The *sdiA*$^+$ and *sdiA* mutant strains for each fusion are indicated in parentheses: ENC_02820 (JLD506/ASD506), ENC_07270 (JLD505/ASD505), ENC_10940 (JLD501/ASD501), ENC_11220 (JLD504/ASD504), ENC_14970 (JLD515/ASD515), ENC_22440 (JLD508/ASD508), ENC_30820 (JLD502/ASD502), and ENC_40870 (JLD500/ASD500).

in ENC_07270, which encodes a hypothetical protein within an operon that encodes a putative type VI secretion system (Durand et al., 2014; Li et al., 2015). JLD506 contains an insertion in ENC_02820, which encodes a prophage integrase. JLD507 and JLD508 each contained an insertion in ENC_22440, which encodes a homolog of CopA, a putative copper-translocating P-type ATPase (Rensing and Grass, 2003; Osman and Cavet, 2011). JLD515 and JLD516 have insertions within the promoter region of ENC_14970, encoding a putative signal transduction protein containing a sensor and diguanylate phosphodiesterase (EAL) domain (Römling et al., 2013).

In *Salmonella*, we have observed differences in the behavior of *sdiA*-regulated fusions at 30°C compared to 37°C, and in motility agar compared to broth or agar plates (Smith and Ahmer, 2003). More ligand-independent SdiA activity is observed at lower temperatures, and more activity in general is observed in motility agar than in broth or agar plates. For *E. cloacae*, therefore, we chose one representative fusion-containing strain for each AHL-responsive locus identified, and tested these representatives under each of these conditions (**Figures 3–8**). Unlike *sdiA*-regulated fusions in *Salmonella*, the *E. cloacae* fusions did not become more ligand-independent at 30°C compared to 37°C. Instead, some fusions were largely ligand-independent under all conditions, while the remainder were ligand-dependent under all conditions. We also tested the response of these fusions to a series of AHL concentrations using oxoC6 and oxoC8. The AHL detection limits of *E. cloacae* are similar to those of *E. coli* and *Salmonella* (**Figure 9**).

AHL-responses are *sdiA*-dependent

We hypothesized that the AHL-dependent responses of the *lux* fusions were dependent on the *sdiA* gene encoded on the chromosome of *E. cloacae*. To test this hypothesis, we constructed an *sdiA* mutation in each of the mTn*5-luxCDABE* fusion strains using two new suicide vectors and a new transposon mutagenesis strategy (see Materials and Methods). Indeed, mutation of *sdiA* in each fusion strain eliminated any responsiveness to AHL in both liquid culture and motility agar (**Table 3**). In one case, *sdiA* in *E. cloacae* acts as a negative regulator of expression. We observed that *sdiA* is required for repression of ENC_02820 since the *sdiA* mutant strain produced more light than the wild-type strain regardless of the presence of AHL, in all of the conditions tested (**Figures 3–8**).

Discussion

SdiA is a LuxR homolog that detects the AHLs produced by other bacteria (Michael et al., 2001; Smith and Ahmer, 2003). To date, SdiA regulon members in *S. enterica* serovar Typhimurium, *E. coli* K-12, EHEC and a plant-pathogenic isolate of *Enterobacter*

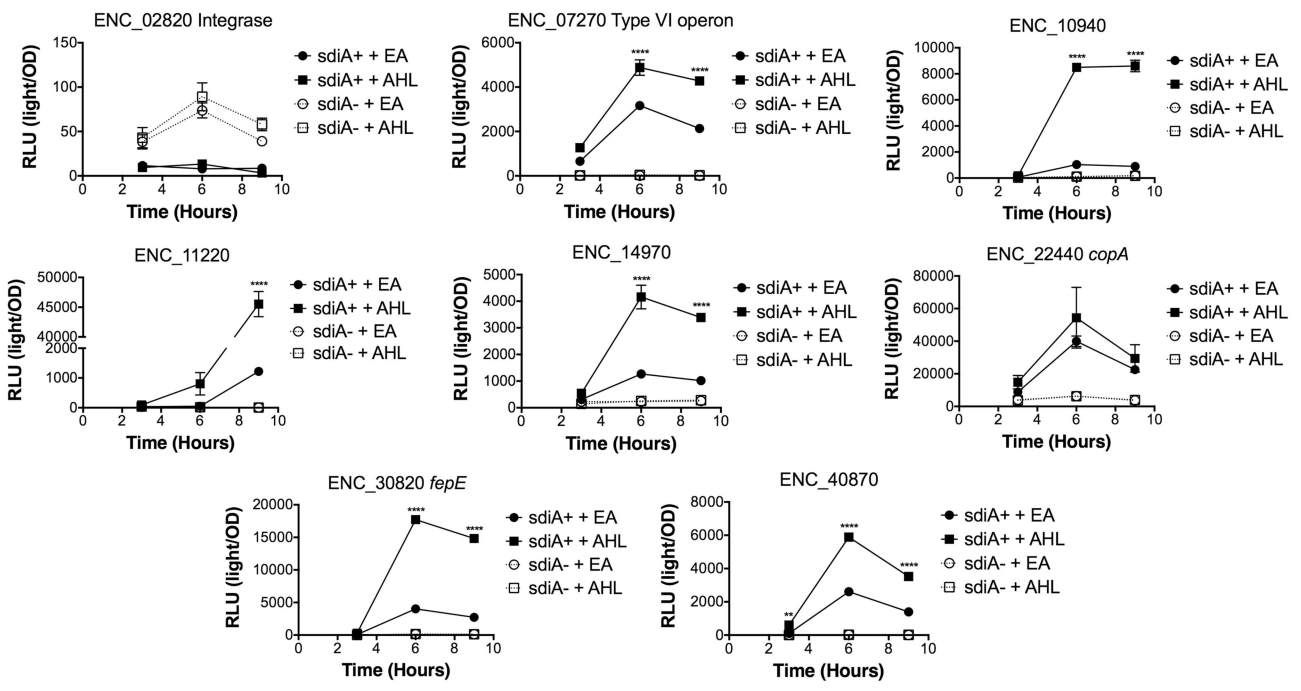

FIGURE 8 | Regulation of AHL-regulated genes in *E. cloacae* in motility agar at 30°C. Expression of mTn*5*/*luxCDABE* fusion strains in either the wild-type (closed symbols) or *sdiA* mutant backgrounds (open symbols) with either 1 μM oxoC6 (squares) or 0.1% ethyl acetate (EA) solvent control (circles) in LB broth. Luminescence is reported in relative light units (light/OD_{590}). Data was collected at 3, 6, and 9 h time points. All data points are the average of three technical replicates and error bars indicate SEM. This is a representative graph of three independent biological replicates. The statistical significance of AHL-dependent changes in gene expression are designated with *p*-values (≤0.05 = *, ≤0.005 = **, ≤0.0005 = ***, ≤0.00005 = ****). The *sdiA*-dependent changes in gene expression were larger than the AHL-dependent changes and were statistically significant ($p \leq 0.00005$) for all fusions in all growth conditions for at least one time point. The *sdiA*$^+$ and *sdiA* mutant strains for each fusion are indicated in parentheses: ENC_02820 (JLD506/ASD506), ENC_07270 (JLD505/ASD505), ENC_10940 (JLD501/ASD501), ENC_11220 (JLD504/ASD504), ENC_14970 (JLD515/ASD515), ENC_22440 (JLD508/ASD508), ENC_30820 (JLD502/ASD502), and ENC_40870 (JLD500/ASD500).

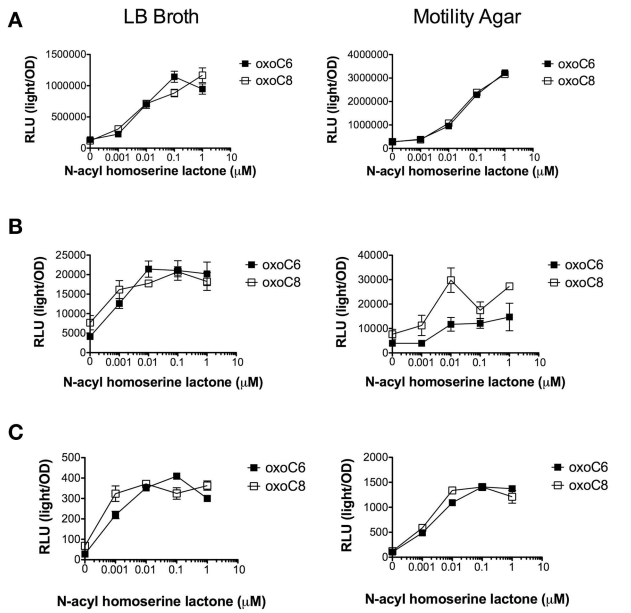

FIGURE 9 | AHL concentration dependent response of *Salmonella*, *E. coli*, and *Enterobacter*. **(A)** Expression of *srgE-luxCDABE* fusion (pJNS25) in *S.* Typhimurium 14028. **(B)** Expression of the *gadW*::Tn*5luxCDABE* fusion in *E. coli* K-12 AL4001. **(C)** Expression of the ENC_40870::Tn*5luxCDABE* fusion in *E. cloacae* JLD500. Assays were done in either LB liquid broth with shaking or motility agar (0.3% agar) at 37°C. Relative light units (light/OD_{590}) after 6 h of growth are indicated for oxoC6 (black squares) or oxoC8 (open squares). All data points are the mean of three biological replicates and error bars indicate SEM.

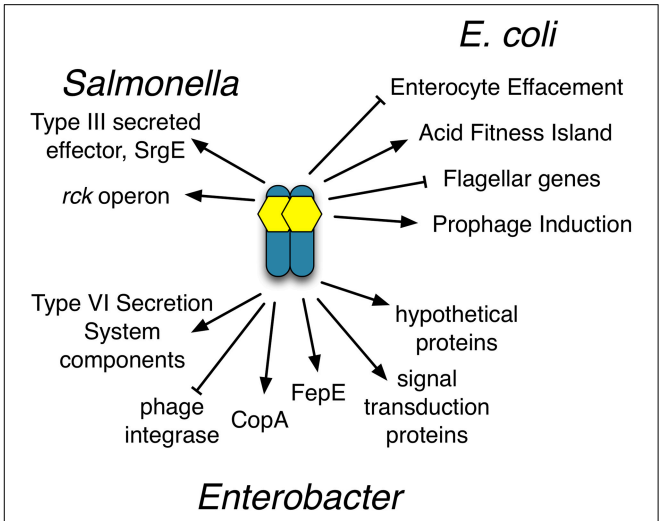

FIGURE 10 | Compilation of the SdiA regulon members in *Salmonella*, EHEC and *Enterobacter*. Diagram of known SdiA regulon members in *Salmonella*, EHEC and *Enterobacter.* Arrows indicate whether SdiA increases the expression of the regulon member and blunt arrows indicate SdiA decreases the expression of the regulon member, either directly or indirectly.

cloacae have been described (Ahmer et al., 1998; Kanamaru et al., 2000; Wei et al., 2001; Suzuki et al., 2002; Smith and Ahmer, 2003; Van Houdt et al., 2006; Lee et al., 2007; Ghosh et al., 2009; Dyszel et al., 2010b; Hughes et al., 2010; Sharma et al., 2010; Shankar et al., 2012; Sharma and Bearson, 2013). Here we report the identification of AHL-responsive and *sdiA*-dependent genes in a mouse isolate of *E. cloacae.* We have previously shown that this *E. cloacae* isolate is not pathogenic and competes with *Salmonella* for colonization of mice (Ali et al., 2014). To identify AHL-responsive genes in this organism, we used a transposon-based genetic screen in which the expression of luciferase by individual mTn*5-luxCBADE* mutants was measured in the presence and absence of AHL. The *sdiA* gene was then mutated in each strain, and the response of every fusion was found to be *sdiA*-dependent (**Figures 3**–**8**). This suggests that SdiA is the only AHL receptor in this isolate of *E. cloacae*.

In *Salmonella*, there is very little SdiA activity in the absence of AHL at 37°C, although some is observed at 30°C (Smith and Ahmer, 2003; Sabag-Daigle et al., 2012). In *E. coli* there seems to be more SdiA activity in the absence of AHL (Dyszel et al., 2010b; Hughes et al., 2010; Sperandio, 2010b). Other work in *E. coli* has shown that SdiA binds target genes *in vivo* in the absence of AHL (Ishihama et al., 2014; Shimada et al., 2014). AHL-independent activity of SdiA was also noted with some, but not all, plasmids used as AHL biosensors in *E. coli* (Lindsay and Ahmer, 2005). However, the *E. cloacae* regulon identified here is very unusual in that SdiA is demonstrating high levels of AHL-independent activity for some fusions but not others. For instance, substantial AHL-independent SdiA activity is observed with ENC_22440 and ENC_07270 (**Figures 3**–**8**). Another fusion was repressed by *sdiA* and this was also independent of AHL (ENC_02820) (**Figures 3**–**8**). It appears that we were fortunate to identify these particular fusions using AHL-responsiveness as the first screen. The LuxR homolog TraR requires AHL for proper structural folding in order to oligomerize into a fully functional dimer structure capable of binding its target promoters (Zhu and Winans, 2001). However, the ligand-independent activity of SdiA suggests that SdiA is properly folded and able to bind target promoters even in the absence of AHL. This may be due to the folding of SdiA around endogenous 1-octanoyl-*rac*-glycerol (Nguyen et al., 2015). The mechanistic differences between ligand-dependent and -independent regulation of genes by SdiA is an interesting topic for further studies.

The role(s) for the SdiA regulon in this isolate of *E. cloacae* is unclear (**Figures 2**, **10**). The *fepE*, *copA*, and type VI secretion genes could be envisioned to have direct interactions with the host, direct interactions with other microbes, or in general survival in the intestinal tract. Alternatively, these genes may play a role outside the host in other environments. In *E. coli*, the induction of lambda prophage is enhanced by AHL in an *sdiA*-dependent manner (Ghosh et al., 2009). The fusion that was repressed by *sdiA* in this study encodes a putative phage integrase, although it does not appear to be encoded within a prophage (**Figure 2**). It would be interesting to determine if SdiA plays a role in phage biology of *E. cloacae*.

In an isolate of *E. cloacae* that promotes the growth of rice roots, SdiA represses biofilm formation and rice root colonization (Shankar et al., 2012). This phenotype is at least partially due to the *sdiA*-dependent repression of the genes encoding curli fimbriae (Shankar et al., 2012). Repression of biofilm formation has been observed in *E. coli* as well (Lee et al.,

2009; Sharma et al., 2010). Interestingly, we did not isolate curli genes in this study. It is not known if curli genes are not regulated by *sdiA* in this isolate, or if we simply missed them, either by chance or due to growth conditions. It would be interesting to determine if the genes identified in this study are regulated by *sdiA* in the *E. cloacae* plant isolate and to determine if the *sdiA* regulon has diverged between the plant and mouse isolates, or if the regulon has remained largely the same.

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Stenotrophomonas maltophilia responds to exogenous AHL signals through the LuxR solo SmoR (Smlt1839)

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Quorum Sensing (QS) mediated by Acyl Homoserine Lactone (AHL) molecules are probably the most widespread and studied among Gram-negative bacteria. Canonical AHL systems are composed by a synthase (LuxI family) and a regulator element (LuxR family), whose genes are usually adjacent in the genome. However, incomplete AHL-QS machinery lacking the synthase LuxI is frequently observed in Proteobacteria, and the regulator element is then referred as LuxR solo. It has been shown that certain LuxR solos participate in interspecific communication by detecting signals produced by different organisms. In the case of *Stenotrophomonas maltophilia*, a preliminary genome sequence analysis revealed numerous putative *luxR* genes, none of them associated to a *luxI* gene. From these, the hypothetical LuxR solo Smlt1839, here designated SmoR, presents a conserved AHL binding domain and a helix-turn-helix DNA binding motif. Its genomic organization—adjacent to *hchA* gene—indicate that SmoR belongs to the new family "LuxR regulator chaperone HchA-associated." AHL-binding assays revealed that SmoR binds to AHLs *in-vitro*, at least to oxo-C8-homoserine lactone, and it regulates operon transcription, likely by recognizing a conserved palindromic regulatory box in the *hchA* upstream region. Supplementation with concentrated supernatants from *Pseudomonas aeruginosa*, which contain significant amounts of AHLs, promoted swarming motility in *S. maltophilia*. Contrarily, no swarming stimulation was observed when the *P. aeruginosa* supernatant was treated with the lactonase AiiA from *Bacillus subtilis*, confirming that AHL contributes to enhance the swarming ability of *S. maltophilia.* Finally, mutation of *smoR* resulted in a swarming alteration and an apparent insensitivity to the exogenous AHLs provided by *P. aeruginosa*. In conclusion, our results demonstrate that *S. maltophilia* senses AHLs produced by neighboring bacteria through the LuxR solo SmoR, regulating population behaviors such as swarming motility.

Keywords: LuxR Orphan, AHL, Acyl-Homoserine lactone, lactonase, quorum sensing, swarming

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Introduction

Bacterial cells can communicate with each other to facilitate their rapid adaptation to fluctuations in the environment. This cell-cell communication mechanism, known as quorum sensing (QS), relies primarily on the production, detection, and response to diffusible signal molecules (also called autoinducers) in a cell-density dependent manner (Fuqua et al., 1994; Whitehead et al., 2001; Fuqua and Greenberg, 2002; Federle and Bassler, 2003). Through this QS communication, numerous bacterial species regulate a variety of functions such as biofilm formation, motility, antibiotic resistance, toxin production, exopolysaccharide synthesis, and extracellular enzyme production among others (Miller and Bassler, 2001). In Gram-negative bacteria, *N*-acyl homoserine lactones (AHLs) are to date the most extensively and best characterized QS signaling molecules. AHL-QS regulation consists of a LuxI-type synthase, which produces signal molecules, and a LuxR-type receptor that binds AHLs and regulates expression of certain genes when signal concentration reaches a critical threshold. LuxR regulators are about 250 residues in length and present two typical domains, the N-terminal autoinducer AHL binding domain (Shadel et al., 1990; Slock et al., 1990) and the C-terminal helix-turn-helix (HTH) DNA-binding domain (Choi and Greenberg, 1991; Fuqua and Winans, 1994). In the presence of AHLs, the N-terminal binding domain interacts with the signal molecule, habilitating the DNA-binding domain to induce transcription of certain genes by binding to their promoters in a region named *luxR* box (Devine et al., 1989; Stevens and Greenberg, 1997). The DNA-binding domain includes three highly conserved aminoacids, while the AHL-binding domain presents six hydrophobic or aromatic residues displaying remarkable variability (18-25%) (Zhang et al., 2002).

The increasing availability of bacterial genome sequences has led to the identification of several LuxR and LuxI homologs. Typically, both *luxI*-type and *luxR*-type genes are located adjacent in the bacterial genome (the cognate *luxR/I* pair). However, *luxR*-type genes without a cognate *luxI*-type in their vicinity are frequently found, and these regulatory elements are then called "orphan" (Fuqua, 2006) or "solo" LuxR (Subramoni and Venturi, 2009a). Recent studies have revealed that "*luxR* solo" genes are widely distributed among bacterial genomes (Hudaiberdiev et al., 2015). LuxR solos present the same modular organization as canonical LuxR, displaying the N-terminal and C-terminal domains. The nature of the signal molecules that bind to the different LuxR solos is quite heterogeneous. It has been shown that the LuxR solo QscR from *Pseudomonas aeruginosa* bind to self-produced AHL signals (Chugani et al., 2001; Lequette et al., 2006), while SdiA of *Salmonella enterica* and *Escherichia coli* respond to exogenous AHL signals (Ahmer et al., 1998; Michael et al., 2001; Ahmer, 2004; Yao et al., 2006). Interestingly, the LuxR solo OryR from *Xanthomonas oryzae* pv. *oryzae* interacts with plant signals, in particular those produced by rice (Ferluga et al., 2007; Ferluga and Venturi, 2009; González et al., 2013). More recently, it has been reported that the human and insect pathogen *Photorhabdus asymbiotica* contains a LuxR solo PauR that is the regulator element of a new QS-system, which is mediated by dialkylresorcinols (DARs) and cyclohexanediones (CHDs) signals (Brameyer et al., 2015). Altogether, this shows that LuxR solos can participate in a wide variety of signaling networks.

Stenotrophomonas maltophilia is an ubiquitous gram-negative bacterium considered an emerging nosocomial pathogen (Brooke, 2012). Moreover, it is frequently found in lungs of cystic-fibrosis (CF) patients (Demko et al., 1998), usually co-isolated with *P. aeruginosa* (Moskowitz et al., 2005). The QS described in *S. maltophilia* is based on the signaling molecule DSF (11-cis-2-decenoic acid), by which it regulates virulence-related processes (Fouhy et al., 2007; Huedo et al., 2014). To date, no *S. maltophilia* strain has been reported to produce AHL and the K279a reference genome contains no *luxI* homolog (Crossman et al., 2008), at least of the usual *luxI* types (Waters and Bassler, 2005). However, sequence analysis reveals that this genome encodes a total of 15 putative LuxR-like proteins, based mainly on homologies of the DNA-binding response domain. From these, only the LuxR solo Smlt1839 showed an N-AHL autoinducer-binding domain. The objective of our study has been to experimentally investigate the role of Smlt1839 in AHL binding and swarming regulation in *S. maltophilia*, in the presence of exogenous and heterologous AHLs.

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in **Table 1**. *E. coli* strains DH5a and BL21 (DE3) were used for general cloning purposes and overexpression of *smoR*, respectively. *S. maltophilia* E77 (Ferrer-Navarro et al., 2013) was used as a model strain to investigate the role of SmoR in detection and response of exogenous AHL signals. *P. aeruginosa* MPAO1 strain was used as an AHL-producer bacterium to evaluate the effect of exogenous signal molecules on swarming motility of *S. maltophilia*. *Agrobacterium tumefaciens* KYC55 (Zhu et al., 2003) was used as a reporter strain to detect AHL production.

E. coli, S. maltophilia and *P. aeruginosa* strains were routinely grown in Luria-Bertani (LB) medium at 37°C. *A. tumefaciens* KYC55 was grown in AT medium (Fuqua and Winans, 1994) at 30°C. When required, the antibiotics were supplemented as follows: ampicillin (Ap) 20 μg/ml (*E. coli*); tetracycline (Tc) 17 μg/ml (*E. coli* and *P. aeruginosa*) or 2 μg/ml (*A. tumefaciens*); erythromycin (Erm) 50 μg/ml (*E. coli*) or 500 μg/ml (*S. maltophilia*); gentamicin (Gm) 10 μg/ml (*E. coli*), 100 μg/ml (*A. tumefaciens*) or 40 μg/ml (*S. maltophilia*); and spectinomycin (Spc) 100 μg/ml (*E. coli* and *A. tumefaciens*).

Sequence Determination and In Silico Analysis

A 5.5 kb fragment containing the ORFs of *smlt1840* and *smlt1839* plus their flanking regions was amplified from *S. maltophilia* strain E77 genomic DNA and subsequently sequenced (Macrogen). The sequence has been used for sequence alignments as well as reference to generate a Δ*smoR* mutant in this model strain. The fragment corresponding to *hchA-smoR* operon and its predicted promoter (1658 bp) was submitted to

TABLE 1 | Primers used in this study.

Primer	Sequence (5′-3′)	Restriction site
P1MutSmoR	AAGCTTTGCCCGGTTCGGTATCGG	*Hind*III
P2MutSmoR	GGATCCTCGCGGCGAGGCACTTCC	*BamH*I
P3MutSmoR	GGATCCCCGGTTCAGCGCCCGGCC	*BamH*I
P4MutSmoR	GAATTCCCCAGCCGCCAGCCCAGC	*EcoR*I
PErm5′	GGATCCGAAACGTAAAAGAAGTTATG	*BamH*I
PErm3′	GGATCCTACAAATTCCCCGTAGGC	*BamH*I
PErm5′rev	GATACTGCACTATCAACACAC	–
PErm3′rev	CTTCCAAGGAGCTAAAGAGGT	–
P1DemSmoR	GTACGTCGGGCGTATCG	–
P2DemSmoR	GCCCTTCTATGCTGG	–
P1ProSmoR	TCTAGACGCACACGCATGGACCG	*Xba*I
P2ProSmoR	GGATCCGAAGGCGTCGCGCTCGG	*BamH*I
P1ExpSmoR	CATATGAGCGATCTGGTGCAGGCG	*Nde*I
P2ExpSmoR	CTCGAGTCAGTCTTCGATCTCGCCT	*Xho*I
PT7up	TAATACGACTCACTATAGGG	–
PT7dw	GCTAGTTATTGCTCAGCGG	–

TABLE 2 | Plasmids used in this study.

Plasmid	Relevant Characteristics	Source
pGEM-Erm	Cloning vector carrying *Erm* resistance gene, *Amp*^{r}, *Erm*^{r}	This work
pEX18Tc	Suicide allelic exchange vector; *Tc*^{r}	Hoang et al., 1998
pEXsmoR	pEX18Tc carrying E77 *smoR* flanking regions interrupted with *Erm* resistance gene, *Tc*^{r}, *Erm*^{r}	This work
pBBR1MCS-5	Broad-host-range cloning vector, *Gm*^{r}	Kovach et al., 1995
pET22b	IPTG inducible expression vector, *Amp*^{r}	Novagen
pET22b-smoR	IPTG inducible expression vector carrying *smoR* ORF, *Amp*^{r}	This work
pBBR1MCS-5-*lacZ*	pBBR1MCS-5 plasmid carrying promoterless *lacZ* gene, *Gm*^{r}	Fried et al., 2012
pBBR1MCS-5-*PsmoR::lacZ*	pBBR1MCS-5 plasmid carrying fusion *PsmoR::lacZ* gene, *Gm*^{r}	This work
pME6000	Broad-host-range cloning vector, *Tc*^{r}	Maurhofer et al., 1998
pME*Plac::aiiA*	pME6000 carrying lactonase *aiiA* gene from *B. subtilis* under the control of *Plac* promoter, *Tc*^{r}	Reimmann et al., 2002

Genbank under the accession number KP691985. Annotation was done using BLAST (Altschul et al., 1990) and intergenic regions were manually inspected for palindromic motifs and *cis* elements that participate in regulating translation. Program RSAT (Thomas-Chollier et al., 2011) was used to scan for a pattern (the palindromic box) within all ORF upstream regions in the K279a genome. A simple screen for *lux*R-like genes using a *S. maltophilia* K279a genomic sequence (AM743169.1) was done by using BLAST and PSI-BLAST (Altschul et al., 1997) to detect remote homologs. Sequences of the *hchA-smoR* operon from other *S. maltophilia* strains were retrieved from their genome sequences at NCBI (http://www.ncbi.nlm.nih.gov/genome/). Translation of ORFs to amino-acid sequences and sequence alignments were done with MEGA 6 (Tamura et al., 2013) and then analyzed with SMART (Letunic et al., 2009) for the identification and annotation of protein domains. Nucleotide and protein sequences were aligned using the ClustalW module implemented in MEGA 6 and manually edited and visualized with BioEdit. Software was run with default parameters unless otherwise stated. Identification of "LuxR-like regulators chaperone HchA associated" in other Proteobacteria was predicted by InterPro (http://www.ebi.ac.uk/interpro/) (Mitchell et al., 2014).

Preparation of Fusion and Expression Vectors

Oligonucleotides used as primers and plasmids used in this study are listed in **Tables 2** and **3**, respectively. The transcriptional fusion construct for the *smoR* promoter in pBBR1MCS-5-*lacZ* (Fried et al., 2012) was generated by amplifying a fragment of 415 bp containing the putative promoter of the operon *hchA-smoR* (*smlt1840-smlt1839*) from *S. maltophilia* E77, using primers P1ProSmoR and P2ProSmoR and FastStart DNA polymerase (Roche). The fragment was digested using *Xba*I and *BamH*I and cloned into their respective restriction sites into pBBR5MCS-5-*lacZ,* generating pBBR5MCS-*PsmoR::LacZ*. This vector was electroporated (Choi et al., 2006) into *S. maltophilia* E77 and transformants were seeded onto LB plates supplemented with 40 μg/ml Gm.

To generate the expression vector for SmoR production in *E. coli*, the ORF of *smlt1839* was amplified using primer pair P1ExpSmoR-P2ExpSmoR and the amplified fragment was digested with *Nde*I and *Xho*I and cloned into their respective restriction sites into pET22b (Novagen), creating pET22b-*smoR*. *E. coli* strain BL21 (DE3) was transformed (Sambrook et al., 1989) with plasmid pET22b-*smoR* and transformants were seeded onto LB plates containing 20 μg/ml Amp.

The vectors pME6000 (Maurhofer et al., 1998) and pME*lacZ::aiiA* (Reimmann et al., 2002)—the latter carrying a transcriptional fusion between *lacZ* promoter and the ORF of the lactonase AiiA from *Bacillus subtilis* strain A24 (Dong et al., 2000)—were provided by the authors and were used to investigate the effect of the lactonase AiiA on the degradation of the AHL signals from *P. aeruginosa*. Both vectors were electroporated (Choi et al., 2006) into *P. aeruginosa* MPAO1 and transformants were seeded onto LB plates containing 17 μg/ml Tc.

Generation of Δ*smoR* Mutant

S. maltophilia E77 Δ*smoR* mutant was obtained by allelic-exchange recombination using erythromycin as antibiotic-resistance cassette. Briefly, *smoR* upstream and downstream flanking regions (993 and 863 bp, respectively) were amplified by PCR using primer pairs P1MutSmoR-P2MutSmoR (upstream region) and P3MutSmoR-P4MutSmoR (downstream region) and inserted, flanking an erythromycin cassette, into the suicide vector pEX18Tc (Hoang et al., 1998), generating plasmid pEX*smoR*. The erythromycin cassette was previously amplified from plasmid pGEM-Erm (**Table 2**) using primers PErm5′ and

TABLE 3 | Strains used in this study.

Strains	Relevant characteristics	References
S. maltophilia		
E77	Wild type	Ferrer-Navarro et al., 2013
E77 Δ*smoR*	E77 Δ*smoR* (Δ*smlt1839*), Erm^r	This work
E77 pBBR1MCS-5-*lacZ*	E77 harboring vector pBBR1MCS-5-*lacZ,* Gm^r	This work
E77 pBBR1MCS-5-*PsmoR::lacZ*	E77 harboring vector pBBR1MCS-5-*PsmoR::lacZ,* Gm^r,	This work
E77 Δ*smoR* pBBR1MCS-5-*lacZ*	E77 Δ*smoR* harboring vector pBBR1MCS-5-*lacZ,* Gm^r, Erm^r	This work
E77 Δ*smoR* pBBR1MCS-5-*PsmoR::lacZ*	E77 Δ*smoR* harboring vector pBBR1MCS-5-*PsmoR::lacZ,* Gm^r, Erm^r	This work
E. coli		
DH5α	*recA1 endA1 hsdR17 gyrA96 supE44 thi-1 relA1Δ(lacZYA-argF)U169 deoR Φ80dlacZΔM15*	Lab. Collection
DH5α pEX*smoR*	DH5α harboring vector pEX*smoR,* Tc^r, Erm^r	This work
BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdSλ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	Novagen
BL21 (DE3) pET22b	BL21 (DE3) harboring pET22b, Amp^r	This work
BL21 (DE3) pET22b-*smoR*	BL21 (DE3) harboring pET22b-*smoR*, Amp^r	This work
P. aeruginosa		
MPAO1	Wild type	Jacobs et al., 2003
MPAO1 pME600	MPAO1 harboring pME600, Tc^r	
MPAO1 pME*Plac::aiiA*	MPAO1 harboring pME-*Plac::aiiA,* Tc^r	This work
A. tumefaciens		
KYC55	KYC55 harboring vectors pJZ384, pJZ410 and pJZ372 Spc^r, Gm^r, Tc^r	Zhu et al., 2003

PErm3′. *S. maltophilia* E77 was electroporated (Choi et al., 2006) with the suicide vector pEX*smoR* and transformants were seeded onto LB plates containing 500 μg/mL Erm and subsequently streaked onto LB plates containing 17 μg/mL Tc to discard single cross-over events. *smoR* deletion was also verified by PCR using primer combinations P1DemSmoR-PErm5′rev (for upstream region) and P2DemSmoR-PErm3′rev (for downstream region). The obtained fragments were subsequently verified by sequencing (Macrogen).

Measuring ß-Galactosidase Activity

To evaluate the expression levels of *hchA-smoR* promoter, ß-galactosidase assays were performed for the strains E77 wild type and Δ*smoR* mutant harboring either the vectors pBBR1MCS-5-*PsmoR:lacZ* or pBBR1MCS-5-*lacZ*—the latter used as a control– during growth in LB medium at 30°C, following the protocol described by Miller (1972). All bacterial cultures were started with an initial inoculum corresponding to an optical density at 550 nm (OD_{550}) of 0.05. To determine the activity of the *hchA-smoR* promoter during growth curve, 0.1 ml-samples were taken at different times from 4 to 48 h. To investigate the effect of the presence of AHL molecules in the activity of the *hchA-smoR* promoter, initial cultures were supplemented with various synthetic AHLs (Cayman Chemical) –C6-HSL, oxo-C8-HSL, C8-HSL, and C10-HSL– with different concentrations (1 up to 10 μM), and 0.1 ml samples were taken and measured after 24 h and 48 h of incubation at 30°C. After analyzing the data we determined β-galactosidase specific activities in Miller Units (Miller, 1972). All AHL stocks were solubilized in 70% acetonitrile/water acidified with 0.1 M HCl final concentration. All experiments were performed by triplicate and comparison of ß-galactosidae activity was performed by One-Way analysis of variance (ANOVA) with a Bonferroni's multiple comparison post-test.

Extraction, Thin Layer Chromatography and Bioassay of AHLs

To evaluate AHL produced by *P. aeruginosa,* 150 ml culture supernatants of strain MPAO1, or MPAO1 transformed with either pME6000 or pME*lacZ::aiiA* grown in LB at 37°C for 24 h (OD_{550} of about 2), were extracted with 300 ml of acidified ethyl acetate (0.1% acetic acid). The organic phase was evaporated to dryness using a rotary evaporator, and the residues were dissolved in an appropriate volume of acidified ethyl acetate. 5 μl aliquots of dissolved ethyl acetate residues were spotted onto C18 reverse-phase plate (Merck) (Shaw et al., 1997) and separated with methanol:water (60:40, vol/vol) as running solvent. TLC plates were subsequently air-dried for at least 1 h and overlaid with 100 ml of unsolidified warm AT medium containing 0.8% agar, 60 μg/ml X-Gal and the AHL reporter strain KYC55 to an OD_{550} of ca. 0.8. TLC plates were incubated overnight at 30°C, and AHL activity was identified by the presence of blue spots. 2 μl of the aforementioned synthetic AHLs were also tested in TLC coupled to bioassay and used as a control.

AHL Binding Assay

The AHL binding assay was performed as described (Subramoni and Venturi, 2009b), with few modifications. 20 ml cultures of *E. coli* BL21 (DE3) harboring either pET22b or pET22b-*smoR* were grown at 37°C in LB medium containing 10 μg/ml Amp to an OD_{550} of 0.1. Bacterial cultures were then supplemented with different AHL molecules (C6-HSL, oxo-C8-HSL, C8-HSL and C10-HSL) at 10 and 20 μM final concentration and cultures were incubated until reaching an OD_{550} of 0.6. SmoR production was induced with 1 μM final concentration of IPTG and the cultures were additionally incubated for 3.5 h. OD_{550} was measured and the cultures were adjusted to contain an equal number of cells per mL and subsequently centrifuged. Cell pellets were washed three times with 10 ml of PBS and cellular suspensions were extracted twice with the same volume of acidified ethyl acetate. The extracts were then dried, dissolved in ethyl acetate and analyzed by TLC coupled to AHL bioassay, as described above. An aliquot of the corresponding induced culture was previously removed to control the identity of the overproduced recombinant protein (see Supplementary Figure S1).

Swarming Assay

Swarming motility was assayed on BM2 medium plates (62 mM potassium phosphate buffer, pH = 7, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.5% [wt/vol] casamino acids, supplemented with glucose 0.4% and solidified with 0.5% BD Difco Noble agar) (Overhage et al., 2007). Plates containing 20 ml of fresh swarm medium were dried under a laminar-flow hood for 20 min before pin-inoculation. When indicated, solidified swarm plates were supplemented with 10 μl of concentrated culture supernatant—extracted as described above– of *P. aeruginosa* MPAO1 and its derivative strains, as indicated in figure captions. Inoculated swarm plates were sealed to maintain the humidity and incubated at 30°C up to five days. Swarming experiments were done in triplicate and representative images are shown.

Results

Smlt1839 Contains both the AHL- and DNA-Binding LuxR Domains

It is known that certain non AHL-producing bacteria are able to sense AHLs and regulate various biological functions in response to signals produced by others through diverse LuxR-like regulators (Patankar and González, 2009). The genome of *S. maltophilia* strain K279a (Crossman et al., 2008) was revisited for the presence of genes encoding putative LuxR-like regulators. Besides the eight genes already annotated as two-component-system response regulators of the LuxR family, a total of seven additional hypothetical LuxR regulators were identified (**Table 4**), none of them associated to a *luxI* homolog. All these LuxR-solo candidates were examined in detail for the presence of the typical N-terminal AHL-binding domain (PFAM 03472) and the C-terminal helix-turn-helix (HTH) DNA-binding domain (PFAM 00196) (Miller and Bassler, 2001). From these, only the gene *smlt1839* was found to encode for a protein—here named SmoR (*Stenotrophomonas maltophilia* orphan regulator)—containing both conserved domains (**Table 4**). A subsequent protein alignment with known orphan regulators from distinct Proteobacteria including PpoR from *P. putida*, SdiA from *S. enterica*, OryR from *X. oryzae* pv. *oryzae,* and TraR from *A. tumefaciens*, revealed that at the N-terminal domain four out of six residues involved in AHL binding (Patankar and González, 2009) are conserved in SmoR (**Figure 1**). Concerning the C-terminal HTH domain, the three residues responsible for DNA binding (Hanzelka and Greenberg, 1995; Fuqua et al., 1996) are also conserved in SmoR (**Figure 1**). Further protein BLAST analysis revealed that SmoR is largely conserved among *S. maltophilia* (data not shown). These results suggest that the conserved regulator SmoR (Smlt1839) could be implicated in signaling systems in *S. maltophilia*.

S. maltophilia SmoR Binds AHLs

It has been demonstrated that various LuxR solos containing the AHL-binding domain are able to bind to one or more AHL signal molecules. To determine whether in *S. maltophilia* the regulator SmoR could bind to any of these signals, an AHL-binding assay was performed. The appropriate overexpression of *S. maltophilia smoR* in *E. coli* strain BL21 (DE3) was validated by MALDI-MS analysis prior to initiate the AHL-binding assay (see Supplementary Figure S1).

E. coli BL21 (DE3) harboring either the empty vector pET-22b or the one overproducing SmoR were grown in a rich medium supplemented with a variety of AHLs (see Materials and Methods). After the appropriate incubation time, the culture supernatant was removed and the cell pellet was washed and subsequently extracted with acidified ethyl acetate. Concentrated cell extracts were visualized by TLC coupled to the AHL bioassay, resulting in the detection of the signal oxo-C8-HSL (**Figure 2**). Likewise, it was observed that the detection depends on AHL concentration, since the culture supplemented with 20 μM oxo-C8-HSL presented a more intense spot compared to that

TABLE 4 | Hypothetical LuxR-like regulators annotated in the genome of *S. maltophilia* strain K279a.

Locus ID	Lenght	N-ter Domain	C-ter Domain	Annotation in K279a
Smlt1839	234	AHL	LuxR HTH	LuxR family transcriptional regulator
Smlt0195	212	REC	LuxR HTH	LuxR family two component response regulator
Smlt0389	223	REC	LuxR HTH	Two component transcriptional regulator, LuxR family
Smlt2299	210	REC	LuxR HTH	Response regulator protein LuxR family
Smlt2366	208	REC	LuxR HTH	Two-component response regulator, LuxR family
Smlt4224	212	REC	LuxR HTH	LuxR family two-component response regulator
Smlt0367	200	REC	LuxR HTH	Two-component system response regulator, LuxR family
Smlt0400	254	REC	LuxR HTH	Two-component response regulator transcriptional regulator
Smlt0881	213	REC	LuxR HTH	Two-component response regulator transcriptional regulator
Smlt1255	213	REC	LuxR HTH	Two-component response regulator transcriptional regulator
Smlt1788	215	REC	LuxR HTH	Two-component response regulator transcriptional regulator
Smlt2595	224	REC	LuxR HTH	Two-component response regulator transcriptional regulator
Smlt2658	213	REC	LuxR HTH	Two-component response regulator transcriptional regulator
Smlt2891	217	REC	LuxR HTH	Two-component response regulator transcriptional regulator
Smlt4624	221	REC	LuxR HTH	Two component system response regulator

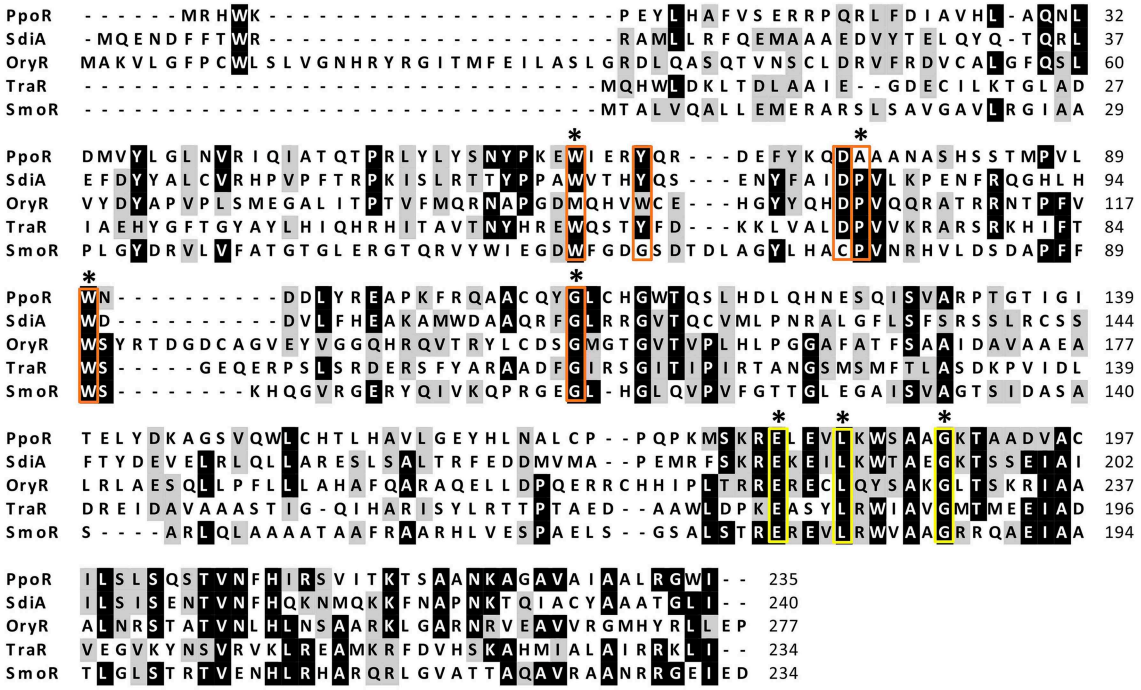

FIGURE 1 | Protein alignment of orthologs LuxR solos from diverse Proteobacteria; PpoR (FM992078): *Pseudomonas putida* strain RD8MR3; SdiA (AAC08299.1): *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain LT2; OryR (AAR91700.1): *Xanthomonas oryzae* pv. *oryzae*; TraR (AAZ50597.1): *A. tumefaciens*; SmoR: *S. maltophilia* E77 (KP691985). Red boxes highlight amino acids implicated in AHL binding and yellow boxes indicate residues involved in DNA binding (HTH). From these, conserved amino acids in E77 are marked with an asterisk.

supplemented with 10 μM. On the other hand, cell extracts from *E. coli* cells containing the empty vector did not show detectable AHL activity (**Figure 2**). The experiment was performed by triplicate and non-systematic binding was observed for the other AHLs tested. Overall, this indicates that *S. maltophilia* could sense AHL signal molecules –in particular oxo-C8-HSL– through the LuxR solo SmoR.

hchA and *smoR* Are Part of the Same Operon, and Operon Expression is Growth-phase- and AHL-Dependent

In *S. maltophilia* K279a, the gene encoding the regulator SmoR is localized downstream of the gene encoding for the chaperone HchA (Smlt1840), separated by only five nucleotides, indicating that both genes could form the operon *hchA-smoR*. This genetic organization is conserved in all available *S. maltophilia* genomes and also in the clinical strain E77 used in the present study. The upstream genes to *hchA* in strains D457 and JV3 are oriented in the opposite direction, indicating that there must be a promoter preceding *hchA* and confirming the existence of a bicistronic operon. Interestingly, this operon has been observed only in few Gammaproteobacteria, including *Pseudomonas* spp., *Vibrio* spp., *Acinetobacter* spp., *Serratia* spp., among few others, as predicted by InterPro (Family IPR019941). Accordingly, these regulator elements are annotated as "LuxR chaperone HchA-associated". In *E. coli*, the gene *hchA* encodes for the chaperone Hsp31, which

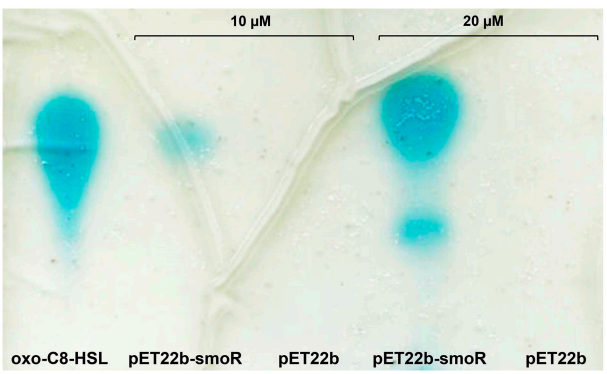

FIGURE 2 | Representative image of AHL binding assay done for *E. coli* BL21 (DE3) harboring the expression vector pET22b-smoR or the empty vector pET22b grown in LB supplemented with AHLs at concentration of either 10 or 20 μM. Blue spots correspond to AHL signal. oxo-C8-HSL was used as control.

displays a high protein identity (60%) to *S. maltophilia* HchA. However, *E. coli hchA* is an isolated gene in this bacterial genome. It has been shown that Hsp31 is a glyoxalase that plays a central role in detoxification of dicarbonyl radicals (Subedi et al., 2011) as well as in the response to bacterial stress such as heat shock (Mujacic et al., 2004; Mujacic and Baneyx, 2006) and acidic

conditions (Mujacic and Baneyx, 2007). In these mentioned studies it has been reported that transcription of *hchA* is induced at high population density.

In *S. maltophilia* E77 the upstream *hchA-smoR* operon region was examined for the presence of a putative promoter. Despite canonical core promoter elements are not found in this region, DNA sequence alignment of this region from different *S. maltophilia* strains revealed a conserved ribosome-binding site and a palindromic conserved motif spanning from positions −75 to −56 with respect to the translational start site (**Figure 3A**). The genome of *S. maltophilia* K279a was also evaluated for the presence of this palindromic motif in other promoter gene regions. Curiously, only a highly similar box (68.75% identity) was found in the intergenic region between *smlt2137* (position −143 to −159) and *smlt2138* (position −15 to −31). Those genes encode for a universal stress-family protein (Smlt2137) and for the transcriptional regulator NfxB (Smlt2138) (Shiba et al., 1995), respectively.

The *hchA-smoR* upstream region including the palindromic sequence motif was fused to *lacZ* gene and used in ß-galactosidase experiments to corroborate the existence of a promoter. The expression levels of the fusion construct *PsmoR::lacZ* were monitored in *S. maltophilia* strain E77 wild type and its derivative Δ*smoR* mutant, in order to determine whether the expression pattern of the operon was similar to that previously described for the *hchA* gene in *E. coli* (Mujacic and Baneyx, 2006, 2007). Additionally, the role of SmoR in such expression was also evaluated, since the autoregulation of LuxR proteins is relatively common (Shadel and Baldwin, 1992; Chatterjee et al., 1996; Minogue et al., 2002).

The results from the ß-galactosidase experiments indicate that the promoter activity is slight at low cell densities and increases with bacterial-growth rate, showing a maximum at 48 h (stationary phase, **Figure 3B**). Although this tendency was observed in both wild type and Δ*smoR* backgrounds, the absence of SmoR led to increased levels of expression compared to the wild type strain E77, specially at late stationary phase of growth (**Figure 3B**) ($P < 0.01$). Under these standard conditions (LB and 30°C) all tested strains showed similar growth curves reaching stationary phase by 24 h approximately. These results suggest that operon components would act at high cell densities, participating likely in this sort of stress response as observed for Hsp31 in *E. coli.*

Since we observed *in vitro* that SmoR bind the signal oxo-C8-HSL (**Figure 2**) and it is known that some active LuxR-like proteins can autoregulate their own expression, we wanted to investigate the role of SmoR on the regulation of *hchA-smoR* operon expression in the presence of AHLs. To do that, β-galactosidase assays were performed for the same strains supplemented with various AHLs (see Materials and Methods).

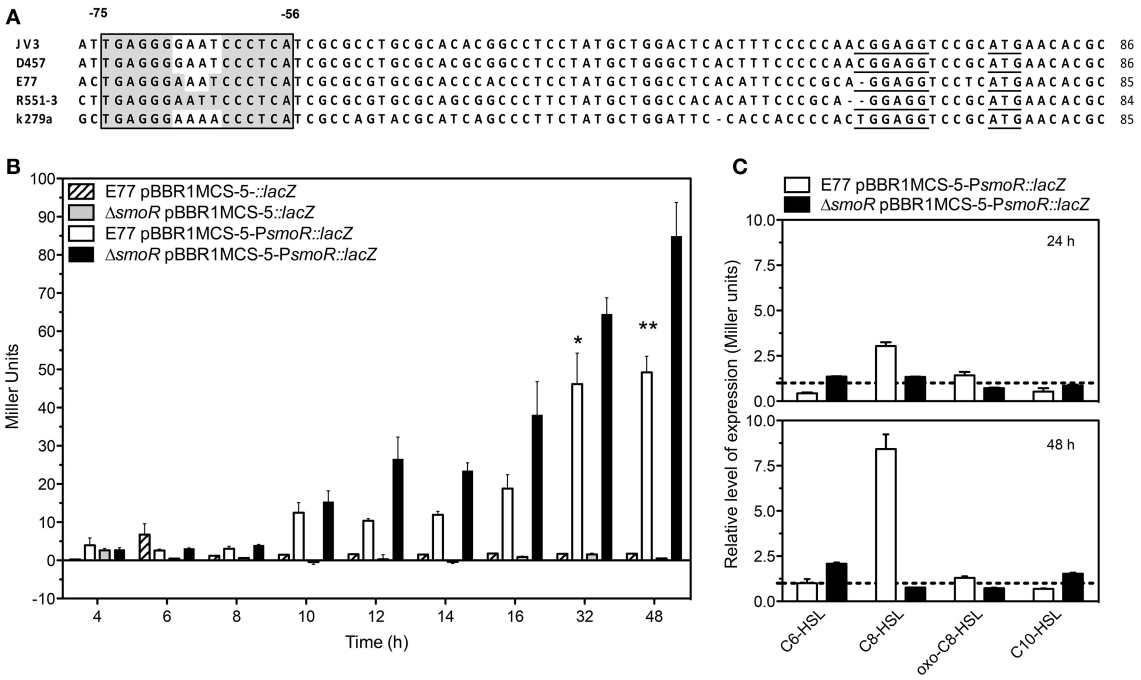

FIGURE 3 | (A) Alignment of the *hchA-smoR* promoter from different *S. maltophilia* strains. Gray shadow indicates the palindromic motif. Underlined sequences correspond to the putative ribosome binding site (RBS) and the start codon (ATG). **(B)** ß-galactosidase assay expressed in Miller Units of E77 WT and Δ*smoR* mutant both harboring either pBBR1MCS-5*::lacZ* or pBBR1MCS-5-*PsmoR::lacZ* monitored during 48 h. Under these conditions all tested strains showed similar growth curves. **(C)** ß-galactosidase activity of E77 wild type and Δ*smoR* mutant both harboring vector pBBR1MCS-5-*PsmoR::lacZ* grown in LB supplemented with diverse AHLs at 1 μM concentration each and incubated at 30°C for 24 or 48 h. Relative expression values are reported as Miller units of β-galactosidase activity in cells grown under the indicated growth conditions divided by the values in cells grown in LB broth without AHLs. *$P < 0.05$; **$p < 0.01$.

The results showed that the expression of the operon was also modulated by the presence of these signal molecules. In particular, supplementation of E77 wild type with 1 μM C8-HSL resulted into approximately threefold and 8-fold operon activation at 24 and 48 h post induction respectively, compared to the expression levels of the supplemented Δ*smoR* mutant (**Figure 3C**). This indicates that SmoR is involved in AHL-dependent operon-expression regulation.

AHLs Produced by *P. aeruginosa* Promote Swarming Motility in *S. maltophilia*, SmoR Playing a Central Role in this Stimulation

It is known that AHL signals modulate several biological functions not only in AHL-producing bacteria, but also in certain species lacking typical *luxI/luxR* systems which are still able to respond to exogenous AHLs through diverse orphan LuxR. One of the behaviors that have raised more interest recently and is commonly regulated by AHL-QS is swarming motility (Daniels et al., 2004). Swarming motility is a rapid and coordinated translocation of a bacterial population across semi-solid surfaces, which frequently requires quorum sensing-mediated synchronization (Kearns, 2010).

Since *S. maltophilia* frequently cohabit with AHL-producing bacteria –i.e., *P. aeruginosa* (Moskowitz et al., 2005)– we investigated the effect that *P. aeruginosa* AHLs could have on *S. maltophilia* regulation, more specifically on swarming motility. To that end, we supplemented *S. maltophilia* swarming plates with concentrated supernatants of *P. aeruginosa* strain MPAO1 wild type and MPAO1 harboring the plasmid pME*Plac::aiiA*, which expresses the lactonase AiiA from *B. subtilis* (Dong et al., 2000). In order to corroborate the effect of the lactonase AiiA in AHL degradation, TLC followed by AHL bioassay was performed in parallel to swarming assays. As shown in **Figure 4**, expression of AiiA led to a decrease of *P. aeruginosa* AHL production and, interestingly, it resulted in a drastic reduction of *S. maltophilia* swarming stimulation. These results would indicate that AHL signals produced by *P. aeruginosa* promote swarming motility in *S. maltophilia.*

Several evidences drive us to suggest that SmoR was responsible for the observed AHL-mediated swarming stimulation in *S. maltophilia* strain E77. It has been shown that, in the related bacteria *X. oryzae* pv. *oryzae*, the LuxR solo OryR also regulates certain motility processes, including swarming (González et al., 2013). In order to test such hypothesis in *S. maltophilia*, MPAO1 concentrated supernatants were spotted onto swarming plates seeded with the strains E77 wild type and the Δ*smoR* mutant. The results further demonstrate that, as observed before, the MPAO1 concentrated supernatant significantly promotes the swarming ability of E77 (**Figure 5**). To the contrary, mutation of *smoR* resulted into a loss of swarming motility and supplementation with *P. aeruginosa* concentrated supernatant produced only a minor stimulation compared to E77 WT, perhaps due to the surfactant character of the AHL signals and other hydrophobic molecules present in its supernatant. These results strongly suggest that AHLs produced exogenously promote swarming motility in *S. maltophilia* and SmoR plays a central role in such stimulation (**Figure 5**).

Discussion

The interest in LuxR-solo investigation has grown among microbiologists, since these regulatory elements may unveil novel signaling systems. The increment of public sequenced genomes has permitted scientists to screen an extraordinary number of bacterial genomes and identify new regulator elements. It recently has been shown in bacteria that 75% of the annotated *luxR*-type genes encode for a "LuxR solo" (Hudaiberdiev et al., 2015), which is a surprisingly high proportion. To date, it is known that various LuxR can regulate a range of biological function in response to: endogenous AHLs (Chugani et al., 2001; Lequette et al., 2006); exogenous AHLs produced by neighboring bacteria (Ahmer et al., 1998; Michael et al., 2001; Ahmer, 2004; Yao et al., 2006); the novel bacterial signaling molecules dialkylresorcinols (DARs) and cyclohexanediones (CHDs) (Brameyer et al., 2015); or even signals produced by plants (Ferluga et al., 2007; Ferluga and Venturi, 2009). Considering the high heterogeneity and complexity of LuxR solos in bacteria, it is highly probable that new regulatory networks are there to discover.

The aim of the present study was to identify and investigate putative LuxR solos in *S. maltophilia.* After screening the genome of the model strain K279a (Crossman et al., 2008), only the protein encoded by the *smlt1839* gene (SmoR) was found to display the two typical LuxR domains: the autoinducer-binding domain (Shadel et al., 1990; Slock et al., 1990) and the DNA-binding helix-turn-helix (HTH) domain (Choi and Greenberg, 1991; Fuqua and Winans, 1994). Analysis at the amino-acid sequence level revealed that from the 9 crucial residues (Whitehead et al., 2001; Zhang et al., 2002), seven are conserved in SmoR. Diverse structural and functional analyses of several LuxR regulators have revealed that, while the DNA-binding domain (HTH) is widely conserved, the autoinducer-binding domain presents substantial variability, likely to adjust to a range of signal molecules (Vannini et al., 2002; Zhang et al., 2002; Yao et al., 2006; Bottomley et al., 2007). This is the situation observed in *S. maltophilia* SmoR, where the AHL-binding domain presents substitutions in residues 62 and 74, while the HTH domain is perfectly conserved (**Figure 1**). It has been reported that in *A. tumefaciens*, the LuxR solo TraR also binds to oxo-C8-HSL through a hydrophobic cavity composed of six conserved residues (Vannini et al., 2002; Zhang et al., 2002). Single-mutation analysis of some of these residues does not render AHL-binding incompetent TraR, but elevated signal concentrations—5000 to 10,000-fold– are then needed to achieve oxo-C8-HSL binding (Koch et al., 2005).

Additionally, it has been demonstrated that in *X. oryzae* pv. *oryzae* (*Xoo*), the LuxR solo OryR—which presents different critical amino acids in the AHL-binding domain– recognizes plant signals rather than AHLs (Ferluga et al., 2007; Ferluga and Venturi, 2009; González et al., 2013). Taking into account this observations and considering the variation in the AHL-binding domain of SmoR, it has been suggested that *S. maltophilia* SmoR might also recognize plant signals (Crossman et al., 2008), a possibility that has not been yet validated. We show here

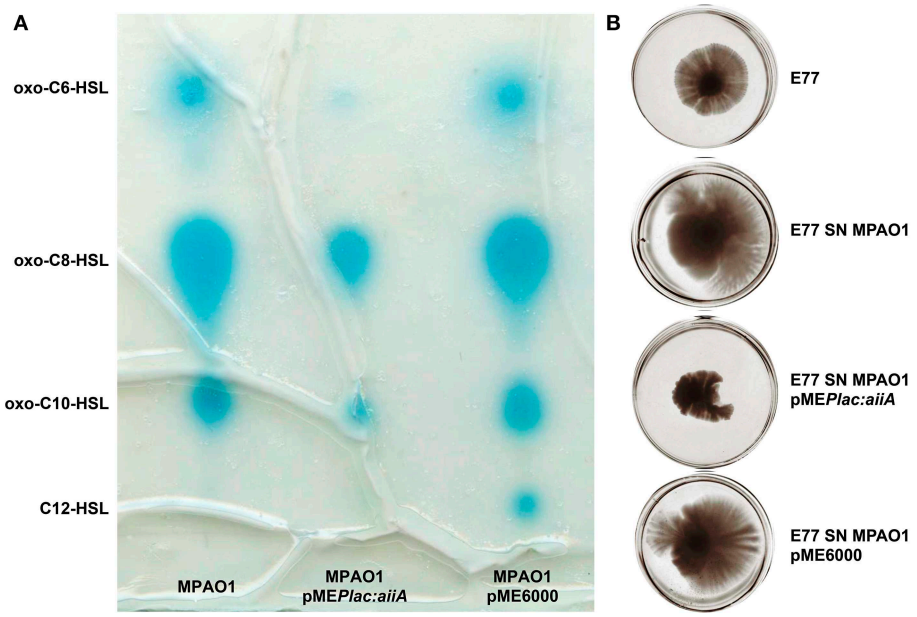

FIGURE 4 | Representative images of (A) TLC coupled to AHL bioassay of concentrated supernatants of *P. aeruginosa* MPAO1, MPAO1 pME*Plac::aiiA,* and MPAO1 pME6000. Blue spots correspond to AHL molecules. **(B)** Swarming motility in BM2 0.5% agar medium plates of *S. maltophilia* E77 supplemented with 10 μl of the same concentrated supernatants (SN) and incubated at 30°C for 5 days.

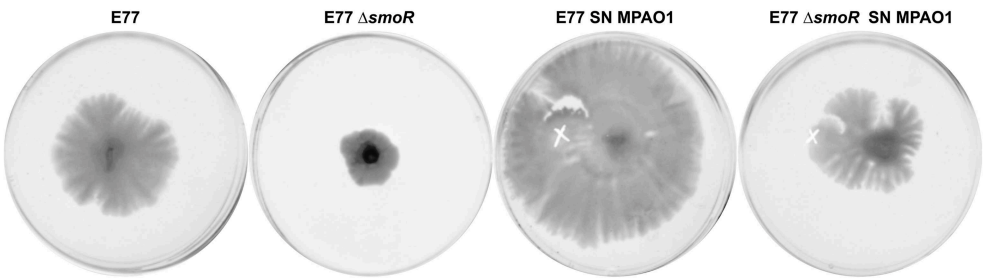

FIGURE 5 | Representative images of swarming motility in BM2 0.5% agar medium plates of *S. maltophilia* E77 WT and Δ*smoR* mutant supplemented with 10 μl of concentrated supernatants (SN) of *P. aeruginosa* strain MPAO1 WT and incubated at 30°C for 5 days. The white cross indicates the zone of the addition of the concentrated supernatants.

that SmoR binds AHLs, in particular the signal oxo-C8-HSL (**Figure 2**).

Genomic organization and sequence analysis have shown that *oryR* and *xccR* are not orthologous to *smoR*. Indeed, SmoR belongs to a recently classified subfamily designated "LuxR-like regulators chaperone HchA associated" as predicted by InterPro (http://www.ebi.ac.uk/interpro/) (Mitchell et al., 2014). Curiously, most bacteria sharing this particular operon display AHL production. However, there has been no functional study on the *hchA-smoR* operon. Nevertheless, the high homology observed between *S. maltophilia* HchA and *E. coli* Hsp31 suggests that, apart from being orthologous genes, both HchA and Hsp31 may regulate similar functions. In the genome of *E. coli*, the *hchA* gene is monocistronic. Its gene product Hsp31 has been widely studied in *E. coli* and its diverse functions have been elucidated. Initially, Hsp31 was reported to be a heat-inducible chaperone, showing feeble protease activity (Sastry et al., 2002; Malki et al., 2003, 2005). Further, it was shown that Hsp31 participates in heat shock and starvation response (Mujacic et al., 2004; Mujacic and Baneyx, 2006) as well as resistance to acid environments, generated during the stationary phase of growth (Mujacic and Baneyx, 2007). Recently it has been demonstrated that Hsp31 is a glyoxalase that participates in the detoxification of dicarbonyl stress (Subedi et al., 2011), which spans even more the functional spectrum of this versatile protein. Overall, this protein appears to act in front of stress environments derived from high cell density, a situation where QS systems are also active. In this line, we have shown that in *S. maltophilia* also a high cell density induces the expression of the *hchA-smoR* operon (**Figure 3B**). This suggests that *S. maltophilia* HchA may also act in front of stress situations such as starvation or accumulation of secondary metabolites, as described for *E. coli*

Hsp31 (Mujacic and Baneyx, 2006, 2007; Subedi et al., 2011). In addition, we have observed that mutation of *smoR* results in a higher activity of operon during growth, which suggests that SmoR may act as a repressor in this situation (**Figure 3B**). On the other hand, we have observed in the wild type background that the presence of the exogenous signal C8-HSL significantly enhance *in vivo* promoter activity (**Figure 3C**). Although no systematic binding was observed for C8-HSL in the AHL-binding assay (data not shown), ß-galactosidase experiments make us reconsider whether SmoR could also bind to this signal, apart from oxo-C8-HSL. A possible explanation could rely on the use of different systems in each technique (note that AHL-binding assay was done in *E. coli*, while ß-galactosidase experiments have been performed in *S. maltophilia*). Therefore, further studies will be necessary to better understand the role of both eight-carbon AHL in the SmoR-dependent regulation. Interestingly, we have observed that the LuxR solos QscR (PA1898) from *P. aeruginosa* and the SmoR are putative orthologs. A new *in vivo* mechanism for LuxR activation depending on the free and signal-bond state has been proposed for QscR (Oinuma and Greenberg, 2011). It has been reported that although QscR binding to the signal 3-oxo-C6-HSL has a slightly effect on its own transcription (Lee et al., 2006), it can stimulate dimerization and activation, while preventing it from proteolysis (Oinuma and Greenberg, 2011; Chugani and Greenberg, 2014). A similar mechanism have been described for TraR regulator, suggesting that this new mechanisms might be widespread among LuxR homologs (Zhu and Winans, 1999, 2001), perhaps including SmoR. Altogether, we hypothesize that the regulator SmoR might bind to the palindromic box and block operon expression, until the presence of signal molecules bind to SmoR and avoid operon repression. Curiously, a highly related palindromic box was also identified between genes *smlt2137* (universal stress family protein) and *smlt2138* (transcriptional regulator *nfxB*) (Mitchell et al., 2014), two proteins that appear to be also involved in stress response. Nevertheless, determining whether SmoR could play an activator or a repressor role as well as which other genes could be under its regulation is something that will require further studies. Overall, our results suggest that in *S. maltophilia,* the *hchA-smoR* operon could have two important functions: (i) detoxification and recycling of secondary metabolites (HchA) and (ii) response to QS signals (SmoR), both deriving from situations of high cell density.

Interactions within microbial populations are common and essential for community maintenance (Ryan and Dow, 2008). *In vitro* studies of microbial consortia represent a first approach to uncover the complex interaction networks that occur between organisms in nature. Interspecific communication between *S. maltophilia* and *P. aeruginosa* has been a subject of investigation in the last years, since these two bacterial species usually share ecological niches and, importantly, they are frequently co-isolated in lungs of cystic fibrosis (CF) patients (Moskowitz et al., 2005). It has been reported that the DSF signal produced by *S. maltophilia* modulates *P. aeruginosa* behavior, including biofilm development and polymixin tolerance (Ryan et al., 2008) as well as virulence and persistence in lungs of CF patients (Twomey et al., 2012). However, as far as we know, the communication between these two potential human pathogens has been only studied in a unidirectional-way. We report here for the first time that *S. maltophilia* can also respond to signaling molecules produced by *P. aeruginosa*. In particular, we have shown that AHLs produced by *P. aeruginosa* stimulate swarming motility in *S. maltophilia* (**Figure 4**), a process in which SmoR might play a central role (**Figure 5**). Nevertheless, we cannot exclude that some other molecules present in the *P. aeruginosa* supernatant, such as rhamnolipids (Caiazza et al., 2005), could also participate in swarming stimulation. If so, it would explain the slightly stimulation observed in the Δ*smoR* mutant, which perhaps could initiate its swarming motility due to the presence of rhamnolipids rather than AHL signals.

It is well established that complex population behaviors such as swarming motility are commonly regulated by AHL-QS systems (Daniels et al., 2004). Furthermore, previous studies have demonstrated that heterologous expression of lactonase AiiA from *B. subtilis* (Dong et al., 2000) reduces AHLs production and swarming motility in *P. aeruginosa* (Reimmann et al., 2002) and *Burkholderia cepacia* species (Wopperer et al., 2006). Regulation of bacterial motility by LuxR solos has been also reported. In *Xoo*, OryR positively regulates swimming and swarming motility by binding to the promoter of numerous flagella genes in response to plant signaling molecules (González et al., 2013).

To date, the only QS system described in *S. maltophilia* is DSF-QS, which is based on the signaling fatty acid molecule 11-cis-2-decenoic acid (Huang and Lee Wong, 2007; Huedo et al., 2014). Previous studies have evidenced that the DSF system regulates several virulence-related processes including bacterial motility, biofilm formation, antibiotic resistance and virulence (Fouhy et al., 2007; Deng et al., 2011; Huedo et al., 2014). In the related bacterium *Burkholderia cenocepacia,* both QS systems co-exist: the DSF –designated BDSF– (Boon et al., 2008; Deng et al., 2010) and the AHL (Wopperer et al., 2006) systems. Interestingly, it has been shown that certain biological functions such as motility, biofilm formation and virulence, are co-regulated by the BDSF- and AHL-dependent QS systems in *B. cenocepacia* (Deng et al., 2009, 2013). Although AHL production has not been reported in *S. maltophilia*, our findings suggest that, besides DSF, exogenous AHL signals could also regulate QS-related phenotypes—as observed for swarming motility–, by interacting with the LuxR solo SmoR.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2015.00041/abstract

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An evolving perspective on the *Pseudomonas aeruginosa* orphan quorum sensing regulator QscR

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Many *Proteobacteria* govern responses to changes in cell density by using acyl-homoserine lactone (AHL) quorum-sensing (QS) signaling. Similar to the LuxI-LuxR system described in *Vibrio fischeri*, a minimal AHL QS circuit comprises a pair of genes, a *luxI*-type synthase gene encoding an enzyme that synthesizes an AHL and a *luxR*-type AHL-responsive transcription regulator gene. In most bacteria that utilize AHL QS, cognate *luxI* and *luxR* homologs are found in proximity to each other on the chromosome. However, a number of recent reports have identified *luxR* homologs that are not linked to *luxI* homologs; in some cases *luxR* homologs have been identified in bacteria that have no *luxI* homologs. A *luxR* homolog without a linked *luxI* homologs is termed an orphan or solo. One of the first reports of an orphan was on QscR in *Pseudomonas aeruginosa*. The *qscR* gene was revealed by whole genome sequencing and has been studied in some detail. *P. aeruginosa* encodes two AHL synthases and three AHL responsive receptors, LasI-LasR form a cognate synthase-receptor pair as do RhlI-RhlR. QscR lacks a linked synthase and responds to the LasI-generated AHL. QS regulation of gene expression in *P. aeruginosa* employs multiple signals and occurs in the context of other interconnected regulatory circuits that control diverse physiological functions. QscR affects virulence of *P. aeruginosa*, and although it shows sensitivity to the LasI-generated AHL, 3-oxo-dodecanoylhomoserine lactone, it's specificity is relaxed compared to LasR and can respond equally well to several AHLs. QscR controls a set of genes that overlaps the set regulated by LasR. QscR is comparatively easy to purify and study *in vitro*, and has become a model for understanding the biochemistry of LuxR homologs. In fact there is a crystal structure of QscR bound to the LasI-generated AHL. Here, we review the current state of research concerning QscR and highlight recent advances in our understanding of its structure and biochemistry.

Keywords: gene activation, cell-cell signaling, sociomicrobiology, acylhomoserine lactone, bacterial communication

THE *PSEUDOMONAS AERUGINOSA* QUORUM SENSING CIRCUIT

Quorum sensing (QS) is a cell-to-cell signaling mechanism that allows microbes to adjust their cooperation strategies according to local cell densities. Often QS systems operate by the extracellular release, dissemination, and uptake of acyl-homoserine lactone (AHL) molecules which, upon attaining a threshold concentration affect the activation or repression of target genes. QS systems have been identified in a wide variety of bacterial species where they allow cells to act cooperatively in varied behaviors such as pathogenesis, accessing nutrients, and organizing in groups as biofilms (Fuqua et al., 1996; Miller and Bassler, 2001).

One of the most intensively studied QS systems is that of *Pseudomonas aeruginosa*, a Gram-negative bacterium found in terrestrial and aquatic environments and as an opportunistic pathogen of a range of eukaryotes. *P. aeruginosa* causes infections among immune-compromised humans and in patients with burn wounds or with the genetic disorder cystic fibrosis, where it establishes chronic and stubborn infections. QS appears to play a vital role in the biology and pathogenesis of *P. aeruginosa*. The QS regulatory circuit in *P. aeruginosa* comprises two sub-circuits, Las and Rhl, which utilize the N-acyl homoserine lactone signal molecules 3OC12-HSL and C4-HSL, respectively (Passador et al., 1993; Ochsner et al., 1994; Pearson et al., 1994, 1995; Ochsner and Reiser, 1995). Each circuit includes an AHL signal synthase gene (*lasI* or *rhlI*), and another gene (*lasR* or *rhlR*) encoding a cognate receptor, which regulates target gene expression. The hierarchically dominant Las system positively regulates the Rhl system (Latifi et al., 1996; Pesci et al., 1997). Transcriptome studies suggest that over 300 genes—between 6 and 10% of the genome—are under QS control (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). In particular, the Las and Rhl systems are known to regulate the production of multiple extracellular virulence factors including elastase, alkaline protease, pyocyanin, and hydrogen cyanide (Passador et al., 1993; Brint and Ohman, 1995; Van Delden and Iglewski, 1998). Sequencing of the *P. aeruginosa* genome revealed the presence of a third LuxR homolog encoded by the ORF PA1898 (Stover et al., 2000). Unlike LasR and RhlR this homolog, later termed QscR, does not have an associated synthase gene.

A LuxR-TYPE REGULATOR WITHOUT A LINKED LuxI-TYPE SYNTHASE

Sequence alignment shows that QscR has a conserved amino-terminal AHL-binding domain and a conserved carboxy-terminal DNA-binding domain typical of the LuxR family of proteins (**Figure 1**) (Chugani et al., 2001). QscR is most closely related to BviR from *Burkholderia vietnamiensis* and an uncharacterized LuxR homolog from a methane oxidizing bacterium (**Figure 1**). QscR is not particularly closely related to LasR, RhlR, or to other orphan LuxR homologs.

Initial genetic studies of QscR suggested that this protein functions to modulate the activity of the Las and Rhl regulons. Both 3O-C12-HSL and C4-HSL accumulated to higher levels earlier in cultures of a *qscR* mutant compared to the wild type. Consistent with this finding, *lasI* and *rhlI* were both prematurely transcribed in the *qscR* mutant. Additional quorum-sensing-controlled genes representative of the Las and Rhl regulons were also expressed early and more strongly in the *qscR* mutant. Thus, QscR appeared to modulate the dynamics of the existing regulatory network by transiently repressing quorum controlled genes early in the growth phase. Cultures of the *qscR* deletion mutant overproduced the virulence factor pyocyanin, and production was evident at a lower culture density than wild type PAO1. The mutant was hypervirulent in the fruit fly *Drosophila melanogaster* infection model (Chugani et al., 2001).

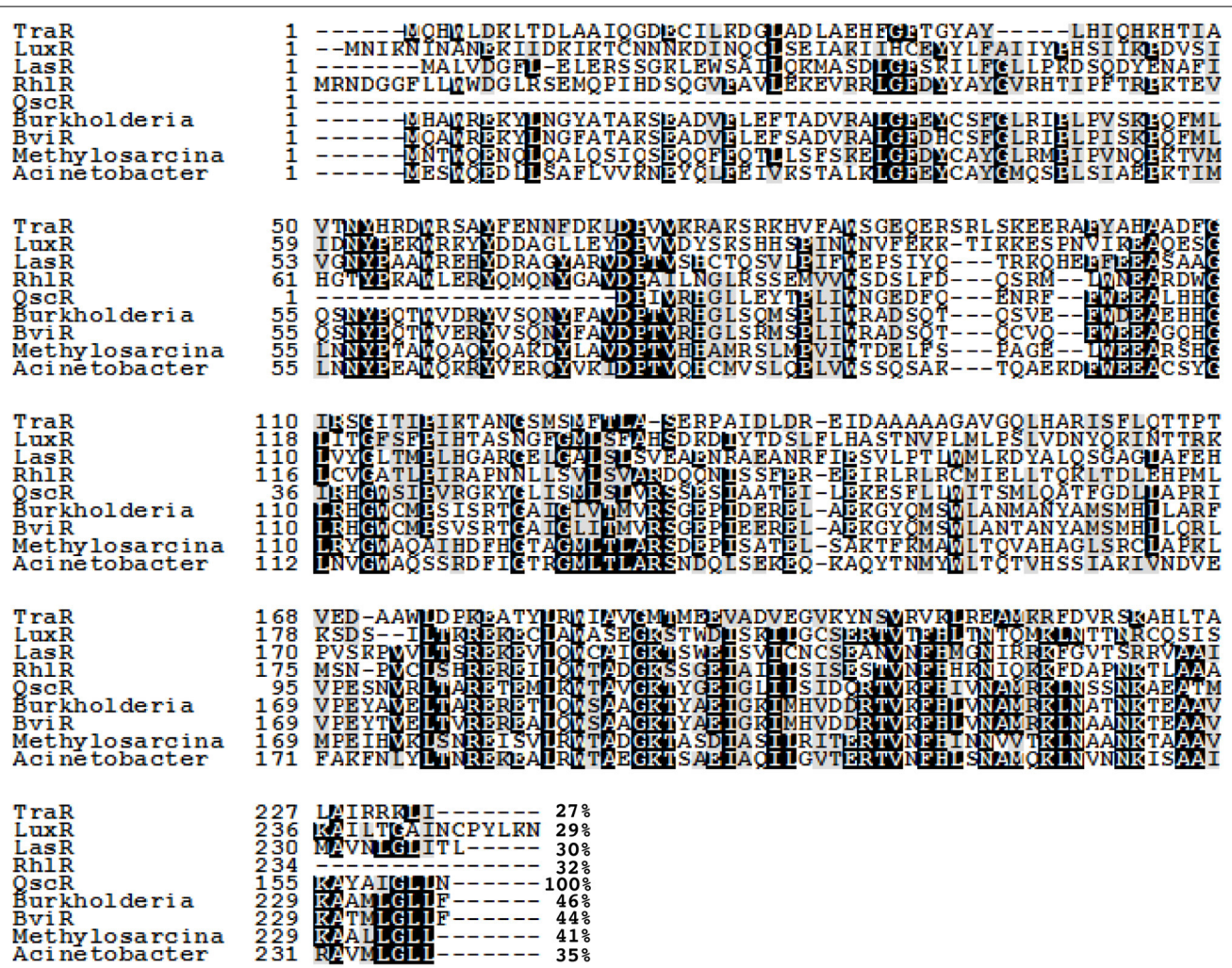

FIGURE 1 | An alignment of the QscR sequence with previously characterized LuxR homologs TraR, LuxR, RhlR, LasR, and BviR. We have also included sequences of three ORFs annotated as LuxR-family transcriptional regulators showing significant identity to QscR; *Methylosarcina lacus* (41% identity), *Burkholderia ambifaria* (46% identity), and *Acinetobacter baumannii* (35% identity). Conserved amino acids are shaded in black. Gray shading indicates that 100% of the residues are similar at that position. The numbers at the end of each sequence indicate the percent identity with QscR. The alignment was generated by using the MUSCLE multiple sequence alignment program and the degree of residue shading was determined by using Boxshade. The sequences used in the alignment and their GenBank or NCBI Reference Sequence (RefSeq) accession numbers are *Agrobacterium tumefaciens* TraR (RefSeq: YP_001967610.1), *V. fischeri* LuxR (GenBank: M96844), *P. aeruginosa* LasR (GenBank: M59425), *P. aeruginosa* RhlR (GenBank: L08962), *Burkholderia cepacia* BviR(GenBank: AAK35156.1), *Burkholderia ambifaria* (RefSeq: WP_006749592.1), *Methylosarcina lacus* (RefSeq: WP_024298126.1), and *Acinetobacter baumannii* (GenBank: EXS59053.1).

QscR RESPONDS TO MULTIPLE AHLs

Preceding studies with LuxR homologs had shown that purification of active, soluble protein required the presence of the cognate AHL during growth (Zhu and Winans, 1999; Schuster et al., 2004; Urbanowski et al., 2004). Overproduction of His-tagged QscR in a *P. aeruginosa* strain producing 3OC12-HSL yielded soluble QscR (Lee et al., 2006). There was a direct correlation between the soluble fraction and the presence of 3OC12-HSL but not C4-HSL, the second *P. aeruginosa* AHL. The *qscR* gene is divergently transcribed from PA1897, which codes for a hypothetical protein, and the *qscR*-PA1897 intergenic region includes an inverted repeat showing similarity to known AHL binding sites. DNase I footprinting analysis with purified QscR and added 3OC12-HSL identified this inverted repeat as a bona fide QscR binding site. This was further confirmed by gel-shift experiments, which also showed that binding of QscR was cooperative. This study seemed to suggest that the *in vitro* DNA-binding activity of QscR was strongly dependent upon exogenously added 3OC12-HSL. Experiments using a transcriptional reporter in *Escherichia coli* confirmed that PA1897 was directly activated by QscR-3OC12-HSL. An *in silico* search of the PAO1 genome identified a second gene, PA5351 that encodes rubredoxin 1, that had a putative QscR binding site in its promoter. Although QscR-3OC12-HSL can bind to this site, the binding was not cooperative and the apparent binding affinity was lower than the affinity for the PA1897 binding site. A limited *in vitro* analysis of the promoters of PA1897 and selected LasR-regulated genes revealed that despite significant similarity between the binding sites and use of a common ligand by the two transcription factors, QscR is unable to activate LasR-dependent genes and vice versa. Thus, although QscR appears to transiently repress a subset of LasR and RhlR regulated genes, this regulation appears to be largely indirect and the mechanism by which this occurs remains unclear. A particularly intriguing finding was that QscR has a broad signal specificity. It can activate a PA1897–*lacZ* reporter at nM concentrations of C8, C10, 3OC10, C12, 3OC12, and C14-HSLs (**Figure 2**). This suggests that in certain mixed speices populations QscR may integrate multispecies signaling by responding to AHLs produced by other bacteria.

In an *in vivo* chemical cross-linking analysis using *E.coli* producing QscR with either LasR or RhlR in the absence of AHL, Ledgham et al. (2003) identified apparent QscR-LasR and QscR-RhlR heterodimers. Based on fluorescence anisotropy analysis of the *E. coli* cells, it was proposed that QscR exists as an oligomer in *E. coli* and that it is destabilized by addition of either 3O-C12-HSL or C4-HSL. This work added to the possible explanations for the QscR-mediated delays in expression of several LasR- and RhlR-activated genes. QscR might directly interact with LasR- or RhlR-controlled promoters, QscR might bind to 3OC12-HSL, the LasR signal, or C4-HSL, the RhlR signal, or QscR might form inactive heterodimers with LasR or RhlR.

THE QscR REGULON INCLUDES GENES THAT ARE DIRECTLY AND INDIRECTLY REGULATED

For a more comprehensive analysis of the QscR regulon, the transcriptomes of the *qscR* mutant and PAO1 were compared at several points during growth by using microarrays (Lequette et al., 2006). This study identified 424 genes that were QscR-controlled.

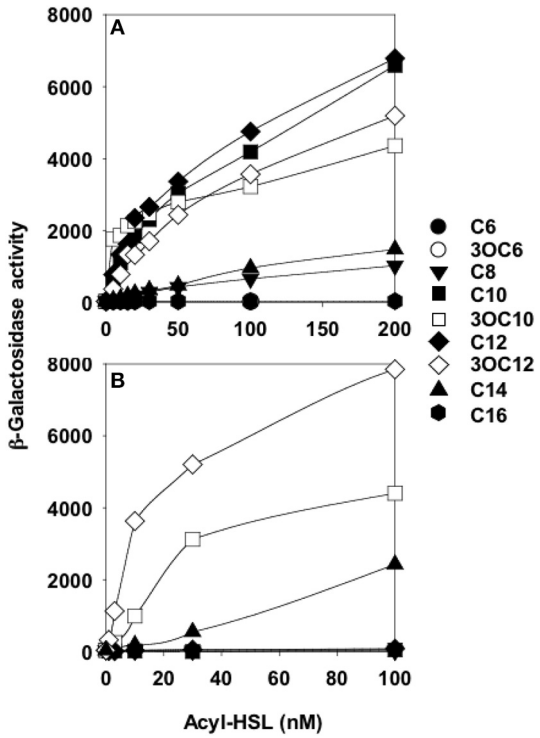

FIGURE 2 | QscR, unlike LasR, responds to multiple AHLs. Shown are *lacZ* reporter expression levels for **(A)** *E. coli* containing a *qscR* expression vector and the PA1897–*lacZ* reporter and **(B)** *E. coli* containing a *lasR* expression vector and the *lasI*–*lacZ* reporter. The acyl chains of each AHL are indicated on the right from Lee et al. (2006).

Among these, 76 genes were induced and a majority (329 genes) were repressed by QscR. It was conceivable that a subset of genes, particularly repressed genes, was regulated indirectly, perhaps through the formation of heterodimers with LasR or RhlR or through signal sequestration; mechanisms independent of the ability of QscR to bind DNA. In contrast, genes that were activated by QscR and genes that were not part of the LasR or RhlR regulons were more likely directly regulated by QscR and dependent on its DNA binding activity. To identify this set of genes, *P. aeruginosa qscR* mutants containing an inducible chromosomal copy of either full-length *qscR* or a *qscR* allele lacking the DNA-binding domain were transcriptionally profiled under inducing conditions. A set of 38 QscR-regulated genes identified in the previous microarray experiment also required the QscR DNA-binding domain. Some of these genes were activated and some were repressed by elevated expression of full-length *qscR*.

BIOCHEMICAL ANALYSIS OF QscR SUGGESTS A NEW MODEL FOR LuxR HOMOLOGS

A biochemical analysis of purified native QscR provided unexpected insights into the properties of QscR that pointed to a new model for LuxR homologs in their free and signal-bound states (Oinuma and Greenberg, 2011). As observed in a previous study using His-tagged QscR (Lee et al., 2006), *E. coli* grown in the presence of 3OC12-HSL yielded higher levels of soluble native

QscR compared to cells grown without added 3OC12-HSL. QscR seemed to retain 3OC12-HSL throughout the purification process and was therefore able to bind target DNA in the absence of added 3OC12-HSL. However, DNA binding was significantly stimulated by addition of 3OC12-HSL to the reaction buffer. These seemingly curious findings can be explained by the equilibrium binding dynamics of QscR and 3OC12-HSL. Dilution of purified QscR-3OC12-HSL into DNA-binding buffer likely causes a shift in equilibrium toward the ligand-free form of QscR and a release of 3OC12-HSL. As expected then, addition of 3OC12-HSL to the reaction stimulates DNA binding.

As shown previously with His-tagged QscR (Lee et al., 2006), gel filtration experiments with native QscR showed that at a low concentration it exists as a monomer in solution. However, at higher concentrations QscR seemed to dimerize in a concentration dependent manner.

Although previous work showed that 3OC6-HSL was not able to activate transcription of the PA1897-*lacZ* reporter fusion in recombinant *E. coli* (Lee et al., 2006), the presence of 3OC6-HSL did facilitate production of soluble QscR. Experiments with QscR purified from *E.coli* grown in the presence of 3OC6-HSL indicated that ligand-free QscR is unstable and that acyl-HSL binding seems to stabilize the protein. Evidence exists in favor of the idea that other LuxR homologs may also be stabilized by acyl-HSLs both *in vitro* and *in vivo* (Zhu and Winans, 1999, 2001). Purification of several other LuxR homologs in the soluble form seems to be contingent upon the presence of their cognate acyl-HSLs. The TraR protein in *Agrobacterium tumefaciens* is targeted for proteolysis in the absence of its ligand 3OC8-HSL (Zhu and Winans, 2001). In fact, studies with TraR supported a view that LuxR family members may be unable to fold into an active polypeptide in the absence of an appropriate AHL.

Experiments done with purified QscR-3OC6-HSL point to an alternate model for LuxR homolog states *in vivo*. Reporter gene fusion experiments showed that QscR-3OC6-HSL is able to activate transcription only slightly. Purified QscR-3OC6-HSL remained soluble although it could bind target DNA only at high concentrations of the AHL. Further experiments showed that QscR binds 3OC6-HSL with a low affinity and that it was possible to replace 3OC6-HSL with other AHL ligands before QscR aggregated into an inactive form. For example, QscR that had dissociated from 3OC6-HSL upon dilution could bind target DNA if 3OC12-HSL was present in the reaction buffer. The fact that QscR could disengage from 3OC6-HSL and bind DNA in the presence of another AHL questioned the prevailing view that in order to maintain a functional conformation, nascent polypeptides of LuxR homologs are required to fold around their cognate AHLs (Zhu and Winans, 1999, 2001; Schuster et al., 2004). A more likely model, which was consistent with the new findings, proposed that the nascent polypeptides fold into functional monomers that are relatively unstable (**Figure 3**). The monomers can bind their cognate AHLs and can form homodimers that bind DNA targets with a high affinity. However, in the absence of suitable AHLs the protein returns to its monomeric state and the monomers either refold into non-functional aggregates or are subject to proteolysis.

The availability of soluble QscR through purification in presence of 3OC6-HSL also made assays for activity and binding

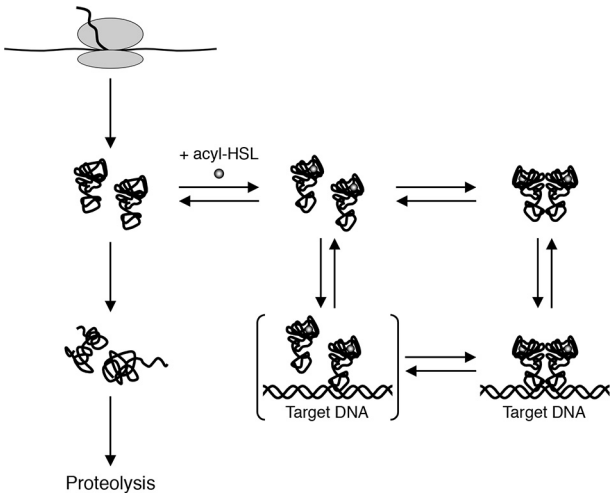

FIGURE 3 | A model for biochemical states of QscR *in vivo*. Nascent QscR polypeptides can fold into active forms and exist as monomers at low concentrations. The monomers can either refold into inactive forms that are subsequently proteolyzed or they can bind AHLs and form homodimers capable of binding target DNA from Oinuma and Greenberg (2011).

dynamics in presence of other AHLs feasible. Purified QscR-3OC6-HSL was used to estimate binding affinities of QscR for other AHLs and for target DNA. Results from gel-shift DNA-binding experiments showed that QscR has a similar binding affinity for 3OC12-, C12-, and C10-HSLs. The affinity for 3OC10-HSL is lower than for the other AHLs. For all tested AHLs, binding of QscR to target DNA appeared to be cooperative (Oinuma and Greenberg, 2011).

CRYSTAL STRUCTURE OF QscR-3OC12-HSL

Detailed biochemical and structural studies of LuxR homologs have often been hampered due to protein instability in absence of ligand or protein insolubility at high concentrations. The biochemical characterization of QscR provided a basis for obtaining a substantial amount of soluble and stable QscR. It was thus feasible to determine the crystal structure of full-length QscR when bound to 3OC12-HSL (**Figure 4**). Previously, structures of full-lengthTraR (*Agrobacterium tumefaciens*) bound to 3OC8-HSL and target DNA, full-length CviR (*Chromobacterium violaceum*) bound to antagonist, and the N-terminal AHL-binding domains of LasR (*P. aeruginosa*) and SdiA (*E. coli*) had been reported (Vannini et al., 2002; Zhang et al., 2002; Yao et al., 2006; Bottomley et al., 2007; Chen et al., 2011). The crystal structure of QscR revealed that the dimer bound to 3OC12-HSL has some shared and some unique features when compared with other available LuxR structures (Lintz et al., 2011). A feature shared with CviR but not with TraR, is the almost symmetric cross-subunit conformation of the QscR homodimer that allows for an extensive dimerization interface. Mutational analyses of QscR confirmed the *in vivo* functional relevance of the interface and suggested that dimerization is important for the response of QscR to AHLs. Further, modeling of QscR-DNA interactions suggests that the DNA-binding domains in the

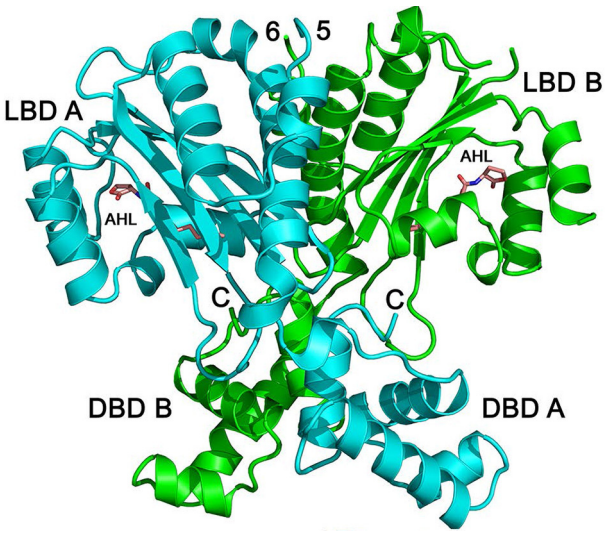

FIGURE 4 | Structure of QscR bound to 3OC12-HSL. The QscR monomers are shown in cyan and green and 3OC12-HSL is shown in orange. The ligand-binding domain (LBD) and the DNA- binding domain (DBD) are also indicated for each chain from Lintz et al. (2011).

cross-subunit conformation are poised to bind DNA. Because the CviR structure was determined with bound antagonists and not AHLs, the generalized relevance of the cross-subunit architecture is unclear. The results with QscR seem to suggest that this structural similarity may be a more generalized feature of LuxR homologs.

As expected of two LuxR-type proteins that recognize the same ligand, the binding pocket surface areas, packing densities, and pocket volumes are almost identical in QscR and LasR. As noted earlier, QscR has a relaxed specificity for AHLs compared to LasR (Lee et al., 2006). A comparison with equivalent residues in other known LuxR structures suggested that Ser56 of QscR influences its specificity for AHLs allowing both 3-oxo-substituted and unsubstituted AHLs to bind. It was proposed that differences in the interactions involving the 3-oxo-position of the acyl chain may form the basis of the relaxed signal specificity relative to LasR.

The ligand-binding domains of 3OC12-HSL-bound QscR and LasR also show a conserved "internalized" conformation of the AHL in the binding pocket whereby the acyl chain of 3OC12-HSL is similarly embedded in a cavity near the region that forms the ligand-binding and the DNA-binding domain interface in QscR (Bottomley et al., 2007; Zou and Nair, 2009). This conformation, which influences the interactions between the ligand-binding and the DNA-binding domains is not observed in AHLs or antagonists with short acyl chains. Thus, it is likely that the acyl-chain length plays a role in the mode of AHL binding. In contrast to other LuxR-type proteins, homodimers of both QscR and LasR have almost identical interactions between the AHL-binding domains. Taken together, the data suggest that the conformation of the QscR and LasR-bound AHL likely promotes dimerization and subsequent binding of the dimer to DNA through an allosteric mechanism.

A structural comparison of 3OC12-HSL-bound QscR with TraR-3OC8-HSL-DNA revealed some interesting differences. While QscR-3OC12-HSL is nearly symmetric, the subunit architecture of TraR-3OC8-HSL bound to DNA is asymmetric and unlike QscR, shows fewer interactions between the ligand-binding and DNA-binding domains. Evidence from structural analyses of TraR bound to its antiactivator TraM suggests that ligand-bound TraR is also unlikely to adopt the cross-subunit architecture observed with QscR. The linker between the ligand-binding and the DNA-binding domains may have evolved for distinct functions in QscR and TraR. In QscR and perhaps LasR, the linker functions to allow extensive contacts with the ligand-binding and DNA-binding domains whereas in case of TraR, the linker probably evolved to bind TraM and not necessarily to interact with the ligand-binding and DNA-binding domains. Thus, the differences in architectures of full-length LuxR proteins may be reflective of differences in their physiological activities and interactions with other regulatory factors.

While the structure of CviR bound to antagonists resembled QscR-3OC12-HSL in the near symmetry of the cross-subunit architecture, unlike QscR, which is poised to bind DNA, the DNA-binding domains of CviR are in a conformation that prevents DNA binding. The structural studies suggest that this architecture would allow conversion of CviR from the inactive form to a form that resembles QscR through only a small configurational alteration that permits interaction between the C-terminal dimerization helices. Further studies will be required to determine whether this configuration persists in active forms of the proteins when bound to target DNA.

A few groups have used a biochemical approach to generate tools that could aid in the study of QscR. Liu et al. (2010) used *in vitro* and *in vivo* assays to identify several furanones that inhibit 3OC12-HSL-dependent activation of QscR. In another study, Mattmann et al. (2011) designed several highly active QscR-selective agonists and antagonists. This study also found that the most potent antagonists for QscR had sterically bulky acyl chains (for example, N-benzoyl acyl groups) and that agonists had branched lipophilic acyl groups. More recently, Weng et al. (2014) reported that a synthetic ligand with an open ring structure, N-decanoyl-l-homoserine benzyl ester, was able to activate QscR. This work suggested that the acyl chain may be more critical for ligand binding to QscR than the lactone ring.

CONCLUSIONS

P. aeruginosa QS is one of the best understood cell-cell signaling systems in bacteria. It has two synthase and receptor pairs that allow it to respond to self-generated AHL signals and a third orphan receptor with no cognate synthase. In this review we have summarized advances in our understanding of the *P. aeruginosa* orphan or solo QS receptor QscR. Although much has been learned, especially about the biochemistry and structure of QscR, many questions still remain unanswered. While it is clear that QscR influences the expression of genes controlled by both the Las and the Rhl systems, the precise mechanism by which this is affected remains unclear. Bacterial populations in nature exist as dynamic and often polymicrobial communities. QscR responds not only to the *P. aeruginosa* signal 3OC12-HSL but also

to multiple other AHLs. It thus has the potential to significantly widen the scope of QS regulation in *P. aeruginosa* through integration of signals produced by other cohabiting species. Although intriguing, the complex nature of microbial growth in mixed communities makes it challenging to test this possibility under laboratory conditions.

A complex regulatory network such as the *P. aeruginosa* QS system operates on a global scale. It controls the expression of numerous genes and is in turn also subject to regulation by other modulators. For example, *lasR* transcription is modulated by Vfr and RsaL (Fagerlind et al., 2003; Viretta and Fussenegger, 2004; Ward et al., 2004). Liang et al. (2012) showed that VqsR binds to the *qscR* promoter region and negatively regulates *qscR*. It is likely that future studies will identify additional modulators of QscR activity or synthesis. It is also important to note that although QS controlled genes can also be regulated at a translational or post-transcriptional level, much of the available information on QscR and *P. aeruginosa* QS is based on transcriptome analyses. Another level of regulation of QS systems involving sRNAs has been described in *Vibrio fischeri* and *Vibrio harveyi* (Lenz et al., 2004; Tu and Bassler, 2007). The possibility of similar regulatory mechanisms in *P. aeruginosa* will have to be considered as well.

QscR has been characterized in great detail by biochemical methods and through structural analysis. These studies have revealed a wealth of information and established a framework for further understanding of not only QscR but other LuxR homologs as well. Because QscR and LasR respond to the same ligand, recent reports of QscR-specific modulators including small molecules and synthetic AHLs provide a means to selectively understand the role of QscR within the *P. aeruginosa* QS system. This approach could help address the larger question regarding the utility of a QS circuit that includes two signal receptors with a shared ligand.

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Corrigendum: An evolving perspective on the *Pseudomonas aeruginosa* orphan quorum sensing regulator QscR

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A corrigendum on

An evolving perspective on the *Pseudomonas aeruginosa* orphan quorum sensing regulator QscR
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Figure 1 of the article by Chugani and Greenberg contains errors in the QscR sequence used in the alignment, which we hereby rectify. In the original figure the N-terminal portion of QscR shows as dashes. We resubmit **Figure 1** with corrections in the sequence.

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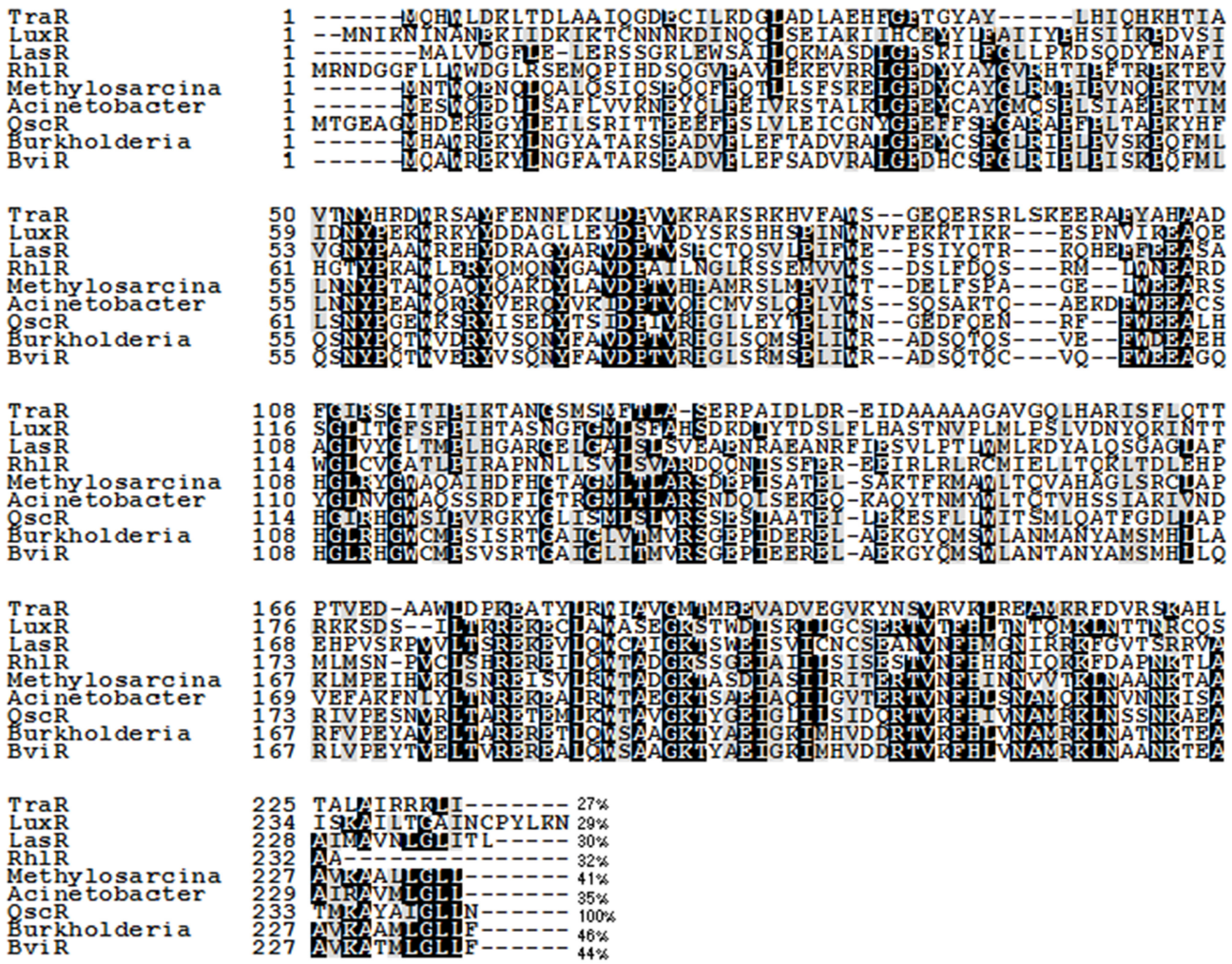

FIGURE 1 | An alignment of the QscR sequence with previously characterized LuxR homologs TraR, LuxR, RhlR, LasR, and BviR. We have also included sequences of three ORFs annotated as LuxR-family transcriptional regulators showing significant identity to QscR; *Methylosarcina lacus* (41% identity), *Burkholderia ambifaria* (46% identity), and *Acinetobacter baumannii* (35% identity). Conserved amino acids are shaded in black. Gray shading indicates that 100% of the residues are similar at that position. The numbers at the end of each sequence indicate the percent identity with QscR. The alignment was generated by using the MUSCLE multiple sequence alignment program and the degree of residue shading was determined by using Boxshade. The sequences used in the alignment and their GenBank or NCBI Reference Sequence (RefSeq) accession numbers are *Agrobacterium tumefaciens* TraR (RefSeq: YP_001967610.1), *V. fischeri* LuxR (GenBank: M96844), *P. aeruginosa* LasR (GenBank: M59425), *P. aeruginosa* RhlR (GenBank: L08962), *Burkholderia cepacia* BviR (GenBank: AAK35156.1), *Burkholderia ambifaria* (RefSeq: WP_006749592.1), *Methylosarcina lacus* (RefSeq: WP_024298126.1), and *Acinetobacter baumannii* (GenBank: EXS59053.1).

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XocR, a LuxR solo required for virulence in *Xanthomonas oryzae* pv. *oryzicola*

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Xanthomonas oryzae pv. *oryzicola* (*Xoc*) causes bacterial leaf streak (BLS) in rice, a serious bacterial disease of rice in Asia and parts of Africa. The virulence mechanisms of *Xoc* are not entirely clear and control measures for BLS are poorly developed. The solo LuxR proteins are widespread and shown to be involved in virulence in some plant associated bacteria (PAB). Here, we have cloned and characterized a PAB LuxR solo from *Xoc*, named as XocR. Mutation of *xocR* almost completely impaired the virulence ability of *Xoc* on host rice, but did not alter the ability to trigger HR (hypersensitive response, a programmed cell death) on non-host (plant) tobacco, suggesting the diversity of function of *xocR* in host and non-host plants. We also provide evidence to show that *xocR* is involved in the regulation of growth-independent cell motility in response to a yet-to-be-identified rice signal, as mutation of *xocR* impaired cell swimming motility of wild-type Rs105 in the presence but not absence of rice macerate. We further found that *xocR* regulated the transcription of two characterized virulence-associated genes (*recN* and *trpE*) in the presence of rice macerate. The promoter regions of *recN* and *trpE* possessed a potential binding motif (an imperfect *pip* box-like element) of XocR, raising the possibility that XocR might directly bind the promoter regions of these two genes to regulate their transcriptional activity. Our studies add a new member of PAB LuxR solos and also provide new insights into the role of PAB LuxR solo in the virulence of *Xanthomonas* species.

Keywords: *Xoc*, QS, LuxR, virulence, motility

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Introduction

Quorum sensing (QS) is a cell-density dependent cell-cell communication system that relies on small chemical signal molecules to control bacterial behavior and coordinate gene expression in a cell-density dependent manner (Fuqua et al., 1994). The QS-controlled bacterial behaviors are diverse, including biofilm formation, cell motility, antibiotic production, light production and sporulation (Nealson et al., 1970; Danhorn and Fuqua, 2007; Goo et al., 2010; Qian et al., 2014). In Gram-negative bacteria, the *N*-acyl homoserine lactones (AHLs) are a major class of signal molecules. This signal is composed of a homoserine lactone ring carrying C_4-C_{18} acyl chains and is thought to function as intraspecies communication (Fuqua et al., 2001; Fuqua and Greenberg, 2002; Ng and Bassler, 2009).

A typical AHL QS system is composed of homologs of the LuxI and LuxR proteins that were first identified in *Vibrio fischeri* (Nealson et al., 1970; Fuqua and Winans, 1996). LuxI homologsare AHL synthases and direct the synthesis of autoinducer molecules. A LuxR-family protein that is part of a QS system has a *N*-terminal AHL-binding and a C-terminal DNA-binding domains. These LuxR-family proteins can bind AHLs, and consequently, the stable LuxR-AHL complex binds at specific gene regulatory sequences called as *lux* box to activate or repress the transcription of target genes (Fuqua et al., 2001). Notably, these two proteins are in most cases genetically adjacent to each other in a canonical AHL QS system.

Additional LuxR-family proteins similar to QS LuxR homologs are being identified by analysis of sequenced bacterial genomes and many of these are without a cognate LuxI protein. These LuxR proteins lacking a genetically linked LuxI have been termed LuxR orphans or solos (Fuqua, 2006; Patankar and Gonzalez, 2009; Subramoni and Venturi, 2009; Gonzalez et al., 2013). Studies so far show that some LuxR solos respond to endogenous AHLs (e.g., QscR from *Pseudomonas aeruginosa*) or to exogenous AHLs produced by neighboring bacteria, such as the SdiA from *Salmonella enterica* and *E. coli* to regulate target genes (Ahmer, 2004; Fuqua, 2006).

Recently, a sub-group of LuxR solos has been identified to be very common in several plant-associated bacteria (PAB); these PAB LuxR solos do not bind AHLs, but respond to yet unidentified plant signal(s)/compound(s) (Ferluga et al., 2007; Chatnaparat et al., 2012). PAB LuxR solos differ in the conservation of one or two of the six invariant amino acids in the AHL-binding domain that have been reported to be important for signal/ligand binding (Gonzalez and Venturi, 2013). Examples of this subfamily are required for full virulence in several phytopathogenic bacteria and include XccR of *Xanthomonas campestris* (Zhang et al., 2007); OryR of *X. oryzae* (Ferluga et al., 2007); XagR of *X. axonopodis* (Chatnaparat et al., 2012) and PsaR2 of *P. syringae* pv. *actinidiae* (Patel et al., 2014).

Bacterial leaf streak (BLS) caused by *X. oryzae* pv. *oryzicola* (*Xoc*) is an important disease of rice in Asia and parts of Africa (Nino-Liu et al., 2006). *Xoc* is a Gram-negative bacterium which produces a characteristic yellow pigment. This pathogen penetrates the rice leaf mainly through stomata, multiplies in the substomatal cavity, and colonizes the parenchyma apoplast causing interveinal lesions (Wang et al., 2007). *Xoc* can also gain access through wounds, but does not invade the xylem because it is restricted by the mesophyll tissue apoplast (Nino-Liu et al., 2006). We identified a PAB LuxR solo homolog in the genome of *Xoc* strain BLS256 and designated it as XocR. In the present study, the role of *xocR* in virulence as well as cell motility is presented and discussed.

Materials and Methods

Bacterial Strains, Culture Media, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *Xoc* strains were grown at 28°C in nutrient broth (NB) medium (beef extract, 3 g/l; yeast extract, 1 g/l; polypeptone, 5 g/l; sucrose, 10 g/l) or on nutrient agar (NA). Medium containing macerated rice material was prepared as described previously (Gonzalez et al., 2013). *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium or on LB agar plates. When required, antibiotics were added to the medium at the following final concentrations: 50 μg/mL kanamycin (Km) for *E. coli* and *Xoc*, 10 μg/mL gentamicin (Gm) for *E. coli* and *Xoc*, and 100 μg/mL rifampicin (Rif) for *Xoc*.

Generation of the xocR Deletion Mutant in X. Oryzae Pv. Oryzicola

Deletion mutants were generated as described previously (Qian et al., 2013b). The *Xoc* wild-type Rs105 was used as the parental strain to generate the in-frame deletion mutant via allelic homologous recombination. In-frame deletion of *xocR* was performed as described previously (Figure S1A). Briefly, two *xocR* flanking regions were generated by polymerase chain reaction (PCR) using the primer pairs *xocR*-1F/*xocR*-1R and *xocR*-2F/*xocR*-2R (Table S2). The *xocR*-1 fragment (digested with *Bam*HI and *Hin*dIII) and the *xocR*-2 fragment (digested with *Hin*dIII and *Xba*I) were ligated into *Bam*HI/*Xba*I-digested pK18mob*sacB* (Schafer et al., 1994). This construct, designated pK18-xocR, was transformed into the wild-type Rs105 by electroporation. Transconjugants were selected on NA plates without sucrose but with Rif (100 μg/mL) and Km (50 μg/mL). Positive colonies were plated on NA plates containing 10% (w/v) sucrose and Rif (100 μg/mL) to select for resolution of the construct by a second cross-over event. The resulting mutant, containing the *xocR* in-frame deletion, was confirmed by PCR (Figure S1B). One of the confirmed mutants, named as Δ*xocR*, was selected for further study.

Complementation of the xocR Mutant

The generation of complemented strains was performed as described previously (Qian et al., 2013a). Briefly, a 1664-bp DNA fragment containing *xocR* and its predicted promoter region was amplified from wild-type Rs105 with xocRH-F/xocRH-R primers (Table S2). The PCR fragment was digested with the *Bam*HI/*Xba*I enzyme and cloned into *Bam*HI/*Xba*I-digested pBBR-MCS5 (Kovach et al., 1995) resulting in the complemented construct (pBBR-XocR). This construct was transformed into Δ*xocR* competent cells by electroporation. Finally, one positive complemented strain named Δ*xocR*(*xocR*) was selected on NA plates with Rif and Gm, and verified by PCR method. This final complemented strain, named as Δ*xocR* (*xocR*) was used for further study.

Pathogenicity Testing and Determination of Bacterial Load *in planta*

The pathogenicity testing and determination of bacterial load assays were performed as described previously (Guo et al., 2012; Qian et al., 2013b). Briefly, *Xoc* strains were incubated in NB broth with appropriate antibiotics at 28°C until the growth reached to exponential phase ($OD_{600} = 0.5$). Then, cells were pelleted by centrifugation at 3099 × g and suspended in an equal volume of sterilized ddH_2O. The final

cell suspension was inoculated into the leaves of 2-week-old rice plants (Shanyou-63, susceptible to BLS) using needless syringe. Water-soaking symptoms were measured 7 days after inoculation. Twenty-five leaves were inoculated for each *Xoc* strain in each treatment. The same experiment was performed three times.

Growth of each *Xoc* strain in rice leaf tissue was detected by homogenizing five inoculated leaves in 9-mL sterile water. The leaves were cut in 6-mm sections around the inoculation spots on days 0, 7 after inoculation (Lee et al., 2008). Diluted homogenates were plated on NA plates supplemented with Rif (for the wild type and mutant). The number of bacterial colonies on these plates was counted after 2 days of incubation at 28°C (Feng et al., 2009). Each diluted homogenate was plated on three plates, respectively. Three replicates for each treatment were used, and the experiment was performed three times.

Hypersensitive Response (HR) Assay

The hypersensitive response assay was performed as described previously (Zou et al., 2006; Qian et al., 2013b). *Xoc* strains were incubated in NB broth with appropriate antibiotics at 28°C until exponential phase (OD_{600} = 0.5). Cells were pelleted by centrifugation at 3099 × g and suspended in different volume of sterilized ddH_2O resulting at three different concentrations of cell suspension (OD_{600} = 0.2, 0.4, and 0.8). These three cell suspensions were used for HR tests. Briefly, *Xoc* strains were infiltrated into the leaves of greenhouse-grown tobacco (*Nicotiana tabacum* L. cv. *Samsun*), and the results were observed after 48 hours of infiltration. Four leaves were inoculated for each *Xoc* strain in each treatment. The same experiment was performed three times.

Determination of Bacterial Growth Ability *in vitro*

Bacterial growth was monitored as described previously (Qian et al., 2013b). To investigate bacterial growth *in vitro,* we tested the growth rate of Δ*xocR* in nutrient-rich broth (NB) and NB with rice macerate medium. In brief, *Xoc* strains were pre-incubated in NB broth at 28°C with shaking at 200 rpm, until the growth was reached to OD_{600} of 1.0. Cells were pelleted by centrifugation at 3099 × g and suspended in an equal volume of sterilized water. Then, 1 mL of cell suspension was inoculated into 100 mL of two testing media. All inoculation broths were grown at 28°C with shaking at 200 rpm and the OD_{600}value was determined every 4 h until bacterial growth reached to the stationary stage. The experiments were performed three times, and each involves three replicates.

Determination of Cell Motility, EPS Production, Protease Activity, and Biofilm Formation between Wild-type Strain and the xocR Mutant in the Absence or Presence of Rice Macerate

Swimming motility assay was performed as described previously (Gonzalez et al., 2013). In brief, *Xoc* strains were grown in nutrient-rich broth (NB) broth at 28°C with shaking at 200 rpm, until the value of OD_{600} reached 1.0, and then 3 μl of each strain was inoculated onto the surface of the motility plates, including NA containing 0.3% soft agar with and without of rice macerate. Cell motility was examined at 72 h after incubation at 28°C. Five independent motility assays were performed for each strain.

EPS production of the bacterium was measured as described previously with some modifications (Tang et al., 1991). Briefly, *Xoc* strains were pre-incubated in NB broth at 28°C with shaking at 200 rpm, until the value of OD_{600} to be 1.0. Cells were pelleted by centrifugation at 3099 × g and suspended in an equal volume of sterilized ddH_2O. Then, 0.5 mL of cell suspension was inoculated into 50 mL of NB or NB with rice macerate. These cultures were grown at 28°C with shaking at 200 rpm for 5 days. EPS was precipitated from the culture supernatant with two volumes of ethanol and dried to a constant weight at 80°C. The difference between the two weights was used to estimate the production of EPS per milliliter of culture. Each experiment was performed three times, and each treatment involved three replicates.

To measure extracellular protease activity, *Xoc* strains were incubated in NB medium at 28°C with shaking at 200 rpm, until the OD_{600} to be 1.0. Then, 3 μL of bacterial culture was spotted on NA plates or NA with rice macerate. Both media contained 1% (m/v) skim milk powder. After 48 h of incubation at 28°C, protease activity was assessed according to the hydrolytic zones around the bacterial colonies (Ryan et al., 2006). Each treatment involves three replications, and the same experiment was performed three times.

To test biofilm formation on abiotic surfaces, *Xoc* strains were pre-incubated in NB broth at 28°C with shaking at 200 rpm, until the value of OD_{600} to be 1.0. Cells were pelleted by centrifugation at 3099 × g and suspended in an equal volume of sterilized ddH_2O. Then, 30 μL of cell suspension was inoculated into 3 mL of NB or NB with rice macerate. These cultures were incubated at 28°C without shaking for 5 days. After gentle removing the suspension, two volume of 10% crystal violet solution was added and treated for 1 h, then the glass tubes were gently washed three times with sterile ddH_2O and air dried for 1 h; then, 3 mL of 40% methanol and 10% glacial acetic acid was added to glass tubes to dissolve the crystal violet stain (Koczan et al., 2009). The dissolved crystal violet was quantified through spectrophotometry at an absorbance of 575 nm by using a Safire microplate reader (Tecan, Research Triangle Park, NC). Each experiment included three replicates and experiments were repeated three times.

Real-Time PCR

The assays of RNA extraction, cDNA synthesis and real-time PCR were performed as described previously (Chatnaparat et al., 2012; Qian et al., 2013b). Quantitative real-time reverse transcription PCR (qRT-PCR) was carried out using a SYBR Premix EX Tag™ II kit (TaKaRa Bio, Shiga, Japan) in an ABI PRISM 7500 Real-Time PCR System (FApplied Biosystems, Foster City, CA, USA). As the endogenous control, 16S rRNA was used. The primer sequences were listed in Table S3. RNA was extracted from *Xoc* wild-type strain Rs105 and Δ*xocR* by using the RNAiso Plus Reagent (TaKaRa Bio) following the manufacturer's instructions. To remove genomic DNA, the eluted RNA samples were treated with RNase inhibitors and DNaseI (TaKaRa Bio). RNA integrity was confirmed by electrophoresis on 1.2% agarose gels. Then,

2 μg of each RNA sample was used to synthesize cDNA with a cDNA Synthesis kit (TaKaRa). The same experiment was performed three times. The transcriptional levels of *XOC_1737* and *XOC_4211* in the wild-type Rs105 and the *xocR*-deletion mutant were also assessed and compared.

Results and Discussion

XocR of *Xoc* Belongs to a Sub-Family of PAB LuxR Solos

To examine whether the genome of *Xoc* has potential LuxR solos, OryR which is a PAB LuxR solo from *Xanthomonas oryzae* pv. *oryzae* (*Xoo*; which is a very closely species to *Xoc*), was selected as a subject to perform a BlastP search in the genome of *Xoc* strain BLS256 (Accession number: NC_017267). This led to the identification of a corresponding ortholog, termed as XocR (XOC_1422), which shared 97% similarity/identity with OryR in sequence at amino-acid level. To determine whether XocR is a member of the PAB LuxR solos, the sequence features of XocR and its genomic organization in *Xoc* were further analyzed. XocR possessed a typical AHL-binding domain at the N terminus and a DNA-binding HTH domain at the C terminus (**Figure 1**). Furthermore, *Xoc* genome did not have a cognate *luxI* and hence XocR is a LuxR solo in *Xoc*. Importantly, as like other PAB LuxR solos, XocR had an imperfect AHL-binding domain with substitutions two of the highly conserved amino acids in the AHL binding domain (Covaceuszach et al., 2013; Gonzalez and Venturi, 2013). More specifically, W57 and Y61, the amino acid with the number in respect to TraR, a canonical QS LuxR protein in *Agrobacterium tumefaciens* (Covaceuszach et al., 2013; Gonzalez and Venturi, 2013), were substituted by methionine (M) and tryptophan (W), respectively (**Figure 1A**). Collectively, these results indicated that XocR belongs to the sub-family of PAB LuxR solos, which binds and responds to the yet-to-be-identified plant-derived compound(s). Further analysis was carried out to identify additional four genes that were genetically adjacent to *xocR* in the genome of *Xoc* strain BLS256. Specifically, *XOC_1420* and *XOC_1421*, which encodes a hypothetical protein and an amino acid transporter, respectively, were located closely in the upstream of *xocR* (**Figure 1B**). This organization is similar to gene arrangement in *Xoo* (**Figure 1B**). However, we also found a difference in the nucleotide sequence of two genes located downstream of *xocR* compared to those of *Xoo*. The observed difference between *Xoo* and *Xoc* was the presence of a gene (*XOC_1424*) encoding a hypothetical protein located closely to the *pip* gene in *Xoc*, which was absent in *Xoo*. In *Xoo* on the other hand at the similar position, a locus (*XOO1270*) encoding a protein that putatively functions as an amino acid transporter was located adjacent to the *pip* gene (**Figure 1B**). Our results suggest that XocR is a member of a sub-family of PAB LuxR solos. Notably, although *Xoo* and *Xoc* are two pathovars of *X. oryzae*, XocR of *Xoc* also exhibited distinctive features in sequence (e.g., sequence organization) with that of well-characterized OryR from *Xoo*. These findings prompted us to test the potential role of *xocR* in the virulence of *Xoc* as well as other important pathogenesis related traits (see below).

xocR is Required for *Xoc* Virulence in Host Rice, but Is Not Necessary for Trigging HR in Non-Host (Plant) Tobacco

In order to gain insight into the role of *xocR* in *Xoc* virulence, we generated the *xocR* in-frame deletion mutant as well as the corresponding complemented strain (Figure S1). Pathogenicity tests showed that mutation of *xocR* almost completely impaired in virulence, whereas the complemented strain regained virulence to wild-type levels (**Figures 2A,B**). This finding suggested that *xocR* was required for *Xoc* virulence on host rice. We were then interested to determine whether *xocR* was also required for triggering the HR (a programmed cell death) in non-host (plant) tobacco. For this purpose, three different cell concentrations of wild-type Rs105 and the *xocR* mutant were infiltrated into the leaves of tobacco. We determined that mutation of *xocR* did not alter the ability to trigger HR in tobacco under all testing conditions and behaved as the wild-type (Figure S2). This point indicated that *xocR* was not involved in trigging HR in non-host (plant) tobacco. Our results also provide a clue to reveal the diversity of function of *xocR* in host and non-host plants. The PAB LuxR solos have been shown to mediate virulence in diverse phytopathogenic bacteria by binding yet-to-be-identified plant signal/compound; these are XccR of *X. campestris* binding a signal present in cabbage (Zhang et al., 2007), OryR of *X. oryzae* binding a signal compound present in rice (Ferluga and Venturi, 2009) and XagR of *X. axonopodis* binding a signal molecules in soybean, rice and cabbage but not in tobacco (Chatnaparat et al., 2012). These previous observations raised the possibility that binding a signal/compound in rice leaves, but not in tobacco by XocR probably contributed to probable roles of XocR in host rice and non-host tobacco.

Mutation of xocR does not Affect the Growth Ability *in planta* and *in vitro*

The finding of virulence deficiency of the *xocR* mutant prompted us to examine whether this trait is associated with the *in planta* growth capacity of the mutant. To test this, we recovered the bacterial cells from the infected rice leaves after inoculation of 7 days. As shown in Figure S3, a mutation in *xocR* did not alter the bacterial cell populations when compared to the ones of the wild-type strain Rs105 *in planta,* as the bacterial numbers recovered from infected rice leaves between wild type and the *xocR* mutant were similar under our testing conditions. To further study growth ability, the *in vitro* growth profiles in different media of wild-type Rs105 and the *xocR* mutant were determined. As shown in Figure S4, it was clearly observed that the *xocR* mutant always displayed wild-type growth ability either in nutrient-rich medium or NB with rice macerate medium. Collectively, these results indicate that the virulence deficiency of the *xocR* mutant on host rice was probably not due to the *in planta* growth impairment. However, our result is not consistent with the finding on the role of PsaR2 in plant colonization, which is another PAB LuxR solos from *P. syringae* pv. *actinidiae*, a pathogen of kiwifruit (Patel et al., 2014). In this bacterium, mutation of *psaR2* impaired the ability of *in planta* colonization and virulence. These findings therefore suggest that PAB LuxR solos may play different roles in plant colonization in different phytopathogens.

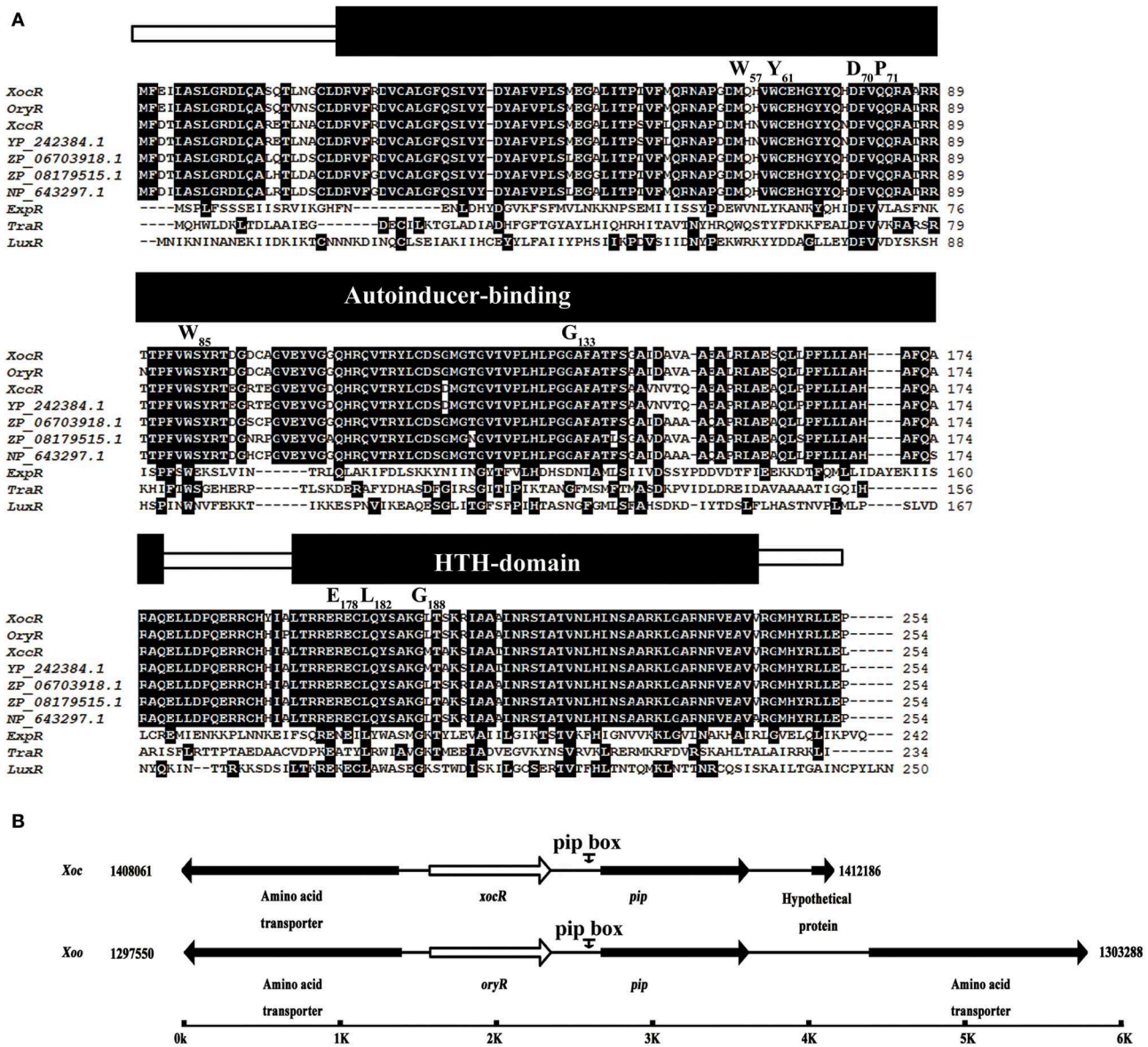

FIGURE 1 | Sequence characterization of XocR in *Xanthomonas oryzae* pv. *oryzicola*. (A) Alignment analysis of LuxR-type transcriptional regulators. Exception to XocR and ExpR, other solo LuxRs used in this figure are described previously (Gonzalez and Venturi, 2013), and they are: OryR, *Xanthomonas oryzae* pv. *oryzae* KACC10331; XccR, *X. campestris* pv. *campestris* 8004; YP_242384.1, *X. gardneri* ATCC 19865; ZP_06703918.1, *X. fuscans* subsp. *aurantifolii* ICPB 11122; ZP_08179515.1, *X. vesicatoria* ATCC 35937; NP_643297.1 *X. axonopodis* pv. *citri* 306; QS LuxR: ExpR of *Pectobacterium carotovorum* subsp. *carotovorum*; TraR of *Agrobacterium tumefaciens*; LuxR of *Vibrio fischeri*. The nine highly conserved amino acids are highlighted in black and their positions with respect to TraR are indicated above. **(B)** Comparison of *xocR* and *oryR* organization in the genome of *X. oryzae* pv. *oryzicola* BLS256 and *X. oryzae* pv. *oryzae* KACC10331, respectively. *xocR* and *oryR* were both highlighted in white.

XocR is Involved in the Regulation of Cell Swimming Motility in the Presence of Rice Signal

The finding that virulence deficiency of the *xocR* mutant was not associated with its growth impairment *in planta*, suggested that XocR may utilize a novel mechanism(s) to regulate virulence in *Xoc*. This prompted us to examine whether any of the four well-studied virulence-associated traits, including cell motility, EPS production, biofilm formation and extracellular protease activity were impaired in the *xocR* mutant, resulting in virulence deficiency. We therefore determined the ability of the *xocR* mutant in regulating these phenotypes. As shown in Figure S5, we did not find any visible difference in the tested phenotypes between wild-type Rs105 and the *xocR* mutant in the absence of rice macerate. As reported previously, the regulation of the PAB LuxR solos with regard to phenotypes occurs to the greatest extent in response to the presence of plant signal(s) (Chatnaparat et al., 2012; Gonzalez et al., 2013). To test this possibility, we re-examined these four tested virulence-associated traits

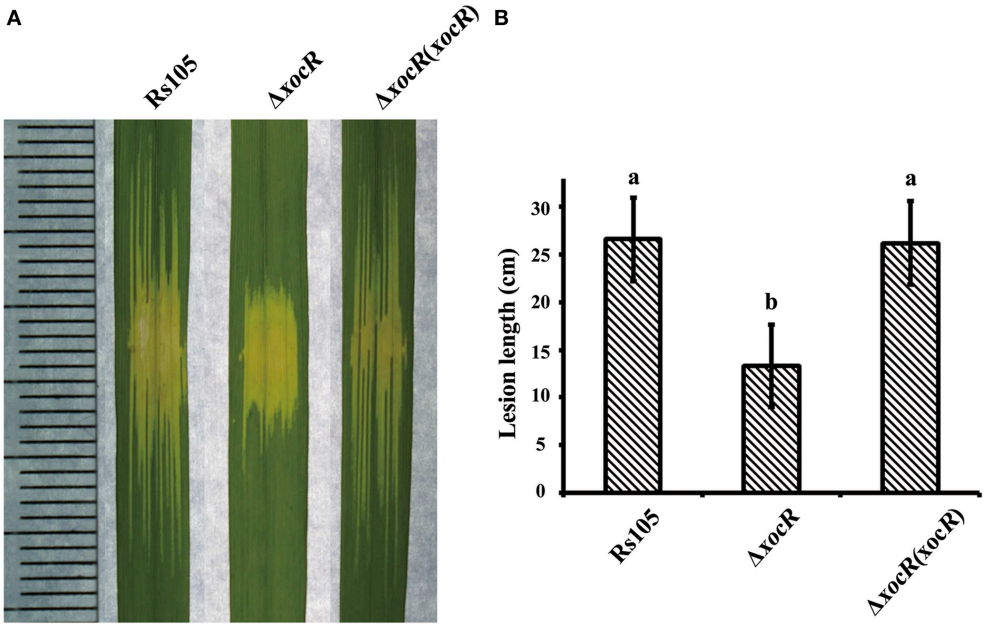

FIGURE 2 | Mutation of *xocR* impaired the virulence of *Xanthomonas oryzae* pv. *oryzicola*. **(A)** Representative result of water-soaking lesion lengths on the rice seedling leaves (cv. Shanyou63, 2-week old) by infiltration with wild-type Rs105 and the *xocR* mutant. **(B)** Calculated data of water-soaking lesion lengths on the leaves of rice seedling leaves. Rs105, wild-type strain; Δ*xocR*, the *xocR* deletion mutant; Δ*xocR* (*xocR*), the complemented strain of the *xocR* deletion mutant. Three replicates were used for each treatment, and the experiment was performed three times. Vertical bars represent standard errors. Different letters above data bars indicate a significant difference between the wild-type strain and tested mutants ($P < 0.05$; *t*-test).

between wild-type Rs105 and the *xocR* mutant in the presence of rice macerate. Using this procedure, we observed that *xocR* was involved in the regulation of cell motility and there was no difference in the other three phenotypes (EPS production, biofilm formation and extracellualr protease) (Figure S6). As shown in **Figure 3**, we observed that a mutation in *xocR* significantly reduced the cell swimming motility of wild-type strain in rich medium supplemented with rice macerate, whereas the cell swimming motility of the *xocR* complemented strain was found to be partially restored to the wild-type level under the same testing conditions (**Figure 3**). Furthermore, we also determined that the *xocR* mutant displayed wild-type growth level in this medium (Figure S4B), suggesting the regulation of *xocR* in cell swimming motility in the presence of rice macerate was not due to any difference in the growth of *Xoc* derivatives. As cell swimming motility can be associated with bacterial growth and EPS production in diverse bacteria (Ali et al., 2000), we tested these phenotypes and determined that the regulation by *xocR* of cell swimming motility was not linked to these two phenotypes in *Xoc* since a mutation of *xocR* did not impair these two traits both in the presence or absence of rice signal(s). Similarly to what occurs in *Xoc*, cell swimming motility is also regulated by OryR in *Xoo* (Gonzalez et al., 2013).

In *Xoo*, OryR was found to positively control the transcriptional expression of a number of movement-related genes, including 30 flagella genes in response to rice macerate (Gonzalez et al., 2013). Further studies showed that OryR directly regulated most probably via a *pip* box-like sequence of the promoter region of *flhF*, the flagella-regulator-encoding gene (Gonzalez et al., 2013). A BlastP search in *Xoc* demonstrated a similar organization of these flagellar genes in *Xoc* (Table S5). This finding raised the possibility that XocR might use pathway/mechanism similar to OryR to modulate cell motility in *Xoc*. It must be noted that *Xoo* is a vascular pathogen unlike *Xoc*, raising the question whether OryR and XocR respond to the same rice signal/compound(s). Identification of the rice signal(s) that interacts with OryR or XocR will give more insight into this point. Nevertheless, our results suggest that response to host signal is important for XocR to regulate cell motility, which seems to facilitate the infection of *Xoc* on host rice.

XocR Controls the Transcription of Two Known Virulence-Associated Genes in the Presence of Rice Signal

In addition to cell motility, we were interested to examine whether XocR regulates other regulatory mechanism(s) to mediate virulence. As described above, XocR most likely binds to a DNA sequence called *pip*-box present in the promoter region of target genes to regulate their transcription. In view of this, an analysis of the promoter sequences of 29 reported virulence-associated genes of *Xoc* was made (Table S4); this led to the discovery of two candidate genes that might be regulated by XocR at transcriptional level, since their promoter regions possessed a potential binding motif (an imperfect *pip* box element) (**Figure 4A**). They are *XOC_1737*, encoding a DNA repair protein (RecN) and *XOC_4211*, encoding an

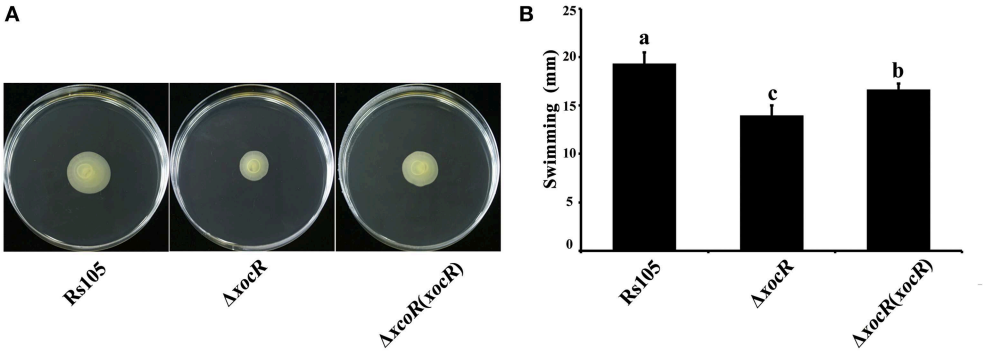

FIGURE 3 | Mutation of *xocR* impaired cell swimming motility of *Xanthomonas oryzae* pv. *oryzicola* in the presence of rice macerate. (A) Representative result of motility haloes of wild-type Rs105 and the *xocR* deletion mutant; **(B)** Calculated data of diameters of the swimming haloes on the plate of NA with rice macerate by tested *Xoc* strains. Rs105, wild-type strain of *X. oryzae* pv. *oryzicola*; Δ*xocR*, *xocR*-deletion mutant of *X. oryzae* pv. *oryzicola*; Δ*xocR* (*xocR*), the complemented strain of the *xocR* deletion mutant. Three replicates were used for each treatment, and the experiment was performed three times. Vertical bars represent standard errors. Different letters above data bars indicate a significant difference between the wild-type strain and tested mutants ($P < 0.05$; *t*-test).

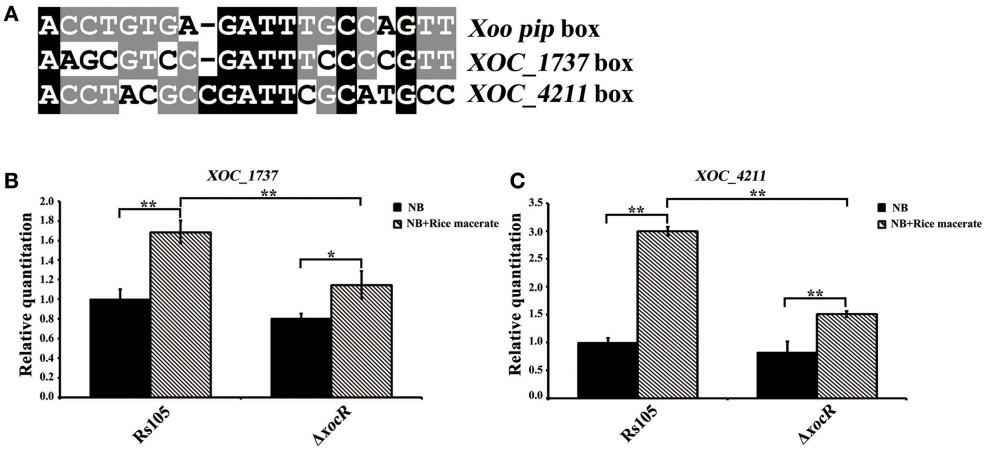

FIGURE 4 | The contribution of rice macerate to the regulation of *xocR* on the transcription of two known virulence-associated genesn *Xanthomonas oryzae* pv. *oryzicola*. (A) Sequence alignment of the characterized *Xoo pip* box and the promoter regions of two characterized virulence-associated genes (*XOC_4211* and *XOC_1737*) in *Xoc*. **(B,C)** Determination of the transcriptional level of *XOC_1737* and *XOC_4211* between wild-type Rs105 and the *xocR* deletion mutant (Δ*xocR*), respectively in the absence or presence of rice macerate. *XOC_1737* encodes a DNA repair protein (RecN) and *XOC_4211* encodes an anthranilate synthase component I (TrpE). Three replicates were used for each treatment, and the experiment was performed three times. Vertical bars represent standard errors. The data was analyzed by One-Way ANOVA. Tukey HSD *post-hoc* comparisons were reported, where $^{**}P < 0.001$, $^{*}P < 0.005$.

anthranilate synthase component I (TrpE). To address whether the transcription of these two genes was controlled by *xocR*, we used qRT-PCR to determine their transcriptional levels in wild-type Rs105 and the *xocR* mutant. As shown in **Figures 4B,C**, compared to control medium (NB), addition of rice macerate significantly induced the transcriptional expression of *recN* (∼1.7-fold) and *trpE* (∼3.0-fold) in the wild-type background. However, the induced transcriptional effect of *recN* and *trpE* by rice macerate was reduced to ∼1.4 and ∼1.8-fold in the *xocR* mutant, respectively, compared to the control medium. Meanwhile, the transcription of *recN* or *trpE* in the wild-type strain and the *xocR* mutant was similar in the absence of rice macerate, whereas in the presence of rice macerate, mission of *xocR* significantly alter the expression pattern of both genes. In details, the expression level of *recN* and *trpE* was reduced to (∼1.5-fold) and (∼2.0-fold), respectively, compared to the wild-type strain in the presence of rice macerate. Although the complemented strain was able to restore the deficiency of the *xocR* mutant in cell motility (**Figure 3**), suggesting the construction of complemented strain is corrected. However, we always found the transcriptional level of both tested genes (*recN* and *trpE*) was highly similar with their cases in the *xocR* mutant under all testing conditions (data not shown). It is possible that the transcriptional level of both genes in the pBBR1-MCS5 vector was relatively high compared to their native levels in the genome, further suggesting the transcriptional regulation of *xocR* on both genes was probably precise

in *Xoc*. Nevertheless, our results collectively suggest that the rice macerate may activate the positive regulation effect of *xocR* on the transcription of two known virulence-associated genes in *Xoc*. It is currently not known whether XocR directly binds the promoter region of *recN* or *trpE* to regulate their transcription in the presence of rice macerate or whether this regulation is indirect. RecN is a highly conserved, SMC (structural maintenance of chromosomes)-like protein in bacteria (Zeigler, 2003). It plays an important role in the repair of DNA double-strand breaks and therefore acts as a critical factor in maintaining genome integrity (Grove et al., 2009). Moreover, RecN also functions as a key component of the SOS response in different bacterial species, including *Haemophilus inlfuenzae*, *Bacillus subtilis* and *Pseudomonas fluorescens* (Alonso et al., 1993; Sweetman et al., 2005; Sanchez et al., 2006; Jin et al., 2007). However, the involvement of *recN* in the virulence of plant pathogenic bacteria is largely unknown. TrpE is one component of microbial anthranilate synthase (AS), which is a member of tryptophan biosynthetic pathway. AS catalyzes the formation of anthranilate from chorismate and ammonia (or glutamine), which is the first committed step branching from shikimate pathway toward the biosynthesis of l-tryptophan (Morollo and Eck, 2001). TrpE is able to catalyze the synthesis of anthranilate with ammonia as the source of nitrogen atom independently (Bauerle et al., 1987). The involvement of *trpE* in the virulence of plant pathogenic bacteria is also poorly understood.

Concluding Remarks

This study reports for the first time the presence of a PAB LuxR solo designated as XocR in *Xoc*. XocR shares high sequence similarity at amino-acid level with OryR from *Xoo*. XocR, responds to rice-derived unidentified compound(s) and regulates virulence on host rice and cell motility similar to OryR. We further provided evidence that XocR mediated *Xoc* virulence through the control of the transcription of two known virulence-associated genes (*recN* or *trpE*) in the presence of rice signal(s). Future studies will focus on the identification of the rice signal(s) that interacts with XocR, and the regulatory mechanisms of XocR on cell motility as well as the transcription of *recN* or *trpE* in *Xoc*.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2015.00037/abstract

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Studies on synthetic LuxR solo hybrids

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A sub-group of LuxR family of proteins that plays important roles in quorum sensing, a process of cell-cell communication, is widespread in proteobacteria. These proteins have a typical modular structure consisting of *N*-ter autoinducer binding and *C*-ter helix-turn-helix (HTH) DNA binding domains. The autoinducer binding domain recognizes signaling molecules which are most often *N*-acyl homoserine lactones (AHLs) but could also be other novel and yet unidentified molecules. In this study we carried out a series of specific domain swapping and promoter activation experiments as a first step to engineer synthetic signaling modules, taking advantage of the modularity and the versatile/diverse signal specificities of LuxR proteins. In our experiments the *N*-ter domains from different LuxR homologs were either interchanged or placed in tandem followed by a *C*-ter domain. The rational design of the hybrid proteins was supported by a structure-based homology modeling studies of three members of the LuxR family (i.e., LasR, RhlR, and OryR being chosen for their unique ligand binding specificities) and of selected chimeras. Our results reveal that these LuxR homologs were able to activate promoter elements that were not their usual targets; we also show that hybrid LuxR proteins retained the ability to recognize the signal specific for their *N*- ter autoinducer binding domain. However, the activity of hybrid LuxR proteins containing two AHL binding domains in tandem appears to depend on the organization and nature of the introduced domains. This study represents advances in the understanding of the modularity of LuxR proteins and provides additional possibilities to use hybrid proteins in both basic and applied synthetic biology based research.

Keywords: LuxR, quorum sensing, LuxR solo, lux box, bacteria, signaling

Introduction

Quorum sensing (QS) is a system of bacterial communication which involves the synthesis and detection of chemical molecules called autoinducers to regulate community-specific traits in a cell density dependent manner (Engebrecht et al., 1983; Kaplan and Greenberg, 1987; Fuqua et al., 1994; Fuqua and Greenberg, 2002). Most commonly the canonical QS system in Gram-negative bacteria is composed of genetically coupled *luxI* and *luxR* homologs. The LuxR homolog is the transcriptional regulator that recognizes and binds, at threshold concentration, to the autoinducer

molecules [generally *N*-acyl homoserine lactones (AHLs)] synthesized by the LuxI homolog to regulate gene expression (Fuqua et al., 1994; Zhu and Winans, 2001; Fuqua and Greenberg, 2002; Schaefer et al., 2013). LuxR homologs are known to have a modular structure consisting of *N*-ter autoinducer binding domain and *C*-ter helix-turn-helix (HTH) DNA binding domain (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995). Most of the LuxR homologs require binding to the signal molecule for correct folding/dimerization/stabilization and subsequent activation of target promoter regions (Zhu and Winans, 2001; Vannini et al., 2002; Zhang et al., 2002). Some LuxR proteins are able to fold/dimerize in the absence of the autoinducer to repress the promoter region of target genes but are de-repressed on binding the ligand due to conformational changes (Pearson et al., 1997; Minogue et al., 2002; Medina et al., 2003; Ventre et al., 2003; Steindler et al., 2009). LuxR homologs usually but not always bind to 20-bp palindromic sequence in the promoter regions of target genes centered at the −42.5 position from the transcriptional start site called the *lux* box; it is known that six key nucleotides in the *lux* box are crucial for the complete interaction between the LuxR homologs and the target DNA (Devine et al., 1989; Egland and Greenberg, 1999; Urbanowski et al., 2004; Schuster and Greenberg, 2007; Antunes et al., 2008). Therefore, the two domains of the LuxR protein, the presence of appropriate ligand and important features of the target promoter element are central for LuxR mediated gene regulation.

LuxR homologs without a cognate autoinducer synthase LuxI, namely LuxR solos or orphans, are now known to be common among members of proteobacteria (Chugani et al., 2001; Fuqua, 2006; Subramoni and Venturi, 2009; Subramoni et al., 2011). In some bacteria their presence greatly increases the regulon that is influenced by QS as LuxR solos may bind to endogenous or exogenous AHLs or even novel signals. In other bacteria lacking the canonical QS system, LuxR solos are thought to play a role in interspecies communication (Case et al., 2008; Patankar and Gonzalez, 2009b; Subramoni and Venturi, 2009; Schaefer et al., 2013; Patel et al., 2014). LuxR solos possess the same modular domain organization as the LuxRs of canonical QS systems. Well-studied AHL-binding LuxR solos include QscR of *Pseudomonas aeruginosa* (Chugani et al., 2001; Ledgham et al., 2003; Fuqua, 2006) and SdiA of *Salmonella enterica* and *Escherichia coli* (Ahmer et al., 1998; Michael et al., 2001; Ahmer, 2004). A sub group of LuxR solos are able to respond to yet unknown plant signal molecules (PSM) instead of detecting AHLs. These proteins are found in plant-associated bacteria (PAB) and show changes in one or two highly conserved amino acids of the autoinducer domain which are thought to be important for signal recognition based on structural studies on TraR (Nasser and Reverchon, 2007). These solos were first described in two species from the *Xanthomonas* genus (Ferluga et al., 2007; Zhang et al., 2007). Later additional proteins belonging to this group were described in other bacterial species (Patankar and Gonzalez, 2009a; Subramoni et al., 2011; Chatnaparat et al., 2012) and they are also present in bacteria associated with *Populus deltoides* (Schaefer et al., 2013). *In silico* modeling studies of these LuxR solos based on the crystallographic structures from different LuxR homologs point to the existence of at least three structural patches in the autoinducer binding domain that are involved in signal recognition (Vannini et al., 2002; Zhang et al., 2002; Yao et al., 2006; Bottomley et al., 2007; Chen et al., 2011; Lintz et al., 2011; Covaceuszach et al., 2013; Patel et al., 2014).

In this study we aim to exploit molecular modeling studies for the rational design of synthetic constructs of the LuxR proteins which differ in signal and promoter binding in order to assess the functionality of the LuxR hybrid proteins. We evaluated the ability of a PAB LuxR solo (i.e., OryR) to bind target promoter regions usually activated by canonical LuxR and vice-versa (i.e., canonical LuxR solos activating PAB LuxR solo target promoter region). We also designed synthetic hybrid variants Ola, Lao, and RhLas by exchanging the *N*-ter autoinducer domains of OryR, LasR, and RhlR and evaluated their ability to bind and regulate specific promoter regions in the presence of the new cognate signal. Finally different synthetic LuxR hybrid variants Dahl1 and Dahl2 containing two *N*-ter autoinducer domains and one *C*-ter DNA binding domain were assessed for their capacity to respond to one or two AHLs signals activating target promoters. This study provides new information about the modularity and signal specificity of LuxR proteins and opens new options for the use of LuxR hybrids in engineering bacterial synthetic regulation systems.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Pseudomonas aeruginosa PUPa3 and *Escherichia coli* strains used in this study are listed in Table S1. *P. aeruginosa* PUPa3 and its derivatives were routinely grown in Luria Bertani (LB) broth and when required antibiotics were added in the following concentrations: 100 μg ml^{-1} nitrofurantoin (Nif), 100 μg ml^{-1} ampicillin (Ap), 300 μg ml^{-1} kanamycin (Km), 100 μg ml^{-1} gentamycin (Gm), 100 μg ml^{-1} tetracycline (Tc) and 250 μg ml^{-1} chloramphenicol (Cm). *E. coli* DH5α and M15 were routinely grown in LB broth and antibiotics were added in the following concentrations when required: 50 μg ml^{-1} Km, 10 μg ml^{-1} Gm, 10 μg ml^{-1} Tc, and 100 μg ml^{-1} Cm. External addition of rice macerate in LB was performed as described by Gonzalez et al. (2013) and AHLs were externally added at a concentration of 1 μM, unless stated otherwise.

Recombinant DNA Techniques, Plasmids Construction, and Gene Synthesis

All recombinant DNA techniques were performed as described in Sambrook and Russell (2001), plasmids were purified by using EuroGold columns (EuroClone, Italy) and plasmid constructs were sequenced by Macrogen (Europe). All plasmids and primers used in this study are described in Table S2.

Gene synthesis was performed by GeneArt® (Life Technologies, Italy) or by GenScript (GenScript, USA) and all nucleotide sequences are listed in Table S3. Construction of pLAO and pOLA was performed in a sequential subcloning

where first the synthesized gene from pMX was cloned in pBBR1MCS2 using HindIII/SacI and then transferred to pBBR1MCS3 using XhoI/SacI. For the construction of pDAHL1 the synthesized gene was transferred from cloning vector pMX to pBBR1MCS2 using HindIII/SacI, resulting in the expression vector pDAHL1. To generate pDAHL2 the synthesized gene was transferred from pUC57 to pBBR1MCS2 using HindIII/SacI, resulting in the creation of pDAHL2. For the generation of pRHLAS amplifications and sequential cuts were performed as follows. First *lasR* and *rhlR* were amplified from *P. aeruginosa* PUPa3 using the pair of primers LasR Fw/LasR Rv and RhlR Fw/RhlR Rv to generate pLASR and pRHLR, respectively. Amplicons were cloned into pGEM® T Easy and sequenced. Constructs were then transferred to pBBR1MCS2 using HindIII/SacI. From this point pLASR was digested using HindIII/BsrBI/SacI generating two fragments which were the AHL-binding domain (approximately 480 bp) and the DNA-binding domain (approximately 230 bp) and the latter eluted and saved. The construct pRHLR was used as template to amplify the AHL-binding domain using the pair of primers T3/RhlRSmaI Rv and the amplicon was cloned in pGEM® T easy. It was then digested with HindIII/SmaI generating a fragment of approximately 520 bp. The AHL-binding domain of RhlR was then cloned HindIII/SacI in frame with the DNA-binding domain of LasR in pBBR1MCS2 generating the construct pRHLAS. All hybrid constructs are schematically represented in **Figure 1**.

β-Galactosidase and β-Glucuronidase Assays

β-galactosidase activities were determined during growth in LB as described by Miller (1972) with the modifications of Stachel et al. (1985). All experiments were performed in triplicate. Statistical analysis was performed using Student's *t*-test. The growth curves of all strains were determined and no significant differences were observed (Figure S3). Only the *E. coli* strain harboring pDAHL1 displayed a slightly longer lag phase under the conditions we tested.

The β-glucuronidase activities of overnight cultures of *E. coli* DH5α or *P. aeruginosa* PUPa3 with the pSS122 reporter plasmid carrying different promoters were determined as follows. Bacterial cells were centrifuged and re-suspended in 600 µl of GUS buffer (50 mM sodium phosphate pH 7.0, 1 mM EDTA, 14.3 mM 2-mercaptoethanol). After this 23 µl of 3% Triton X-100 in GUS buffer and 23 µl of 3% sodium lauryl sarcosinate in GUS buffer were added to the samples, the preparations were incubated at 30°C for 10 min, and then 100 µl of 25 mM *p*-nitrophenyl-β-d-glucuronic acid (PNPG) (Sigma) were added. The reaction was stopped by adding 280 µl of 1 M Na_2CO_3 after sufficient yellow color had developed. Both the optical densities at 595 nm (OD_{595}) of the bacterial cultures and the OD_{415} of the samples, measuring the intensity of the yellow color developed by the β-glucuronidase reaction ($OD_{415PNPG}$), were determined. 1 Miller unit of β-glucuronidase activity was defined as follows: 1 Miller unit $= 1000 \times \{[OD_{415PNPG} - (1.75 \times OD_{595})]/(t \times v \times OD_{595})\}$, where t is the time of the reaction (in minutes), v is the volume of the culture assayed (in milliliters), OD_{595} is the cell density just before the assay, and 1.75 is the correction factor. All measurements were done in triplicate. Statistical analysis was performed using Student's *t*-test.

Homology Modeling

Five web-based servers were exploited to build the 3D homology models and the top-score models generated were then ranked and validated by the protein model quality predictors AIDE (Mereghetti et al., 2008) and ProQ (Wallner and Elofsson, 2003), including PSIPRED (Buchan et al., 2010) for secondary structure prediction.

In details HH-pred (Biegert et al., 2006) produced the top-score model for full length LasR and synthetic hybrids Lao and Ola (being the predicted LGscore and MaxSub values of 5.081, 4.208 and 2.608, 0.459, 0.323, and 0.211, respectively, and the statistical indicators TM-score and RMSD being 0.74, 0.76, and 0.70, and 5.08, 5.68, and 8.42 Å, respectively). The top scored model for full length RlhR was generated by InFold (Roche et al., 2011) (being the predicted LGscore and MaxSub value of 4.445 and 0.503, respectively, and the statistical indicators TM-score and RMSD being 0.71 and 6.13 Å, respectively); while Phyre2 (Kelley and Sternberg, 2009) produced the top scored model for RHLAS (being the predicted LGscore and MaxSub value of 4.624 and 0.495, respectively, and the statistical indicators TM-score and RMSD being 0.67 and 7.01 Å, respectively). All templates that have been used for homology modeling are listed in Table S3.

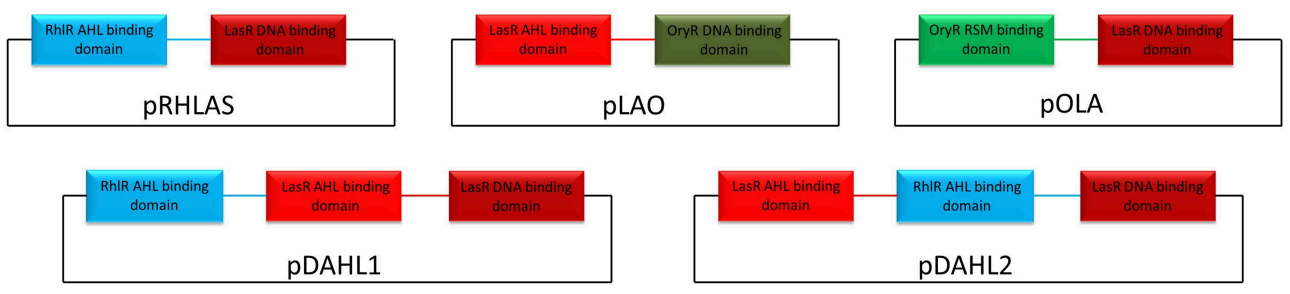

FIGURE 1 | Schematic representation of the hybrid variants exploited in this study. DNA sequences belonging to LasR, RhlR, and OryR are colored in red, light-blue, and green respectively. Linker regions between the functional domains are colored accordingly.

Results

Both AHL and Non-AHL Binding LuxR Proteins Regulate Foreign Promoters

In order to assess whether PAB LuxR solos could bind and activate target promoters of a canonical LuxR protein, β-galactosidase assays were carried out with specific promoter-*lacZ* reporter fusion constructs as described in the Materials and Methods section. All strains harboring the plasmid constructs did not display any significant alteration in growth and in the transition to the stationary phase (Figure S3). In these assays, we tested the ability of the PAB LuxR solo (OryR) of *Xanthomonas oryzae* pv. *oryzae* (Xoo) to activate promoter regions of *lasI* and *rhlI* in *E. coli*. As expected OryR activated reporter expression regulated by the promoter of the Xoo proline iminopeptidase gene (*pip*; pPIP122 or pPIP220) which is known to be its target gene in the presence of rice-plant macerate (Ferluga and Venturi, 2009). Additionally, among the several promoters tested OryR was able to activate the expression of the *lasI* promoter which is known to be regulated by LasR in the presence of OC12-HSL (pLASI220 and pLASI190) (Rampioni et al., 2007; Babic et al., 2010) (**Figure 2A**). In a reverse experiment, LasR and RhlR were expressed in *E. coli* and tested for their ability to bind and activate the promoter region of Xoo *pip* (pPIP220). Reporter expression was not detected in the presence of any of the proteins tested (Figure S1) except by LasR in the presence of its cognate signal OC12-HSL (**Figure 2B**).

Overall these data suggest that the operator elements of the *lasI* promoter and of the promoter regulated by OryR are sufficiently similar to enable recognition and activation by either OryR or LasR.

Domain Switching Maintains Modular Functionality of Hybrid LuxR Proteins

We designed synthetic hybrid proteins variants by using domains of native full-length proteins LasR and RhlR from *P. aeruginosa*, and OryR from *X. oryzae* pv. *oryzae* as described in the Materials and Methods section (**Figure 1**). In order to validate the structural consistency of the synthetic hybrids, the homology models of each native and hybrid protein were constructed and their capability to form a functional dimeric assembly exploited. In detail, the previously obtained OryR model (Covaceuszach et al., 2013) and the homology of full length LasR and RhlR models were compared with those built on the basis of the designed synthetic hybrids, i.e., Rhlas (AHL-binding domain of RhlR and DNA-binding domain of LasR), Lao (AHL-binding domain of LasR and DNA-binding domain of OryR) and Ola (PSM-binding domain of OryR and DNA-binding domain of LasR). As shown in **Figure 3**, in all the hybrids most of the linker after the last alpha-helix of the autoinducer binding domain (in details 7aa in either LasR and OryR and 9aa in RhlR) were maintained to guarantee the control of the orientation of the respective HTH DNA binding domains, allowing specific promoter recognition in response to the cognate signaling molecule bound by the autoinducer binding domain.

In order to evaluate the effects of interchanging the AHL-binding domains of LasR and RhlR hybrid construct pRHLAS was tested for its ability to regulate gene expression in response to cognate signals of both LasR and RhlR. The resulting Rhlas activated expression of the *lasI* promoter only in the presence of C4-HSL (**Figure 4A**). This result shows that by interchanging the AHL domains of QS LuxR homologs the hybrid protein remains functional and retains the ability to respond to the cognate signal recognized by the AHL-binding domain to activate gene expression, in this case the target promoter of LasR.

We were also interested to determine whether the modularity of domains would also be maintained in hybrid proteins that contained autoinducer binding domains with relatively distinct ligand selectivity as for example AHL vs. plant-derived signal molecules. We tested this by interchange of the PSM-binding domain of OryR and the AHL-binding domain of LasR. The hybrid proteins Lao and Ola (**Figures 3E,F** respectively) were

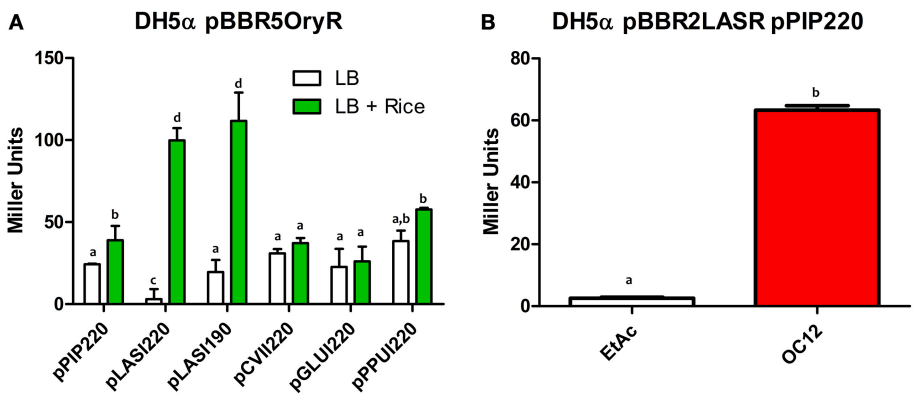

FIGURE 2 | β-galactosidase assays showing reporter expression levels regulated by *pip*, *lasI*, *cvil*, *glul*, and *ppul* promoters in *E. coli*. **(A)** β-galactosidase measurements of promoter activities in the presence of native OryR (pBBR5OryR) of Xoo using plasmids pPIP220, pLASI220, pCVII220, pGLUI220, and pPPUI220, in the absence or presence of total rice macerate at 5% v/v. **(B)** β-galactosidase measurements of *pip* promoter activity in the presence of native LasR (pBBR2LasR) of *P. aeruginosa* strain with pPIP220 in the absence (EtAc) and presence of the cognate signal molecule (OC12-HSL) at a concentration of 1 μM. All experiments were performed on triplicate, the means, and errors are shown and the statistical analysis were calculated using Student's *t*-test ($P = 0.05$). Distinct letters (a–d) indicate statistically different values.

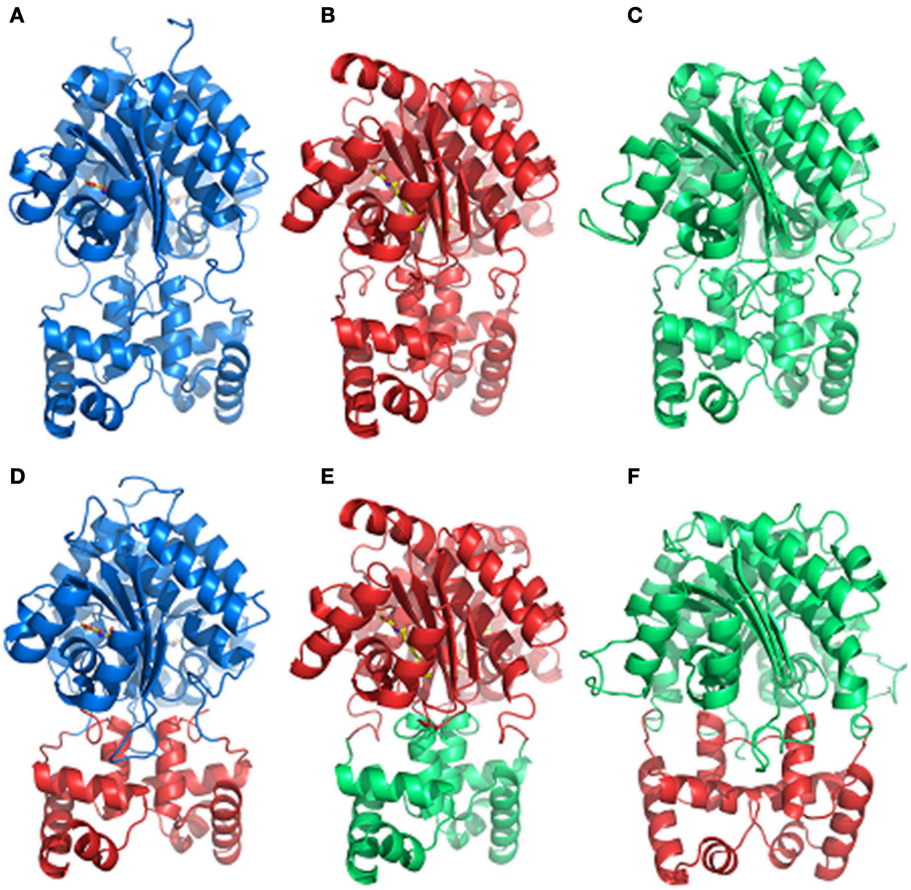

FIGURE 3 | Comparison of the dimeric assembly of the 3D structure-based homology models of the full-length LuxR homologs and of the synthetic hybrids variants. (A) RhlR, **(B)** LasR, **(C)** OryR, **(D)** Rhlas, **(E)** Lao, **(F)** Ola. The color code is as in **Figure 1**. The carbon, nitrogen and oxygen atoms of AHLs, are colored in yellow, blue, and red, respectively. Figures produced by Pymol (DeLano, 2002).

tested for their ability to respond to OC12-HSL and rice macerate, respectively. For both Lao and Ola we observed activation of reporter expression using pPIP122 and pLASI190 (that carry P_{pip} and P_{lasI}, respectively) in the presence of the cognate signals of their *N*-ter domains (**Figures 4B,C**). These results indicate that although activation levels are not as high as in a hybrid protein of two canonical LuxR proteins that responds to AHLs, Lao, and Ola are still functional.

Hybrid LuxR Proteins with Two Autoinducer Binding Domains in Tandem are Functional

Results from our experiments with hybrid LuxR proteins indicated that swapping modular domains of these proteins did not lead to loss of function and actually conferred specificity toward the cognate signal of the interchanged *N*-ter autoinducer binding domain. It was also of interest to test the effects of addition of a second autoinducer binding domain of full-length LuxR proteins and to examine their ability to respond to the cognate signals of both autoinducer binding domains by monitoring reporter expression regulated by a target promoter (i.e., P_{lasI}). The hybrid synthetic LuxR proteins generated as part of this study are represented in **Figure 1**. Their structural consistency was exploited by homology modeling. In detail, the dimeric assembly of RhlR and LasR homology models showed that the respective positions and distances of the joining aminoacids (whose main chain atoms are highlighted by spheres) were consistent with a likely dimeric assembly of Dahl1 and Dahl2 (depicted in **Figures 5A,B**, respectively), especially taking into account the flexibility of the regions at the *N*-ter of the proteins and at the level of the linker regions of the autoinducer binding domains that are combined in these hybrids.

To assess the functionality of Dahl1, in which the *N*-ter AHL-binding domain of RhlR was placed upstream the native LasR, we used C4-HSL alone, OC12-HSL alone or a mixture of both signal molecules. We observed that C4-HSL alone was not able to induce activation of the *lasI* promoter, whereas OC12-HSL induced reporter expression (**Figure 6A**). The combination of both AHLs neither increased nor decreased the levels of promoter activation, compared to having OC12-HSL alone, indicating that the presence of the newly added RhlR AHL-binding domain did not interfere with the activity of the native LasR protein.

We also constructed a similar double AHL-binding domain protein in which the two autoinducer binding domains were

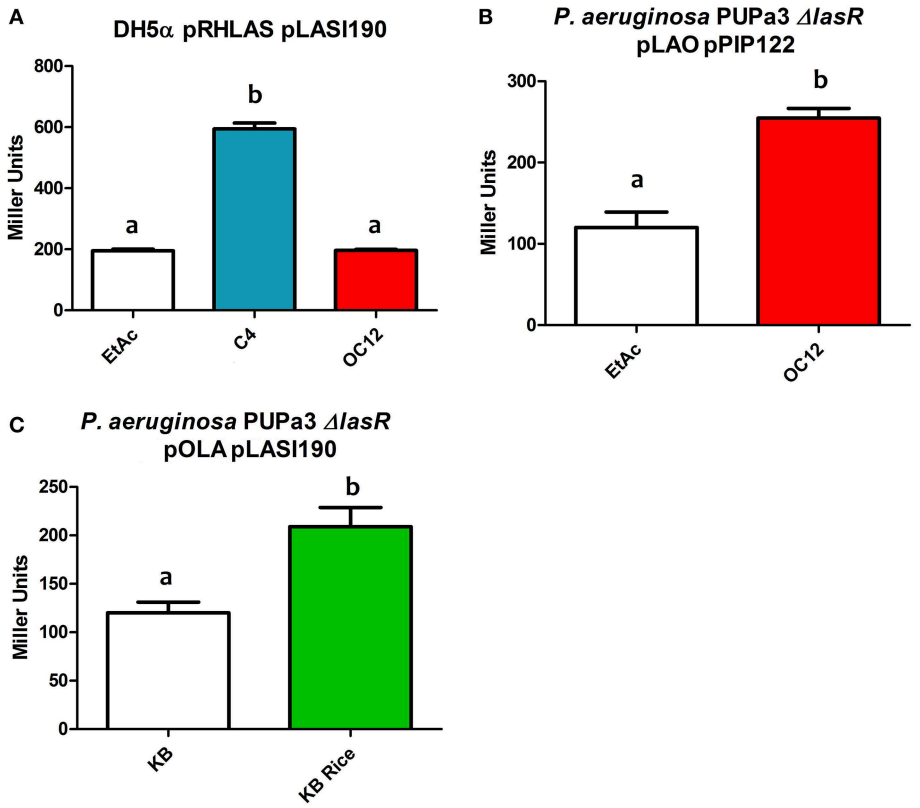

FIGURE 4 | β-galactosidase assays showing reporter expression levels regulated by *lasI* and *pip* promoter in different strains. (A) β-galactosidase measurement of promoter activity in the presence of the hybrid protein Rhlas (pRHLAS) using the reporter plasmid pLASI190 in the absence (EtAc) or in the presence of AHLs (C4 and OC12-HSL) at a concentration of 1 μM in *E. coli*. **(B)** β-galactosidase measurement of promoter activity in the presence of the hybrid protein Lao (pLAO) using the reporter plasmid pPIP122 in the absence or presence of OC12-HSL at the concentration of 1 μM in *P. aeruginosa* PUPa3Δ*lasR*. **(C)** β-galactosidase measurement of promoter activity in the presence of the hybrid protein Ola (pOLA) with plasmid pLASI190 in the absence or presence of total rice macerate at 5% v/v in *P. aeruginosa* PUPa3Δ*lasR*. All experiments were performed on triplicate, the mean, and error are shown and the statistical analysis were calculated using Student's *t*-test ($P \leq 0.05$). Distinct letters (a and b) indicate statistically different values.

inverted with respect to Dahl1, resulting in the Dahl2 hybrid (**Figure 1**). Surprisingly by using Dahl2 we were not able to detect activity when the protein was exposed only to C4-HSL. In presence of only OC12-HSL the promoter expression levels were raised around 1.5 times. However, when both signals were added concomitantly we could measure promoter activation levels up to 3 times higher than in the control experiments (**Figure 6B**). These results suggest that differently from Dahl1, the full activity of this hybrid protein was only possible when both *N*-ter autoinducer binding domains had their cognate signal molecules present.

Discussion

LuxR homologs are important proteins that are involved in gene regulation in Gram-negative bacteria (Whitehead et al., 2001; Fuqua and Greenberg, 2002). Many studies indicate that these proteins usually possess specificity toward one or few cognate molecules conferring a controlled response to self or non-self signals (Zhu and Winans, 2001; Minogue et al., 2002; Urbanowski et al., 2004; Yao et al., 2006; Zhang et al., 2007; Ferluga and Venturi, 2009; Subramoni et al., 2011). Although much is known about which are the cognate signal molecules and the set of genes being regulated by several LuxR homologs, little is known about the modular activity of these proteins (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995; Antunes et al., 2008; Ahumedo et al., 2010; Lintz et al., 2011; Covaceuszach et al., 2013). Our results indicate that the LuxR homolog OryR can activate the target promoter of LasR and vice versa. The analysis of the promoter region of *lasI* and *pip* shows that they are very similar, especially because of the conserved CT(N_{12})AG motif present both in the *las* and the *pip* boxes (Figures S2A,B) (Schuster et al., 2004; Schuster and Greenberg, 2007). Our experiments with hybrid synthetic LuxR proteins variants with exchanged domains of LasR and RhlR showed that the proteins were functional and retained specificity for the cognate signal molecule of the *N*-ter autoinducer binding domain. Furthermore, when two *N*-ter autoinducer binding domains were present in tandem, the hybrid proteins retained their functionality. The activity of these double autoinducer binding domain containing hybrid LuxR

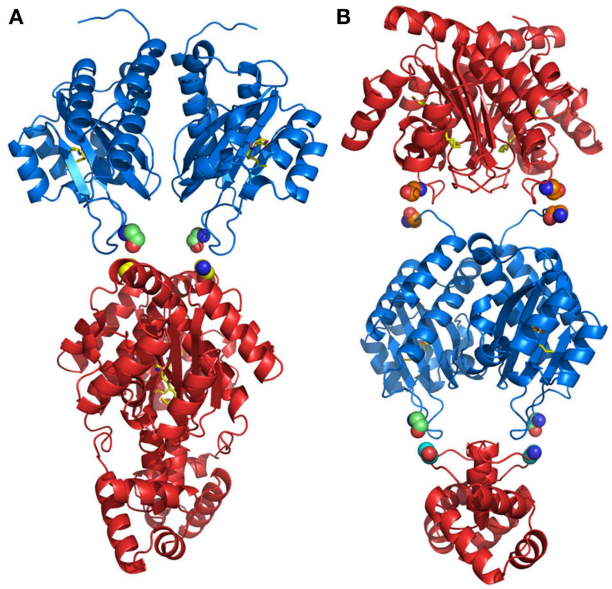

FIGURE 5 | Comparison of the dimeric assembly of the 3D structure-based homology models of the synthetic hybrids containing two AHL-binding domains. (A) Dahl1 and **(B)** Dahl2. The color code is as in **Figure 1**. The corresponding linking flexible regions in the Dahl1 and Dahl2 hybrids variants are colored in green/yellow and in orange and in green/cyan, respectively. The main chain atoms of the residues that are joined together in the synthetic hybrids are shown as spheres. The carbon, nitrogen, and oxygen atoms of AHLs, are colored in yellow, blue, and red, respectively. Figures produced by Pymol (DeLano, 2002).

proteins were however dependent not only on the cognate signal molecule(s) but also on the sequential order of the domains. In fact, synergistic effect due to the presence of both cognate signal molecules was observed for one of the two hybrid proteins, i.e., Dahl2. These observations provide new information regarding the modularity and signal specificity of non-native hybrid LuxR proteins.

The ability of LasR and OryR to regulate foreign promoters in response to their cognate signal molecules suggests that the operator sequences of their promoters are close enough to facilitate promoter activation. Therefore, combinations of either of these promoters and the LuxR homolog proteins with their cognate signal molecules may be exploited for engineering signaling modules or synthetic microbial communities. For example, in the construction of complex regulation systems in synthetic biology involving two or more LuxR homolog proteins that respond to different signal molecules but regulate the same targets. On the other hand cross-promoter specificity of LuxR homolog proteins that respond to AHL and non-AHL signal molecules such as plant derived molecules has important implications for inter-bacterial interactions in a common niche colonized by different bacterial species. Our previous results have demonstrated that 15 different AHLs (including C4-HSL and OC12-HSL) did not result in *pip* promoter activation by OryR and furthermore did not interfere with OryR-activation via the plant signal (Ferluga and Venturi, 2009). In addition biochemical tests have shown that none of the many AHLs tested could solubilize OryR (Ferluga et al., 2007). These results clearly indicate that the two *N*-terminal binding domains do not undergo competition with respect to their ligands making these hybrid proteins specific for a response signal.

LuxR hybrid proteins that had their domains exchanged proved to be still functional when they were presented with the signal molecule known to activate the interchanged *N*-ter autoinducer binding domain. Even swapping the autoinducer binding domain between a protein that responds to plant derived signal molecules (i.e., OryR) and a protein that responds to OC12-HSL (i.e., LasR) conferred the ability to the new hybrid protein to respond to the new cognate signal molecule but kept the specificity toward its native promoter region. The ability to control the molecules, to which the LuxR homolog proteins respond, could allow bacteria to respond not only to its cognate QS signal molecule, but also to an exogenous signal molecule.

The addition of a second ligand binding domain upstream a native LuxR homolog proved to work differently depending on the constructed protein. Dahl1, that is comprised of native LasR with an *N*-ter AHL-binding domain of RhlR added upstream, proved to be functional only when the LasR cognate signal molecule was exogenously provided (i.e., OC12-HSL). On the other hand the construct, in which the AHL-binding domain of RhlR was placed in between the AHL-binding and the HTH-DNA binding domain of LasR (Dahl2), showed full activity only in the presence of both signals. This latter result could pave the way to the construction of hybrid proteins which will respond not to one but to two different AHL signals triggering a second level of control or even leading to a complex regulation system, as for example by adding the PSM-binding domain of OryR in between the two domains of LasR. Our results also shows that by simply adding a second AHL-binding domain in a native LuxR homolog might not always confer a second level of control making clear that more studies are required to elucidate the importance of the *N*- and *C*- ter domains of LuxR homolog for their transcriptional activity. It is tempting to speculate that the outcome of this response is the likely result of the interplay between the protein folding, solubility and stability properties of the autoinducer binding domains in the absence of the cognate AHL that differs in the Dahl1 and Dahl2 hybrid variants. Indeed LasR is highly insoluble in the absence of OC12-AHL, while RhlR is known to be soluble, even if not active, in the absence of C4-AHL. Therefore, in both cases the solubility and the associated functionality of the two hybrids are likely to be strictly dependent on the presence of OC12-AHL, essential for the LasR AHL-binding domain to be soluble. However, in the Dahl1 hybrid the conformational change allowing a proper HTH DNA-binding domain orientation is likely to be only dependent on the LasR AHL-binding domain (due to its proximity to the HTH DNA binding domain) and therefore only on the presence of OC12-AHL. On the contrary in the Dahl2 variant the presence of OC12-AHL that is known to trigger the folding of the LasR AHL-binding domain might be suboptimal in conferring the proper HTH DNA-binding domain orientation due to the presence of the soluble RhlR AHL-binding domain just upstream of the HTH DNA binding domain. The presence instead of both AHLs

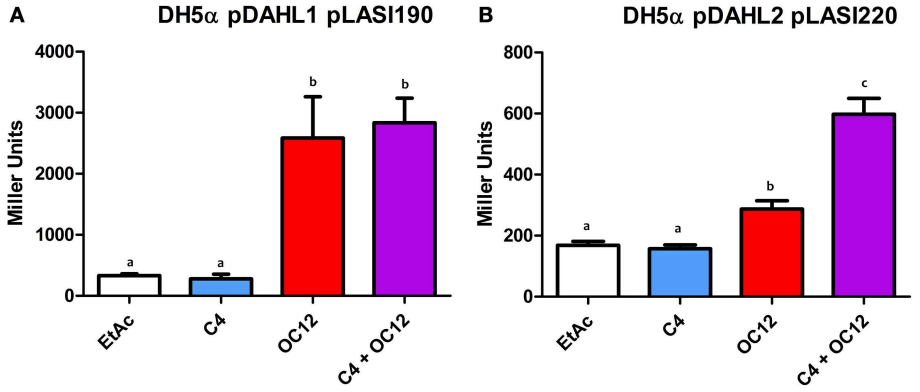

FIGURE 6 | β-galactosidase assays showing reported expression levels regulated by the *lasI* promoter in different conditions. (A) β-galactosidase measurement of the promoter activity in the presence of the hybrid protein Dahl1 (pDAHL1) with reporter plasmid pLASI190 in the absence (EtAc) or in the presence of AHLs (C4 and/or OC12-HSL) at a concentration of 1 μM. **(B)** β-galactosidase measurement of the promoter activity in the presence of the hybrid protein Dahl2 (pDAHL2) with reporter plasmid pLASI220 in the absence or presence of AHLs (C4 and/or OC12-HSL) at the concentration of 1 μM. All experiments were performed on triplicate, the mean, and error are shown and the statistical analysis were calculated using Student's *t*-test ($P \leq 0.05$). Distinct letters (a and b) indicate statistically different values.

might guarantee the overall proper folding and stabilization and therefore the optimal transcriptional activity.

In summary, our study evidences that OryR and LasR possess promoter regions with highly similar *lux*-like boxes. It also shows that domains of different LuxR subfamilies which respond to different signal molecules can be exchanged indicating a most probable common ancestor. Lastly the results obtained here using tandem double AHL-binding domains could pave the way to the design of novel proteins which are functional when they respond to more than one type of AHLs. These results open new avenues for future research on the importance of the modularity of LuxR proteins.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2015.00052/abstract

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LuxR solos in *Photorhabdus* species

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Bacteria communicate via small diffusible molecules to mediate group-coordinated behavior, a process designated as quorum sensing. The basic molecular quorum sensing system of Gram-negative bacteria consists of a LuxI-type autoinducer synthase producing acyl-homoserine lactones (AHLs) as signaling molecules, and a LuxR-type receptor detecting the AHLs to control expression of specific genes. However, many proteobacteria possess one or more unpaired LuxR-type receptors that lack a cognate LuxI-like synthase, referred to as LuxR solos. The enteric and insect pathogenic bacteria of the genus *Photorhabdus* harbor an extraordinarily high number of LuxR solos, more than any other known bacteria, and all lack a LuxI-like synthase. Here, we focus on the presence and the different types of LuxR solos in the three known *Photorhabdus* species using bioinformatics analyses. Generally, the N-terminal signal-binding domain (SBD) of LuxR-type receptors sensing AHLs have a motif of six conserved amino acids that is important for binding and specificity of the signaling molecule. However, this motif is altered in the majority of the *Photorhabdus*-specific LuxR solos, suggesting the use of other signaling molecules than AHLs. Furthermore, all *Photorhabdus* species contain at least one LuxR solo with an intact AHL-binding motif, which might allow the ability to sense AHLs of other bacteria. Moreover, all three species have high AHL-degrading activity caused by the presence of different AHL-lactonases and AHL-acylases, revealing a high quorum quenching activity against other bacteria. However, the majority of the other LuxR solos in *Photorhabdus* have a N-terminal so-called PAS4-domain instead of an AHL-binding domain, containing different amino acid motifs than the AHL-sensors, which potentially allows the recognition of a highly variable range of signaling molecules that can be sensed apart from AHLs. These PAS4-LuxR solos are proposed to be involved in host sensing, and therefore in inter-kingdom signaling. Overall, *Photorhabdus* species are perfect model organisms to study bacterial communication via LuxR solos and their role for a symbiotic and pathogenic life style.

Keywords: LuxR solos, quorum sensing, cell-cell communication, quorum quenching, entomopathogenic bacteria

INTRODUCTION

Photorhabdus spec. are pathogenic enteric bacteria that maintain a mutualistic interaction with heterorhabditid nematodes and can infect a wide variety of insect species. To date, three different *Photorhabdus* species are known: *P. luminescens*, *P. temperata*, and *P. asymbiotica* (Fischer-Le Saux et al., 1999). Whereas the first two species are highly pathogenic toward insects, *P. asymbiotica* additionally infects men (Gerrard et al., 2003). *P. asymbiotica* is associated with severe soft-tissue and systemic infections in humans, and is considered as an emerging threat (Gerrard et al., 2004). Commonly, all *Photorhabdus* bacteria colonize the gut of the infective juvenile stage of *Heterorhabditis* spec. nematodes. Upon entering insect larvae, the nematodes directly inject the bacteria by regurgitation into the insect's hemocoel. Once inside the insect, the bacteria rapidly replicate and quickly establish a lethal septicemia in the host by production of a broad range of different toxins that kill the insect within 48 h. At this stage the dying insect glows, due to bacterial luciferase production. Bioconversion of the insect's body by a huge set of bacterial exoenzymes produces a rich food source both for the bacteria and the nematodes. Furthermore, the bacteria support nematode reproduction probably by providing essential nutrients that are required for efficient nematode proliferation (Han and Ehlers, 2000). Moreover, the bacteria produce several antibiotics to defend the insect cadaver from invasion of other bacteria. When the insect cadaver is depleted, the nematodes and bacteria re-associate and emerge from the carcass in search of a new insect host (see Clarke, 2008; Waterfield et al., 2009, for review). During this complex life cycle the bacteria constantly have to monitor their host environment, and to communicate with each other as well as with their hosts to perfectly adapt to the respective conditions.

Bacterial communication via small molecules to mediate group-coordinated behavior, referred to as quorum sensing (QS), is well recognized. Typically, Gram-negative bacteria use small diffusible molecules, e.g., acyl-homoserine lactones (AHLs) derived from fatty acids, whereas Gram-positive bacteria use

peptide derivatives for communication. However, recently the production of AHLs by a Gram-positive bacterium belonging to the *Exiguobacterium* genera isolated from marine water was discovered (Biswa and Doble, 2013). The prototypical quorum sensing system of Gram-negative bacteria consists of a LuxI-like autoinducer synthase that produces AHLs as signals and a LuxR-type receptor that detects the AHLs to control expression of specific genes (Waters and Bassler, 2005). The AHLs are constantly synthesized by LuxI, and are sensed by the cognate LuxR-like receptor when exceeding a threshold concentration. Upon AHL-binding, LuxR binds to the promoter/operator regions of the target genes/operons causing altered gene expression in response to the cell count. Thus, bacteria respond to AHLs and adapt bacterial group-behavior by regulation of gene expression when the cell density reaches a certain size (quorum). Signaling via AHLs has been linked to diverse phenotypes like the production of virulence factors, motility, antibiotic production, sporulation, bioluminescence or biofilm formation (Waters and Bassler, 2005). LuxI/LuxR based quorum sensing systems have been intensively studied. The first system was described in *V. fischeri* showing that AHLs are used to regulate light production dependent on the cell density (Nealson and Hastings, 1979). Furthermore, LuxR-based cell-cell communication is medically relevant as many pathogenic bacteria use these quorum sensing systems for an effective infection process (Rutherford and Bassler, 2012).

Moreover, many bacterial genomes encode additional LuxR homologs lacking a cognate LuxI synthase. These LuxR homologs are designated as LuxR orphans (Patankar and González, 2009) or LuxR solos (Subramoni and Venturi, 2009). Genome sequencing of 265 proteobacterial genomes revealed that 68 of these encode at least one LuxI and one LuxR homolog (Case et al., 2008). Furthermore, 45 of these 68 genomes encode more LuxR-type proteins than LuxI homologs like *Pseudomonas aeruginosa*, which harbors beside the two classical QS systems, LasI/LasR and RhlI/RhlR, the LuxR solo QscR (Oinuma and Greenberg, 2011). Further 45 genomes do not harbor a *luxI*-like AHL synthase encoding gene, but encode at least one LuxR homolog. The three *Photorhabdus* species all lack a LuxI homolog, but have an extraordinary high number of genes that encode potential LuxR solos and are therefore assumed to have a huge capacity for cell-cell and/or inter-kingdom communication (Heermann and Fuchs, 2008). Therefore, these bacteria are perfect model organisms to study bacterial cell-cell communication via LuxR solos. Here we focus on the function of the multiple LuxR solos of the three known *Photorhabdus* species for their life cycle, their role in cell-cell communication and inter-kingdom signaling, as well as the specificity of signal perception.

MATERIALS AND METHODS

BACTERIAL STRAINS

The strains *Photorhabdus luminescens* TT01 (Fischer-Le Saux et al., 1999), *Photorhabdus temperata* NC19 (Tailliez et al., 2010), *Photorhabdus asymbiotica* ATCC43949 (Wilkonson et al., 2009), and *Vibrio harveyi* BB120 (Bassler et al., 1997) were used in this study.

QUORUM QUENCHING BIOASSAY

Bioluminescence of *Vibrio harveyi* wild-type (BB120) was used as read-out to analyze degradative activity of AHLs in *Photorhabdus* species supernatants. Therefore, *V. harveyi* BB120 strain was grown at 30°C in LM medium [10% (w/v) peptone, 5% (w/v) yeast extract, 20% (w/v) NaCl] supplemented with carbenicillin (100 μg/ml). An overnight culture of *V. harveyi* BB120 was inoculated at Optical Density (OD) at 600 nm = 0.2 in LM and a volume of 80 μl was pipetted into each well of a 96-microtiter plate. Cells were then grown for 4.5 h aerobically at 30°C until the mid-exponential growth phase, the growth phase where bioluminescence naturally occurs (Anetzberger et al., 2012). After 4.5 h of growth a volume of 80 μl of the cell-free supernatant of *P. luminescens*, *P. temperata*, or *P. asymbiotica*, respectively, was added. Thus, *P. luminescens* and *P. temperata* cultures were grown at 30°C, and *P. asymbiotica* culture was grown at 37°C in CASO Medium. After 4 d and 7 d, cells were adjusted to OD 600 nm = 1 before the supernatant was harvested via centrifugation. As control the same volume of LM medium and supernatant of a *V. harveyi* BB120 culture, harvested after 8 h and 1 day of growth, was added. OD and luminescence were monitored every hour with a Sunrise plate reader (Tecan, Crailsheim) and a Centro luminometer (Berthold Technologies, Bad Wildbad), respectively.

BIOINFORMATICS STUDIES

For bioinformatics identification of LuxR solos the genome assembly and annotation reports of *Photorhabdus luminescens* subsp. *laumondii* TT01 (NCBI reference sequence NC_005126.1), of *Photorhabdus temperata* subsp. *khanii* NC19 (BioSample SAMN02597464, 19 contigs, GenBank Assembly ID: GCA_000517265.1), of *Photorhabdus asymbiotica* subsp. *asymbiotica* ATCC43949 (NCBI reference sequence NC_012962.1), and of the *P. asymbiotica* plasmid pPAU1 (NCBI reference sequence NC_012961.1) were used. LuxR solos in the three above-mentioned *Photorhabdus* species were first identified based on the presence of the C-terminal "HTH LUXR" motif (SMART00421) using SMART 7 software (Simple Modular Architecture Research Tool) (Letunic et al., 2012) and BLAST software (Altschul et al., 1990). Protein domains were identified using SMART 7 software and are identified with a maximal *p*-value of 2e-07 for the "PAS_4"-domain (PFAM08448), of 2e-06 for the "HTH LUXR" motif (SMART00421) and of 1.80e-24 for the "Autoind_bind"-domain (PFAM03472). Furthermore, homologous proteins in *P. asymbiotica* were identified using STRING 9.1 database (Franceschini et al., 2013) based on the LuxR solos of *P. luminescens* (Heermann and Fuchs, 2008).

To elucidate the relationship between the LuxR solos of *Photorhabdus* species, the protein sequences of the 100 LuxR solos of the three *Photorhabdus* species, of LuxR from *Vibrio fischeri*, of TraR from *Agrobacterium tumefaciens*, of SdiA from *Escherichia coli* and of QscR and LasR from *Pseudomonas aeruginosa* were aligned and a phylogenetic tree was generated based on the alignment using CLC Mainworkbench 7 (CLC Bio Qiagen, Hilden, Germany). In the next step, the amino acid residues at the positions of the WYDPWG-motif in the

signal-binding domain (SBD) of AHL-sensors were added as metadata layers.

AHL-lactonases and AHL-acylases in the three *Photorhabdus* species were identified using SMART 7 software (Letunic et al., 2012), BLAST software (Altschul et al., 1990) and STRING 9.1 database (Franceschini et al., 2013) based on the presence of known AHL-lactonases and AHL-acylases from *Bacillus cereus, Agrobacterium tumefaciens, Arthrobacter* spec., *Rhodococcus erythropolis, Pseudomonas aeruginosa, Streptomyces* spec., *Dictyoglomus thermophilum*, and *Anabaena* spec. Furthermore, the genomes of the three *Photorhabdus* species were analyzed for the presence of the "Beta-lactamase" motif (PF00144).

RESULTS AND DISCUSSION

PHOTORHABDUS SPECIES HARBOR THREE DIFFERENT TYPES OF LuxR SOLOS

We analyzed the genome of each one representative strain of the three known *Photorhabdus* species *P. luminescens*, *P. temperata*, and *P. asymbiotica* for the presence of genes that encode potential LuxR solos. We found that all three *Photorhabdus* species harbor an exceptionally high number of LuxR solos, with 40, 22, and 38 LuxR solos in *P. luminescens*, *P. asymbiotica*, and *P. temperata*, respectively (**Figure 1**). A previous study revealed that *P. luminescens* has 39 LuxR solos (Heermann and Fuchs, 2008), but SMART 7 software (Letunic et al., 2012) actually identified Plu4288 as LuxR solo as well, which increased the number of LuxR solos to 40 in this organism. These LuxR solos all contain a C-terminal DNA-binding domain (DBD) with a helix-turn-helix motif, the "HTH LUXR" motif, which is typical for LuxR-type proteins and that was used to identify LuxR solos in *Photorhabdus* species. However, LuxR-type receptors are composed of two functional domains, the C-terminal DBD and the N-terminal SBD that are connected with a short linker (Nasser and Reverchon, 2006). Furthermore, the size of the protein rather than the degree of similarity is crucial whether a protein with such a domain organization meets the criteria to be a LuxR homolog, which is about 250 amino acids in length (Fuqua et al., 1994; Subramoni and Venturi, 2009). All the LuxR homologs identified in the three *Photorhabdus* species meet these criteria, however, diverse domains can make up the SBD, which is the domain that is important for signal-sensing specificity of the

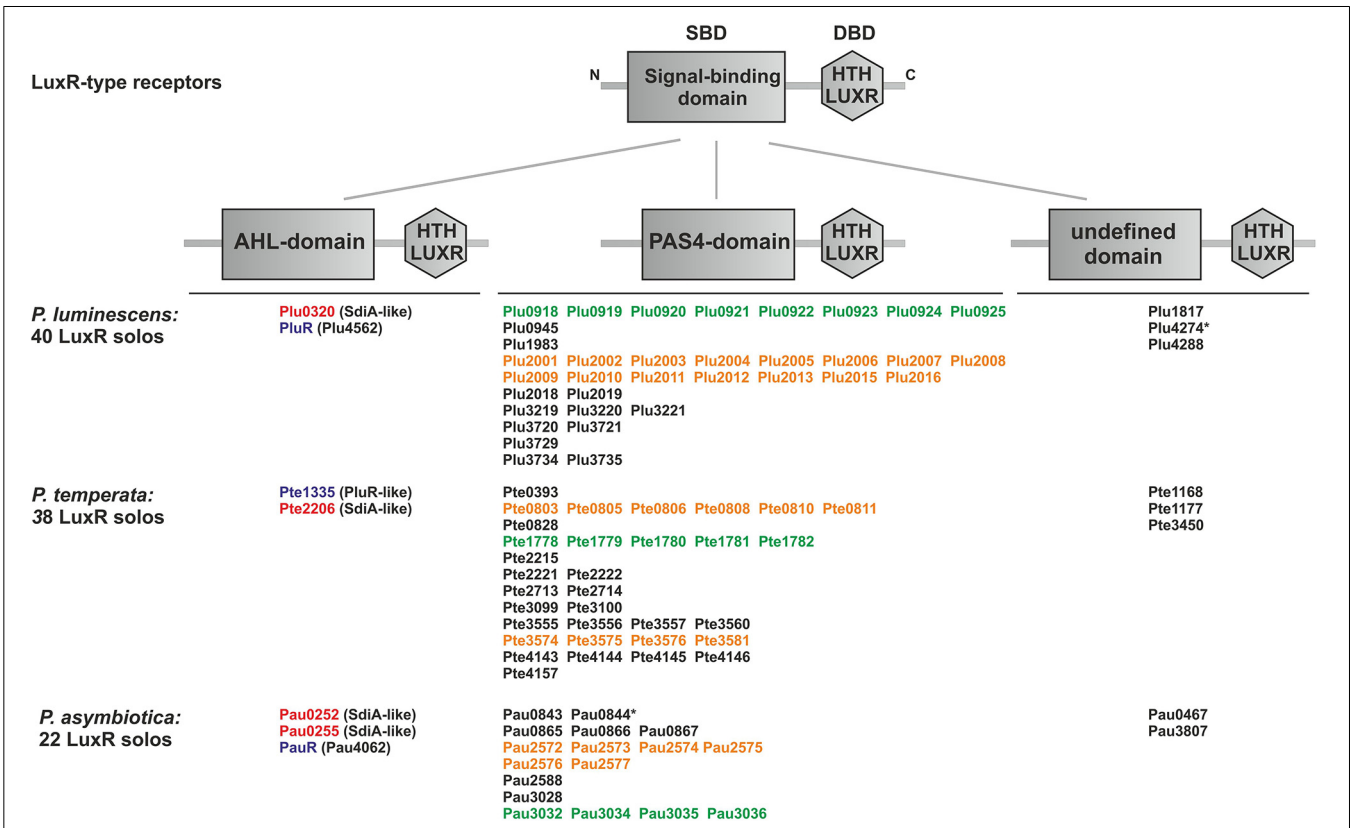

FIGURE 1 | Presence and domain structure of the LuxR solos in *P. luminescens, P. asymbiotica,* and *P. temperata*. LuxR-type receptors share a modular domain structure, with a N-terminal signal-binding domain (SBD) (gray box) and a C-terminal DNA-binding domain (DBD) with the conserved "HTH LUXR" motif (gray hexagon), which is illustrated in the upper part. The N-terminus is marked with an "N" and the C-terminus with a "C." The LuxR solos of *Photorhabdus* species, *P. luminescens* TT01, *P. temperata* NC19, and *P. asymbiotica* ATCC43949, are grouped into three types based on the different N-terminal domain. The "HTH LUXR" motif (SMART00421) is indicated by a hexagon, the AHL-domain (PFAM03472: "Autoind_bind"-domain) and the PAS4-domain (PFAM08448: "PAS_4"-domain) by boxes. LuxR solos marked with an asterisk additionally have an N-terminal transmembrane domain (not illustrated). Homologous proteins or cluster are marked with the colors red, purple, orange and green. LuxR solos were identified using SMART 7 software (Letunic et al., 2012) and BLAST software (Altschul et al., 1990).

receptor. The entire 100 LuxR solos of the three *Photorhabdus* species differ in their N-terminal SBD, for which reason they are grouped into three types: LuxR solos with a PAS4-domain, LuxR solos with an AHL-domain (Autoind_bind) and LuxR solos with yet undefined SBD domains (**Figure 1**). Presumably, these variable SBD-domains enable the bacteria to sense diverse signals, like exogenous AHLs, exogenous or endogenous non-AHLs, or eukaryotic signals, and thereby influence different cellular processes (Subramoni and Venturi, 2009). The overall number of the LuxR solos might therefore reflect the diversity of invertebrate or vertebrate hosts that *Photorhabdus* species can infect or colonize.

LuxR solos containing a PAS4-domain

The majority of the LuxR solos in *Photorhabdus* spec. contain a N-terminal PAS4-domain, which are in sum 80 of the 100 identified LuxR solos. However, the signals sensed by these LuxR solos are yet unidentified. Commonly, PAS-domains are ubiquitous, they appear in archaea, eubacteria and eukarya and are involved in binding of a diverse set of small regulatory molecules either covalently or non-covalently (Hefti et al., 2004). In the fruit fly *Drosophila melanogaster* PAS3 domains have been proved as insect juvenile hormone receptors (Dubrovsky, 2005). The homologous PAS4-domains in *Photorhabdus* are assumed to bind hormone-like molecules and are therefore proposed to be major players in inter-kingdom signaling via the detection of hormone-specific signals from the eukaryotic hosts (Heermann and Fuchs, 2008). As this is most likely, it cannot be excluded that also other bacteria might be sensed via one or more of the PAS4-LuxR receptors. Moreover, the majority of the PAS4-domain-containing LuxR solos in *Photorhabdus* spec. are organized in large gene clusters, which are *plu0915-plu0925* and *plu2001-plu2016* in *P. luminescens*, *pau2572-pau2577*, and *pau3032-pau3036* in *P. asymbiotica* and the operons *pte0803-pte0811*, *pte1178-pte1779*, and *pte3555-pte3581* in *P. temperata*. The function of this genetic clustering as well as the high redundancy of all these PAS4-LuxR solos is unknown to date. Presumably, the high redundancy of PAS4-LuxR solos might be a co-evolutional result by adaptation of the bacteria to the variety of insect hosts they can infect. This idea is underlined by the finding that several LuxR solos from plant-associated bacteria are known to respond to plant signaling molecules and are therefore assumed to have undergone co-evolution with the related host plant (Covaceuszach et al., 2013). Putative signals sensed by the PAS4-LuxR solos might be diffusible substances like hormones. The phytopathogens belonging to the genus *Xanthomonas* use a fatty acid signal belonging to the Diffusible Signal Factor (DSF) family for cell-cell signaling to control the virulence of *Xanthomonas campestris* pv. *campestris* (Xcc) to plants. Recently, a second sensor for DSF was identified, which is a complex sensor kinase having a N-terminal PAS4-domain essential for sensing of DSF (An et al., 2014). This supports the idea that the PAS4-LuxR solos of *Photorhabdus* spec. are adapted to distinct signals from the invertebrate hosts (insects and nematodes) or in case of *P. asymbiotica* additionally to vertebrate hosts, especially humans, to expand or fine-tune their regulatory network.

LuxR solos containing an AHL-domain

Besides the high number of PAS4-LuxR solos, all three *Photorhabdus* species contain at least one LuxR solo that is homologous to the LuxR solo SdiA. *P. luminescens* and *P. temperata* each have one SdiA homolog, Plu0320 and Pte2206, respectively, whereas the human pathogenic *P. asymbiotica* has two SdiA homologs, Pau0252 and Pau0255. The LuxR solos SdiA of *Escherichia coli* and *Salmonella enterica* are known to detect exogenously produced AHLs and therefore these organisms are able to sense mixed microbial communities (Wang et al., 1991; Michael et al., 2001). All four SdiA homologs in *Photorhabdus* spec. have a N-terminal "Autoind_bind" domain (PFAM03472) (**Figure 1**), which is typical for AHL-sensors and responsible for AHL-sensing. Similar to SdiA, the four LuxR solos Plu0320, Pau0252, Pau0255, and Pte2206, respectively, can be assumed to detect AHLs as well. Since no *luxI* gene is present in any of the *Photorhabdus* genomes, it is most likely that these LuxR solos can respond to exogenously produced AHLs, produced by other bacteria, probably of the insect gut, which are then sensed by *Photorhabdus* spec. during the infection process. Moreover, AHLs have never been detected using different analytical chemistry methods (HPLC/MS, GC/MS) in any *Photorhabdus* strain, despite the analysis of >100 strains (Darko Kresovic and Helge B. Bode, data not shown). As the human pathogen *P. asymbiotica* has two putative AHL-sensors (**Figure 1**), it is most likely that AHL-sensing plays an even more important role for vertebrate than invertebrate host infection. Interestingly, also the three homologous LuxR solos PluR (Plu4562) of *P. luminescens*, PauR (Pau4062) of *P. asymbiotica*, and PluR (Pte1335) of *P. temperata* contain a predicted "Autoind_bind" domain like the AHL sensors. However, the LuxR solo PluR senses α-pyrones named photopyrones (PPYs) instead of AHLs (Brachmann et al., 2013), which shows that the presence of an "Autoind_bind" domain is not automatically going along with AHL-sensing. PluR was the first example of a LuxR solo that senses an endogenous signal and is therefore part of a bacterial cell-cell communication system in *P. luminescens* regulating expression of the *pcfABCDEF* operon leading to cell clumping (Brachmann et al., 2013). *P. luminescens* produces eight different PPYs, out of which three (PPYA, PPYB, PPYD) are mainly present in the culture fluid. Among these, PluR senses PPYD with the highest sensitivity in a concentration as low as 3.5 nM. Furthermore, the ketosynthase-like protein PpyS was identified as the PPY synthase and therefore as producer of the signaling molecules. All proteins are also present with high homology in *P. temperata*, which indicates a similar strategy for cell-cell communication in this genus (Brachmann et al., 2013). The insect and human pathogen *P. asymbiotica* harbors a *pcf* regulon that is highly homologous to that of *P. luminescens*. It consists of the *pcfABCDEF* (*pau4068-pau4063*) operon and an adjacent gene encoding a LuxR solo that we named PauR (Pau4062). However, *P. asymbiotica* contains neither a *ppyS* homolog nor a *luxI*-like synthase and does not produce any PPYs or AHLs. Therefore, it is assumed that *P. asymbiotica* uses yet unidentified signaling molecules for cell-cell communication, which are sensed by PauR. As we will focus on below, this is rather due to the presence of a conserved amino acid motif within the SBD, which is intact in the

SdiA homologs, but different in both PluR proteins and PauR. When PluR was originally detected it met the criteria to be defined as LuxR solo. Since PluR has a cognate synthase, but which is different from LuxI, it is thus part of an entire cell-cell communication system. Therefore, it is disputable whether the designation LuxR solo is actually appropriate at least for PluR.

LuxR solos containing a non-defined signal-binding domain

Besides LuxR solos with defined SBDs, all three *Photorhabdus* species harbor LuxR solos with yet unidentified SBDs, namely Plu1817, Plu4274, Plu4288, Pau0467, Pau3804, Pte1168, Pte1177, and Pte3450. In contrast to many other LuxR solos, which are often hydrophobic and membrane associated (Welch et al., 2000), Pau0844 and Plu4274 are predicted to contain a N-terminal transmembrane domain (TM). Although the exact mechanism of DNA-binding by a membrane-integrated protein is not yet fully understood, other examples of those membrane-integrated DNA-binding receptors are known, e.g., the pH-sensor CadC of *Escherichia coli* (Haneburger et al., 2011). The presence of an intact TM within the SBD could give indication for sensing an extremely hydrophobic and non-water-soluble signaling molecule. The LuxR solo Pau0844 is the only LuxR solo that harbors a TM in combination with a PAS4-domain, which might therefore sense an extremely hydrophobic eukaryotic signal. Overall, the LuxR solos with yet undefined SBDs might expand the range of signals that are sensed and therefore the communication network. However, their roles in cell-cell communication and/or inter-kingdom signaling as well as the related signaling molecules are unknown to date.

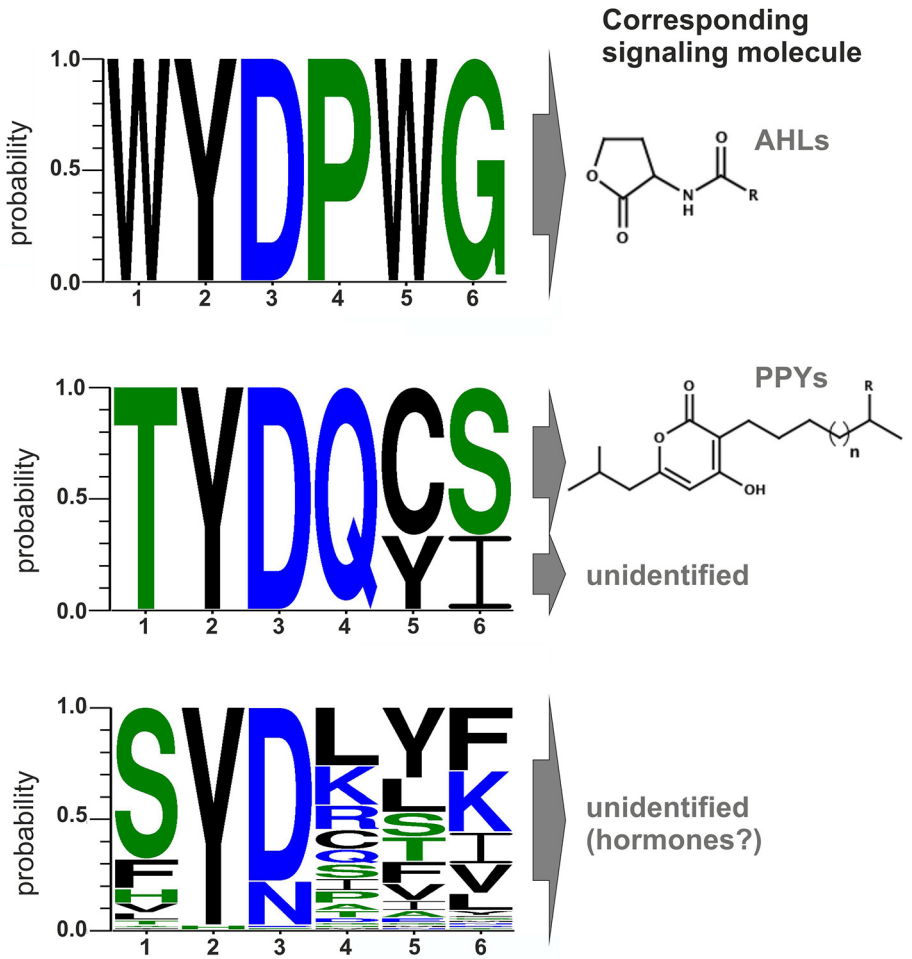

FIGURE 2 | Conserved amino acid motifs of LuxR-type proteins with different domains and their corresponding signaling molecules. Upper part: Motif of the six conserved amino acid positions in typical AHL-sensors. Protein sequences of LuxR from *Vibrio fischeri*, TraR from *Agrobacterium tumefaciens*, SdiA from *Escherichia coli*, QscR and LasR from *Pseudomonas aeruginosa* were used to generate the alignment. **Middle part**: Motif of the six conserved amino acids positions of PluR (Plu4562) from *P. luminescens*, PluR (Pte1335) from *P. temperata* and PauR (Pau4062) from *P. asymbiotica*. PluR from *P. luminescens* and from *P. temperata* are sensing photopyrones as signaling molecule, however this is yet unidentified for PauR. **Lower part:** Motif of the six conserved amino acid positions of the overall 80 PAS4-LuxR solos in all three *Photorhabdus* species, whereas the corresponding signal molecules are yet unknown but possible are eukaryotic hormones. All alignments were generated with CLC Mainworkbench 7 software (CLC Bio Qiagen, Hilden, Germany). The sequence logo was made with WebLogo3 (Crooks et al., 2004).

CONSERVED AMINO ACID MOTIFS IN THE SIGNAL-BINDING DOMAIN OF LuxR SOLOS

Comparison of all LuxR solos of *Photorhabdus* spec. indicates that signal-specificity relies on the SBD, given that the major differences are located in the SBD and the similarities in the DBD. In general, proteins of the LuxR family are less conserved in their amino acid sequence, which only makes up between 18 and 25% identity (Subramoni and Venturi, 2009). However, LuxR-type proteins sensing AHLs harbor six highly conserved amino acid in the N-terminal SBD that are important for signal-binding, signal molecule specificity and shaping the signal-binding pocket. These six conserved amino acids are W57, Y61, D70, P71, W85, and G113 (with respect to TraR), which are either hydrophobic or aromatic and displaying a conserved motif for AHL-sensors (Patankar and González, 2009). Bioinformatics analyses of the amino acids of the *Photorhabdus*-specific LuxR solos at these positions reveals that the conserved WYDPWG-motif of AHL-sensors is only completely present in the SdiA homologs Plu0320 of *P. luminescens*, Pau0252 and Pau0255 of *P. asymbiotica*, and Pte2206 of *P. temperata*, supporting the idea that these LuxR solos sense exogenous AHLs. All other LuxR solos in *Photorhabdus* spec. have at least two substitutions at different positions in the WYDPWG-motif. Remarkably, amino acid Y61 (with respect to TraR) is present in 97% of the LuxR solos of the three *Photorhabdus* species. This amino acid is known to be involved in binding of the acyl chain of the signaling molecule via hydrophobic interactions, e.g., in TraR (Churchill and Chen, 2011) or LuxR (Nasser and Reverchon, 2006). Therefore, there seems to be a common mechanism to arrange distinct signaling molecules in the signal-binding pocket, independent from the exact chemical nature of the signaling molecule. The position D70 (with respect to TraR) is known to be important for binding the amide group of the cognate AHL within AHL-binding LuxR receptors (Churchill and Chen, 2011). This position is also conserved among 80% of the LuxR solos in the three *Photorhabdus* species, whereas the remaining LuxR solos (19%), mainly from *P. asymbiotica*, have the substitution D70N at this position, which might be important to sense eukaryotic signals during vertebrate infection (**Figure 2**). Although not sensing AHLs (Brachmann et al., 2013), the three LuxR solos, PauR and both PluR homologs of *P. asymbiotica*, *P. luminescens*, and *P. temperata*, respectively, have a predicted "Autoind_bind" domain. However, only the two conserved amino acids Y61 and D70 (with respect to TraR) are present in these LuxR solos. Moreover, both PluR proteins share a TYDQCS-motif, which is therefore probably specific for PPY-sensing (**Figure 2**). Individual replacement of Y66 and D75 with alanine within PluR prevented PPY-binding, revealing that these amino acids are important for PPY-binding and/or specificity (Brachmann et al., 2013). In contrast, PauR has a slightly different SBD-motif, which is TYDQYI, indicating that this LuxR solo, although highly homologous to both PluR proteins, senses a signal that is different from PPYs as well as AHLs. Overall, variations in the WYDPWG-motif that is typical for AHL-sensing probably expand the diversity of signals that can be sensed by LuxR-type proteins having an "Autoind_bind" domain. Additionally, the 80 LuxR solos of *Photorhabdus* spec. with a PAS4-domain share a more variable amino acid motif in the SBD, reflecting a higher variety of signals they might sense (**Figure 2**). However, the precise signals that are sensed by PAS4-LuxR solos are yet unknown. The huge diversity of the SBD-motifs of PAS4-LuxR solos in *Photorhabdus* and the variations in the conserved amino acid motifs probably gives the bacteria the capacity to respond to a broad range of signals that occur in the different environments or hosts.

A closer look at the protein sequence and phylogeny of the *Photorhabdus*-specific LuxR solos reveals that related LuxR solos, independent of their host organism, share the similar amino acid motif in the SBD (**Figure 3**). More precisely, LuxR solos encoded by one operon tend to have a similar amino acid motif at the six conserved positions of the SBD. For example the *plu0919-plu0925* operon and the homologous *pte4143-pte4146* operon encoding the respective PAS4-LuxR solos both show replacement of amino acid W85 (with respect to TraR) to L and of G113 (with respect to TraR) either to V or L in the WYDPWG-motif. This in total applies for 14 of 80 LuxR solos that have a PAS4-domain. However, over 30 LuxR solos show a substitution of W85 to Y containing a PAS4-domain. These homologous changes may originate via gene duplications within one species and might give these LuxR solos the ability to sense similar signals. On the other hand, closely related LuxR solos also incline to the same kind of substitution of the amino acid motif in the SBD (**Figure 3**). The homologous PluR LuxR solos, Plu4562 from *P. luminescens* and Pte1335 from *P. temperata*, share the identical substitutions of four conserved amino acids, forming a TYDQCS-motif allowing both to sense PPYs. The closely related LuxR solo PauR from *P. asymbiotica* harbors the slightly different TYDQYI-motif at the similar positions, suggesting binding of a distinct signaling molecule. This could be due to the fact that cell-cell-communication via PPY might be inappropriate when infecting vertebrates. Comparably, the LuxR solos of the novel subfamily of plant-associated bacteria have substitutions in the WYDPWG-motif, which are W57M and Y61W (with respect to TraR) that might allow the binding of plant signal molecules rather than AHLs (Patankar and González, 2009; González and Venturi, 2013). The *P. luminescens* specific PAS4-LuxR solos Plu2018, Plu3221, Plu3720, Plu3729, and Plu3735 are closely related to each other and all have an identical amino acid motif within the SBD, which is SYDLYK. This shows that phylogeny of the LuxR solos correlates with the protein sequence as well as the six conserved amino acid positions within the SBD.

Furthermore, variations of the amino acids at the conserved positions to motifs different from WYDPWG offers the possibility to sense different signals or altering the affinity toward a specific signal, as the latter is true for EsaR from *Pantoea stewartii* (Shong et al., 2013). However, LuxR solos with similar amino acid motifs in the SBD of different species might in turn lead to the binding of the same signaling molecules.

QUORUM QUENCHING VIA AHL-LACTONASES AND AHL-ACYLASES IN *PHOTORHABDUS* SPECIES

As identified above, each *Photorhabdus* species harbor a SdiA-homolog, which are the LuxR solos Plu0320, Pte2206, Pau0252, and Pau0255 of *P. luminescens*, *P. temperata*, *and P. asymbiotica*, respectively, containing an AHL-binding domain. Although

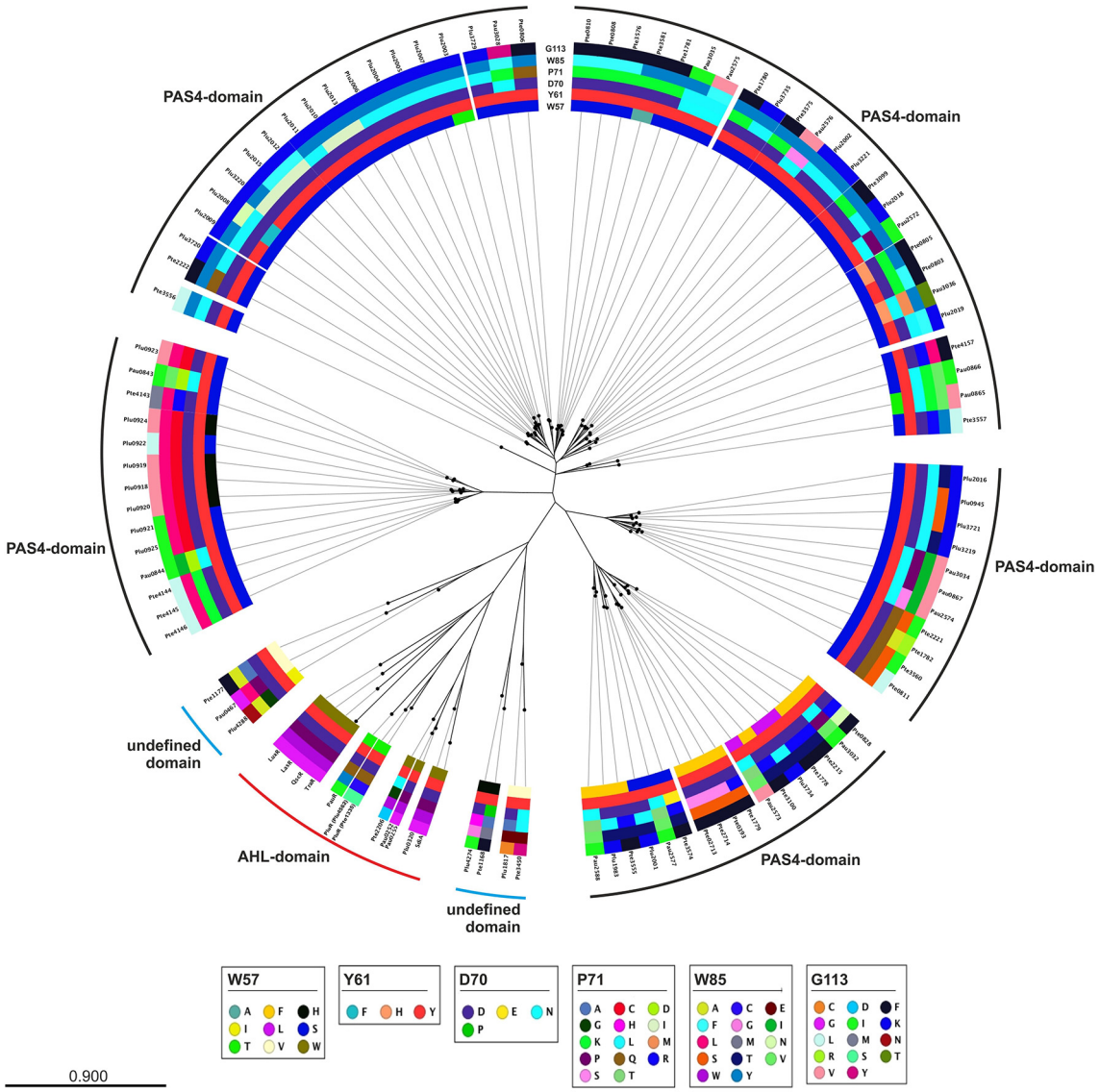

FIGURE 3 | Phylogenetic tree of the LuxR solos present in *P. luminescens, P. temperata,* and *P. asymbiotica*. Protein sequences of the overall LuxR solos of the three *Photorhabdus* species, of LuxR from *Vibrio fischeri*, of TraR from *Agrobacterium tumefaciens*, of SdiA from *Escherichia coli* as well as of QscR and LasR from *Pseudomonas aeruginosa* were aligned and a phylogenetic tree was generated. Based on this alignment the different amino acid motifs at the six conserved positions were identified by deviation from the WYDPWG-motif in the SBD of AHL-sensors. A special focus on the amino acids at positions of the WYDPWG-motif is shown from the inner to the outer circle. The amino acid W57, with respect to TraR, is marked in brown, Y61 in red, D70 in purple, P71 in dark red, W85 in pink and G113 in light pink, however substitutions within this positions are marked in different colors. LuxR-type proteins with an AHL-binding domain are marked with a red dash, LuxR-type proteins with a PAS4-domain are marked with a black dash and LuxR-type proteins with a N-terminal yet undefined domain are marked with a blue dash. Alignment and radial phylogenetic tree was generated with the CLC Mainworkbench 7 (CLC Bio Qiagen, Hilden, Germany). The scale bar indicates the length of the branches.

the functions of these LuxR solos are yet unknown, it might be the sensing of external AHLs. One reason for sensing external AHLs could be to interfere with LuxR quorum sensing systems of their environment by degradation of the QS signaling molecule of neighboring bacteria. This process is designated as quorum quenching (QQ), interferes with the communication of a bacterial population, and can be used to influence their activity, e.g., virulence. For this purpose, many bacteria produce lactonases and acylases, which hydrolyze the lactone ring and cleave the amide bond, respectively, of AHLs. These enzymes are found in both Gram-negative AHL producers and non-AHL producers as well as in Gram-positive bacteria (Williams, 2007). The virulence of the plant pathogens *Pseudomonas solanacearum*, diverse *Erwinia* species and the human pathogen *Pseudomonas aeruginosa* is influenced via QQ enzymes (Dong et al., 2000). We analyzed the genome of all three *Photorhabdus* species for the presence of genes that encode putative AHL-lactonases and AHL-acylases. We identified several homologs of known AHL-lactonase

Table 1 | Homologous AHL-lactonases and AHL-acylases in *Photorhabdus* spec.

Strain	Protein	Enzymatic activity	*P. luminescens* TT01	*P. asymbiotica* ATCC43949	*P. temperata* NC19
B. cereus	AiiA	AHL-lactonase	Plu1749	Pau1378	Pte3377
A. tumefaciens	AiiB	AHL-lactonase	–	AttM/AiiB (Pau0481)	–
A. tumefaciens	AttM	AHL-lactonase	Plu2238	AttM/AiiB (Pau0481)	Pte1161
Arthrobacter spec.	AhlD	AHL-lactonase	Plu2238	–	–
P. aeruginosa	PvdQ	AHL-acylase	Plu3527	–	–
Streptomyces spec.	AhlM	AHL-acylase	Plu3527	–	–
D. thermophilum	AhlA	AHL-acylase	Php (Plu1997)	Php (Pau2581)	Pte3570
Anabaena spec.	AiiC	AHL-acylase	Plu3527	–	–

The AHL-lactonases AiiA from Bacillus cereus *(Wang et al., 2004), AiiB from* Agrobacterium tumefaciens *(Liu et al., 2007), AttM from* Agrobacterium tumefaciens *(Zhang et al., 2002) and AhlD from* Arthrobacter spec. *(Park et al., 2003) were used in this study to identify homologs in the three* Photorhabdus *species. AHL-lactonases have a "Lactamase_B" domain (PF00144) belonging to the metallo-beta-lactamase superfamiliy. Furthermore, Zn^{2+}-dependent hydrolases exist, e.g., AiiB, AhlD, AttM, QsdA, Plu2238, Pau1378, Pte1161, and Pte3377. The acylases PvdQ from* Pseudomonas aeruginosa *(Huang et al., 2003), AhlM from* Streptomyces spec. *(Park et al., 2005), AhlA from* Dictyoglomus thermophilum *(Coil et al., 2014), and AiiC from* Anabaena spec *(Romero et al., 2008). Analyses are based on STRING 9.1 database and NCBI Blast (Altschul et al., 1990) were used in this study to identify homologs in the three* Photorhabdus *species. The AHL-acylases AhlM, AhlA, and AiiC belong to the Ntn-hydrolase superfamily.*

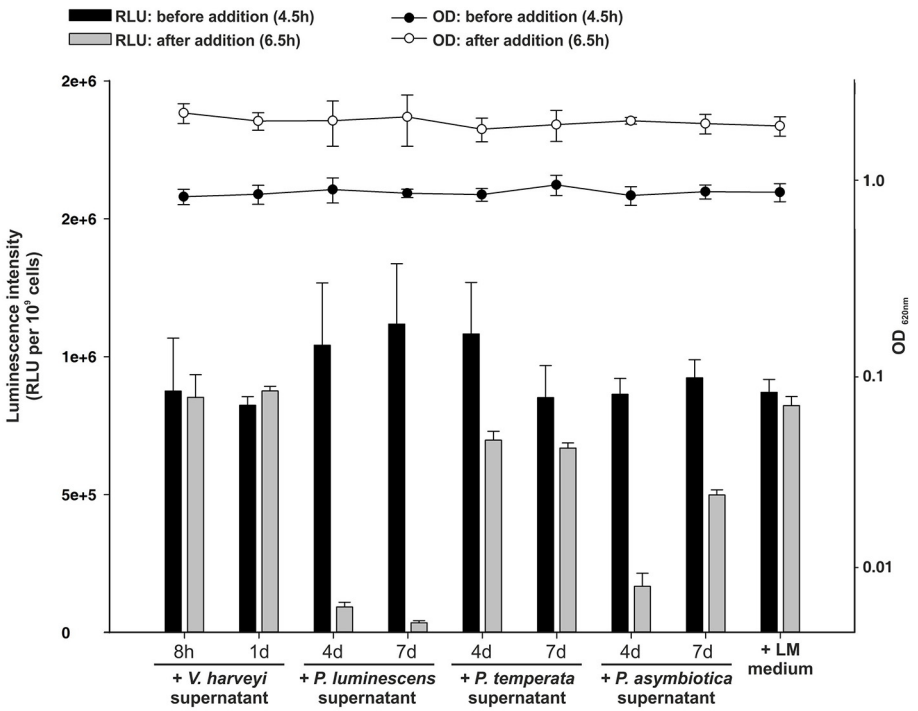

FIGURE 4 | Quorum quenching by *Photorhabdus* species culture fluids. Bioluminescence of *Vibrio harveyi* wild-type BB120 was used as read-out to analyze degradative activity of *Photorhabdus* spec. supernatants. The supernatants of *P. luminescens*, *P. temperata*, and *P. asymbiotica* were harvested after 4 and 7 days of growth and added to the BB120 reporter strain in the mid-exponential growth phase, when bioluminescence occurs. As controls *V. harveyi* supernatant and LM medium, harvested after 8 h and 1 day of growth, was added. Luminescence (RLU) was measured before (black bars) and after (gray bars) the addition of the *Photorhabdus* spec. supernatant and optical density (OD) was measured before (black circles) and after (white circles) the addition of the *Photorhabdus* spec. supernatant. Error bars represent standard deviation of at least three independently performed experiments. RLU, relative light units.

and AHL-acylases in all three *Photorhabdus* species (**Table 1**). Particularly, homologs for the AHL-lactonase AiiA from *Bacillus* spec., which inactivates the QS signal and decreases the virulence of *Erwinia carotovora* (Park et al., 2008), are found in all three *Photorhabdus* species, which are Plu1749, Pau1378, and Pte3377. In addition, AiiA showed a strong enzymatic activity toward distinct AHLs, which vary in length and the substitution at the C3 position of the acyl chain (Wang et al., 2004). Park et al. (2003) demonstrated that the AHL-lactonase AhlD of *Arthrobacter* spp. blocks the communication of other bacteria and

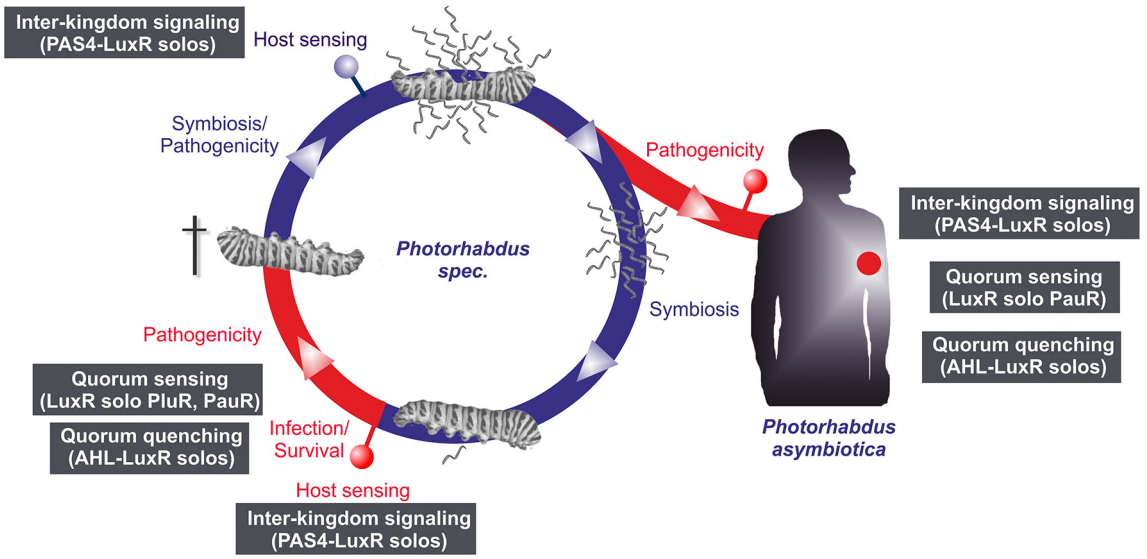

FIGURE 5 | Life and infection cycle of *Photorhabdus* spec. and involvement of the different LuxR solos. Life cycle of *P. luminescens*, *P. temperata*, and *P. asymbiotica* (left panel). The bacteria colonize the upper gut of heterorhabditid nematodes that invade insect larvae. After release into the insect's hemolymph the bacteria produce several toxins that rapidly kill the prey. After death of the insect host, the bacteria degrade the cadaver, and additionally support nematode development. When the cadaver is depleted from nutrients, bacteria and nematode re-associate and leave the carcass in search for a new victim. The pathogenic part of the cycle is drawn in red, the symbiotic part in blue. *P. asymbiotica* can additionally infect humans by inducing systemic and soft tissue infections (right panel). Putative involvement of the different LuxR solos at steps of the infection process or host sensing is indicated by the gray boxes (see text for details).

yet *P. luminescens* could use the same strategy via the homolog Plu2238. The AHL-lactonase AttM of *A. tumefaciens*, encoded on the At plasmid, is important for the attachment to plant cells (Liu et al., 2007) and a homolog is found in each *Photorhabdus* species, which are Plu2238, Pau0481, and Pte1161. However, the plasmid pPAU1 from *P. asymbiotica* harbors no known AHL-lactonase or AHL-acylase or homologs revealing that QQ by *Photorhabdus* is not especially important for vertebrate infection, or that the similar QQ enzymes for vertebrate as well as for invertebrate infection are used. However, we not only detected the presence of these putative AHL-lactonases and AHL-acylases in *Photorhabdus* species. Moreover, we analyzed the potential QQ ability of *Photorhabdus* species culture fluids. Therefore, the culture fluids of all three *Photorhabdus* spec. at different growth phases were examined using the marine bacterium *Vibrio harveyi* and its regulation of bioluminescence via accumulation of the AHLs as reporter. Thereby, all three culture fluids emerged to have high QQ activity, putatively by degradation of the AHL HAI-1 of *V. harveyi* (**Figure 4**). Especially, the culture supernatant of *P. luminescens* had the highest AHL-degrading activity against HAI-1 of *V. harveyi* compared to those of *P. temperata* as well as *P. asymbiotica* (**Figure 4**). However, the number of putative AHL-lactonases and AHL-acylases homologs predicted in all three *Photorhabdus* species is similar (**Table 1**). These differences in QQ could therefore only be explained with the theory that the respective genes might be differently regulated in the three *Photorhabdus* species and that therefore not all enzymes are fully produced or activated under the *in vitro* experimental conditions used. Anyway, our findings indicate that the three *Photorhabdus* species can interfere with the AHL-based quorum sensing systems of other bacteria. This could be an important step in the infection process, probably to shut down virulence of opportunistic pathogens or food competitors that are present in the host gut or in the soil to prevent them from pitching into their prey or to stop them from antibiotic production. The expression of the different AHL-lactonases and/or AHL-acylases encoding genes could be activated via the SdiA-homologous LuxR solos Plu0320, Pte2206, Pau0252, and Pau0255 of *P. luminescens*, *P. temperata*, *and P. asymbiotica*, respectively, after sensing the external AHLs. However, whether the AHL-lactonases and/or AHL-acylases encoding genes are under control of the SdiA-homologous LuxR solos in *Photorhabdus* spec. is indeed possible, but has to be investigated.

Overall, the defense of the dead insect larvae, which is a rich food source, is crucial for the survival of *Photorhabdus* as well as the nematode partner. Thus, disturbing of the ambient mixed microbial community and its communication might be an important step for a successful infection process and reproduction.

CONCLUSIONS

LuxR solos emerge to be more and more important players in cell-cell communication or inter-kingdom-signaling as they offer possibilities using alternative communication molecules to AHLs. In all three *Photorhabdus* species an extraordinary high number of LuxR solos were identified, making them to optimal model organisms to study the function of LuxR solos in bacteria. Thereby, regulation via LuxR solos can be proposed to be important at different steps of the *Photorhabdus* life cycle and infection process (**Figure 5**). However, the majority of the LuxR solos in all three *Photorhabdus* species contain a PAS4-domain, which lends

support for the theory that host sensing is highly important at different steps in the *Photorhabdus* life cycle. In contrast, sensing other bacteria by detecting their AHLs as well as quorum sensing seems to be only important for the pathogenic steps of the life cycle rather than for the symbiosis parts. We have seen that the LuxR solos contain different SBDs, which include diverse amino acid motifs at conserved positions. The diversity of these motifs gives rise to the speculation that signal-binding of all these LuxR solos goes far beyond AHL-signaling, as we have recently demonstrated for PluR for the first time (Brachmann et al., 2013). One can only guess the variety of signals perceived by all these LuxR solos and their function in cell-cell communication and interkingdom-signaling. It will be the goal of the near future research to unravel the various signaling molecules and to correlate them to the specific LuxR solos or amino acid motifs in the SBD of these proteins. The presence of all those different types of LuxR solos gains first insight into the complexity of the communication network between bacteria among each other as well as with their hosts. As most of the LuxR solos that have been investigated so far are involved in regulation of insect or plant pathogenicity, the homologous receptors or the related signaling molecules might be promising specific drug targets in human pathogens as well.

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Identification of potential genetic components involved in the deviant quorum-sensing signaling pathways of *Burkholderia glumae* through a functional genomics approach

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Burkholderia glumae is the chief causal agent for bacterial panicle blight of rice. The acyl-homoserine lactone (AHL)-mediated quorum-sensing (QS) system dependent on a pair of *luxI* and *luxR* homologs, *tofI* and *tofR*, is the primary cell-to-cell signaling mechanism determining the virulence of this bacterium. Production of toxoflavin, a major virulence factor of *B. glumae*, is known to be dependent on the *tofI/tofR* QS system. In our previous study, however, it was observed that *B. glumae* mutants defective in *tofI* or *tofR* produced toxoflavin if they grew on the surface of a solid medium, suggesting that alternative signaling pathways independent of *tofI* or *tofR* are activated in that growth condition for the production of toxoflavin. In this study, potential genetic components involved in the *tofI*- and *tofR*-independent signaling pathways for toxoflavin production were sought through screening random mini-Tn*5* mutants of *B. glumae* to better understand the intercellular signaling pathways of this pathogen. Fifteen and three genes were initially identified as the potential genetic elements of the *tofI*- and *tofR*-independent pathways, respectively. Especially, the ORF (bglu_2g06320) divergently transcribed from *toxJ*, which encodes an orphan LuxR protein and controls toxoflavin biosynthesis, was newly identified in this study as a gene required for the *tofR*-independent toxoflavin production and named as *toxK*. Among those genes, *flhD*, *dgcB*, and *wzyB* were further studied to validate their functions in the *tofI*-independent toxoflavin production, and similar studies were also conducted with *qsmR* and *toxK* for their functions in the *tofR*-independent toxoflavin production. This work provides a foundation for future comprehensive studies of the intercellular signaling systems of *B. glumae* and other related pathogenic bacteria.

Keywords: *Burkholderia glumae*, bacterial panicle blight of rice, bacterial grain rot of rice, quorum-sensing, toxoflavin

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Introduction

Burkholderia glumae causes bacterial panicle blight of rice and produces major virulence factors, such as toxoflavin and lipase, under the control of the quorum sensing (QS) system mediated by the *luxI* homolog, *tofI*, and the *luxR* homolog, *tofR* (Kim et al., 2004, 2007; Devescovi et al., 2007).

Kim et al. (2004) used biosensors and thin layer chromatography to determine the acyl-homoserine lactone (AHL)-type autoinducers of *B. glumae* and found that *N*-octanoyl homoserine lactone (C8-HSL) and *N*-hexanoyl homoserine lactone (C6-HSL) are produced by the LuxI homolog, TofI. C8-HSL is considered as the functional autoinducer binding to TofR for promoting the virulence-related phenotypes including toxoflavin production and flagellum-mediated motility, while the role of C6-HSL is still vague (Kim et al., 2004, 2007). Several important genes for the virulence of *B. glumae* regulated by C8-HSL and its cognate receptor TofR include: the *tox* gene clusters (operons) for toxoflavin biosynthesis (*toxABCDE*) and transport (*toxFGHI*) (Kim et al., 2004; Shingu and Yoneyama, 2004; Suzuki et al., 2004), *lipA* encoding the LipA lipase (Devescovi et al., 2007), genes for flagellar biogenesis and *qsmR* encoding an IclR-type transcriptional regulator (Kim et al., 2007), and *katG* encoding a protective catalase (Chun et al., 2009). In addition, genes for the type III secretion system (T3SS) was demonstrated to be a part of the regulon of the *tofI*/*tofR* QS system (Kang et al., 2008). As these genes under the control of the *tofI*/*tofR* QS is important for the survival, colonization and pathogenesis of *B. glumae*, it will be beneficial to expand the knowledge upon the intercellular signaling network involving *tofI* and *tofR* for gaining a better understanding of the pathogenic behaviors of this pathogen.

In our previous study, a series of deletion mutants of *B. glumae* for *tofI* and *tofR* were generated for comprehensive characterization of the *tofI*/*tofR* QS system, using a Louisiana strain of *B. glumae*, 336gr-1 (Chen et al., 2012). Consistent with the previous studies with mutant derivatives of the *B. glumae* strain BGR1, Δ*tofI* or Δ*tofR* derivatives of *B. glumae* 336gr-1 did not produce toxoflavin in Luria-Bertani (LB) broth (Chen et al., 2012). However, these mutants produced high levels of toxoflavin when they were grown on solid media, including Luria broth (LB) agar and King's B (KB) agar (Chen et al., 2012). These results indicate the presence of previously unknown signaling/regulatory pathways for the production of toxoflavin that are activated in certain growth conditions (e.g., solid media) in *tofI*- and *tofR*-independent manners (Chen et al., 2012). In this study, Δ*tofI* and Δ*tofR* derivatives of *B. glumae* 336gr-1 were randomly mutagenized with mini-Tn*5 Cm* and the mutants showing lost ability of toxoflavin production on LB agar were screened in an attempt to identify and characterize the *tofI*- and *tofR*-independent signaling/regulatory pathways for toxoflavin production. For more sensitive visual detection of altered toxoflavin production by each transposon mutant, a DNA construct that harbors the promoterless *gusA* reporter gene fusion to the promoter region of the toxoflavin biosynthesis operon *toxABCDE*, $P_{toxABCDE}$::*gusA*, was introduced into the Δ*tofI* and Δ*tofR* mutants, and the expression of the fused *gusA* reporter gene in each mutant was monitored based on the blue coloration of each mutant on LB agar containing the substrate of β-glucuronidase, 5-bromo-4-chloro-1*H*-indol-3-yl β-D-glucopyranosiduronic acid (X-gluc). Mutated genes of the primarily screened mutants, which did not show blue coloration on LB agar with X-gluc, were identified by sequencing the flanking regions of mini-Tn*5 Cm* inserted in the genome and the functions of selected genes were further studied in terms of their regulatory roles in toxoflavin production and virulence.

Materials and Methods

Growth Conditions of Bacterial Strains

The bacterial strains and plasmid constructs used in this study are listed in **Table 1**. Media used for routine cultures of bacterial strains were LB broth or LB agar (Sambrook, 2001) with appropriate amendments of antibiotics. Liquid cultures were incubated in a shaking incubator at 200 rpm at 37°C. The antibiotics and their working concentrations used in this study were: ampicillin (Amp), 100 μg/ml; kanamycin (Km), 50 μg/ml; nitrofurantoin (Nit), 100 μg/ml; and gentamycin (Gm), 20 μg/ml. The substrate of β-glucuronidase, X-gluc), was applied at a working concentration of 2 mM.

Recombinant DNA Techniques

General DNA manipulations were conducted following standard methods (Sambrook, 2001). A Strata Clone™ PCR cloning kit (Agilent Technologies, Santa Clara, CA, USA) was used for cloning PCR products into a pSC-A-amp/kan vector. Cloned PCR products in pSC-A-amp/kan were sequenced for confirmation at GeneLab in the LSU School of Veterinary Medicine or at Macrogen USA (http://www.macrogenusa.com) using M13 forward and reverse primers. A GenePulser unit (BioRad Laboratories, Hercules, CA, USA) was used to perform electroporation for transforming *E. coli* competent cells under 1.5 kV, with 1 μl of ligated DNA and 25 μl of competent cells. Triparental mating was used for the transformation of *B. glumae* with DNA constructs (Figurski and Helinski, 1979). Digested DNA fragments were extracted from the agarose gel using GenElute™ Gel extraction kits (Sigma-Aldrich, St. Louis, MO, USA). The concentrations of the purified DNA and RNA were measured using a NanoDrop DN-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All the restriction enzymes used in this study were purchased from New England Biolabs (Beverly, MA, USA). The genomic library used in this study was constructed previously in our laboratory (Karki et al., 2012a).

Construction of pKGpToxA-GUS

To generate the DNA construct for a *gusA* fusion to the promoter region of *toxABCDE* ($P_{toxABCDE}$::*gusA*), the DNA fragment containing 562 bp upstream and 108 bp continuous coding sequences of *toxA* was amplified with the primer set, ToxA PF/ToxA PR (**Table 2**). The resultant PCR product was initially cloned into pSC-A-amp/kan, generating pSC-A-pToxA. The cloned region was then introduced into pBB2GUS, a broad host vector containing a promoterless *gusA*, using the unique *Hin*dIII site to obtain pBB2GUS-pToxA. The region containing the $P_{toxABCDE}$::*gusA* fusion was cut with *Kpn*I and *Spe*I and ligated to the suicide vector, pKNOCK-Gm (Alexeyev, 1999), to generate pKGpToxA-GUS. This DNA construct was then introduced into the Δ*tofI* strain, LSUPB145, and the Δ*tofR* strain, LSUPB289, through tri-parental mating, generating LSUPB178 and LSUPB324, respectively.

Random Mutation of *B. glumae* and Screening of Random *B. glumae* Mutants

Mini-Tn*5 Cm*, a mini-Tn*5* transposon carrying a Cm resistant gene (De Lorenzo et al., 1990), was introduced to LSUPB178

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	References
Escherichia coli		
DH10B	F$^-$ *araD139* Δ(*ara, leu*)7697 Δ*lacX74 galU galK rpsL deoR* ø80d*lacZ*ΔM15 *endA1 nupG recA1 mcrA* Δ(*mrr hsdRMS mcrBC*)	Grant et al., 1990
DH5α	F$^-$ *endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1 deoR* Δ(*lacZYA*-argF)-U169 ø80d*lacZ*ΔM15	Grant et al., 1990
S17-1λpir	*recA thi pro hsdR* [res− mod+][RP4::2-Tc::Mu-Km::Tn*7*] λ *pir* phage lysogen, Sm^r/Tp^r	Simon et al., 1983
Burkholderia glumae		
336gr-1	Wild type strain and the causative isolate of bacterial panicle blight of rice in Crowley, LA	This study
LSUPB22	spontaneous mutant of 336gr-1	This study
LSUPB145	A Δ*tofI* derivative of 336gr-1	Chen et al., 2012
LSUPB169	A Δ*tofR* derivative of 336gr-1	Chen et al., 2012
LSUPB139	A Δ*tofI-tofR* derivative of 336gr-1	Chen et al., 2012
LSUPB286	A Δ*tofM* derivative of 336gr-1	Chen et al., 2012
LSUPB172	A derivative of 336gr-1 carrying pKGpToxA-GUS	This study
LSUPB178	A derivative of Δ*tofI* carrying pKGpToxA-GUS	This study
LSUPB324	A derivative of Δ*tofR* carrying pKGpToxA-GUS	This study
LSUPB503	A *flhD*$^-$ derivative of the Δ*tofI* strain, LSUPB145	This study
LSUPB273	A *qsmR*$^-$ derivative of the wild type strain, 336gr-1	This study
LSUPB275	A *qsmR*$^-$ derivative of the Δ*tofI* strain, LSUPB145	This study
LSUPB277	A *qsmR*$^-$ derivative of the Δ*tofR* strain, LSUPB169	This study
LSUPB445	A *toxK*$^-$ derivative of the Δ*tofR* strain, LSUPB169	This study
LSUPB460	A *dgcB*$^-$ derivative of the wild type strain, 336gr-1	This study
LSUPB462	A *dgcB*$^-$ derivative of the Δ*tofI* strain, LSUPB145	This study
LSUPB464	A *dgcB*$^-$ derivative of the Δ*tofM* strain, LSUPB286	This study
LSUPB515	A *wzyB*$^-$ derivative of the Δ*tofI* strain, LSUPB145	This study
LSUPB516	A *wzyB*$^-$ derivative of the Δ*tofR* strain, LSUPB169	This study
Chromabacterium violaceum		
Chromabacterium violaceum CV026	A biosensor that can detect AHL molecules	McClean et al., 1997
Plasmid		
pSC-A-amp/kan	A blunt PCR cloning vector; f1 *ori*, pUC *ori*, *lacZ*', Km^R, Amp^R	Stratagene
pRK2013::Tn*7*	A helper plasmid; ColE1 *ori*	Ditta et al., 1980
mini-Tn*5 Cm*	A derivative of mini-Tn5 transposon, R6K *ori*, RP4 *oriT*, Cm^R	De Lorenzo et al., 1990
pKNOCK-Km	A suicide vector; R6K *ori*, RP4 *oriT*, Km^R	Alexeyev, 1999
pKNOCK-Gm	A suicide vector; R6K *ori*, RP4 *oriT*, Gm^R	Alexeyev, 1999
pBBR1MCS-2	A broad host range cloning vector, RK2 *ori*, *lacZ*α, Km^R	Kovach et al., 1995
pBB2GUS	a derivative of pBBR1MCS-2 containing a promoterless *gusA*	This study
pSC-A-pToxA	A PCR clone of 682 bp upstream promoter and partial coding region of *toxA* in pSC-A-amp/kan, Amp^R, Km^R	This study
pBB2GUS-pToxA	A subclone of pSC-A-pToxA for the promoter and partial coding region of *toxA* in pBB2GUS at *Hin*dIII site	This study
pKGpToxA-GUS	A subclone of pBB2GUS-pToxA for the promoter and partial coding region of *toxA* gene and *gusA* gene in pKNOCK-Gm at *Kpn*I and *Spe*I sites	This study
PSC-qsmR	A PCR clone of 355-bp internal region of *qsmR* gene in pSC-A-amp/kan, Amp^R, Km^R	This study
PSC-flhD	A PCR clone of 304-bp internal region of *flhD* gene in pSC-A-amp/kan, Amp^R, Km^R	This study
PSC-toxK	A PCR clone of 402-bp internal region of *toxK* gene in pSC-A-amp/kan, Amp^R, Km^R	This study
PSC-dgcB	A PCR clone of 376-bp internal region of *dgcB* gene in pSC-A-amp/kan, Amp^R, Km^R	This study
PSC-wzyB	A PCR clone of 370-bp internal region of *wzyB* gene in pSC-A-amp/kan, Amp^R, Km^R	This study
pKKmqsmR	A subclone of pSC-qsmR for the internal region of *qsmR* gene in pKNOCK-Km at *Kpn*I and *Sac*II sites, Km^R	This study
pKKmflhD	A subclone of pSC-flhD for the internal region of *flhD* gene in pKNOCK-Km at *Eco*RI site, Km^R	This study
pKKmtoxK	A subclone of pSC-toxK for the internal region of *toxK* gene in pKNOCK-Km at *Eco*RI site, Km^R	This study
pKGmdgcB	A subclone of pSC-dgcB for the internal region of *dgcB* gene in pKNOCK-Km at *Spe*I and *Hin*dIII sites, Km^R	This study
pKGmwzyB	A subclone of pSC-wzyB for the internal region of *wzyB* gene in pKNOCK-Gm at *Spe*I and *Kpn*I sites, Km^R	This study

TABLE 2 | The PCR programs and primers used for directional mutation.

Amplified region	Product length	Primers* (5′ 3′)	PCR program
Promoter and partial coding region of *toxA*	682 bp	ToxA PF: AAGCTTTCCCTTCGCTTTTC (*Hin*dIII) ToxA PR: CTCGAGACCAATCATGTGGAA (*Xho*I)	Annealing at 55°C Extension for 1 min
Flanking region of miniTn*5 Cm* insertion	Variable	Y Linker Primer: CTGCTCGAATTCAAGCTTCT Tn5 primer: GGCCAGATCTGATCAAGAGA	Annealing at 58°C Extension for 1 min Kwon and Ricke, 2000
Internal region for *qsmR*	355 bp	QSMRIK FP: CCGCCTCGGTGCTCGAACTG QSMRIK RP: AGCGTATCCTCCAGGGCGGG	Annealing at 60°C Extension for 1 min
Internal region of *flhD*	304 bp	FlhD-1: AATGCTCGCCGAGATCAA FlhD-2: TTAGCGGAGGCTTTCGAC	Annealing at 54°C Extension for 40 s
Internal region of *flhC*	462 bp	FlhC-1: GTGCTCGAGGTCAAGGAAATC FlhC-2: CAGCCCGCAGACGAAAC	Annealing at 54°C Extension for 40 s
Internal region of *toxK*	402 bp	OR-1: GATTCAGGCGGGCTAGTTT OR-2: CGCCGAATACGGCTACTG	Annealing at 54°C Extension for 40 s
Internal region of *dgcB*	376 bp	DGC-FP: CGTAGGTGTCGTTGTACTGCTTGA DGC-RP: ATCATCGTGCTGTCGACCAAGGA	Annealing at 58°C Extension for 30 s
Internal region of *wzyB*	370 bp	Oap-1: ACTCGCACGACATCTTCATC Oap-2: GGGTTCGTGCCGTAATAGAG	Annealing at 53°C Extension for 30 s
Region spanning the potential suicide vector inserted sites in *flhD*	445 bp	FLHD-C1: GCCACAATGACTGCAAGAATATAA FLHD-C2: GCAGATGATGTAGGGAGTGTTAG	Annealing at 53°C Extension for 30 s
Region spanning the potential suicide vector inserted sites in *toxK*	683 bp	toxK-C1: GGCAGCAAATCTCCGTTTATTC toxK-C2: GTACCGGTGCTGGATATGATT	Annealing at 54°C Extension for 45 s
Region spanning the potential suicide vector inserted sites in *qsmR*	863 bp	QsmR-C1: CCAGCGTGGACTTTGTCAT QsmR-C2: CAGTCTCGAGCAGCCATTC	Annealing at 52.5°C Extension for 1 min
Region spanning the potential suicide vector inserted sites in *dgcB*	878 bp	DGCB-C1: ATTGCGCATTCTGAAGGAAAC DGCB-C2: CAGCACGACACCGAACT	Annealing at 55°C Extension for 1 min
Region spanning the potential suicide vector inserted sites in *wzyB*	818 bp	Oap-C1: TGCACTATCACCTCGGTCT Oap-C2: CCAGTCGTGCAGTTCCTC	Annealing at 55°C Extension for 1 min

**The restriction sites added in the primers are underlined.*

(a Δ*tofI* derivative carrying the $P_{toxABCDE}$::*gusA* fusion in the genome) and LSUPB324 (a Δ*tofR* derivative carrying the $P_{toxABCDE}$::*gusA* fusion in the genome) by tri-parental mating for random mutagenesis. After the tri-parental mating, mini-Tn*5 Cm* mutants that did not show blue coloration (indicating no β-glucuronidase gene activity) on $LB_{Gm/Cm/Nir/X-gluc}$ plates after 2 days of incubation at 37°C were collected for identifying the genes disrupted by mini-Tn*5 Cm*.

Identification of Genes Disrupted by Mini-Tn5 Cm

The mutated genes of the screened mutants were identified following a previously developed method (Kwon and Ricke, 2000) with some modifications. Briefly, genomic DNA of mutant strains were extracted using a GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). Genomic DNA (ca. 4 mg) was digested with two restriction enzymes, PstI and NlaIII, and the digested DNA fragments were ligated to the Y-shaped linker. Then, Y Linker Primer, CTGCTC-GAATTCAAGCTTCT (specific to Y linker sequence), and Tn5 primer, GGCCAGATCTGATCAAGAGA (specific to transposon), were used to amplify the DNA sequences flanking where the transposon was inserted. The amplified PCR products were sequenced and the sequence data were BLAST-searched against the National Center for Biotechnology Information (NCBI) database for identification of the sequenced region.

Directional Mutation for Validation of Gene Functions

A 300—400 bp internal DNA sequence of a gene of interest was amplified using a primer set indicated in **Table 2** and cloned into a pKNOCK suicide vector, pKNOCK-Km or pKNOCK-Gm (Alexeyev, 1999). Specifically, PCR products for 355 bp of *qsmR*, 304 bp of *flhD*, 402 bp of *toxK*, 376 bp of *dgcB* and 370 bp of *wzyB* were cloned into PSC-A-amp/kan to generate PSC-qsmR, PSC-flhD, PSC-toxK, PSC-dgcB and PSC-wzyB, respectively.*Kpn*I and *Sac*II sites were used to transfer the *qsmR* region in PSC-qsmR to pKNOCK-Km to generate pKKmqsmR. *EcoR*I site was used to move the *flhD* and *toxK* regions in PSC-flhD and PSC-toxK to pKNOCK-Km vector, generating pKKmflhD and pKKmtoxK, respectively. *Spe*I and *Hin*dIII were used to transfer the *dgcB* region from pSC-dgcB to pKNOCK-Gm, generating pKGmdgcB. *Spe*I and *Kpn*I were used to transfer the *wzyB* region from pSC-wzyB to pKNOCK-Gm, generating pKGmwzyB. Each final DNA construct in a pKNOCK suicide vector was introduced into *B. glumae* strains by tri-parental mating. Diagnostic PCR was conducted to confirm if the internal sequence of each gene was successfully integrated to the bacterial genome through homologous recombination. Primer pairs listed in **Table 2**, FLHD-C1/FLHD-C2, toxK-C1/toxK-C2, QsmR-C1/QsmR-C2, DGCB-C1/DGCB-C2, and Oap-C1/Oap-C2, were used for the diagnostic PCRs to confirm

the targeted mutations of *flhD*, *toxK*, *qsmR*, *dgcB*, and *wzyB*, respectively.

Quantification of Toxoflavin Production by *B. glumae* Strains Grown on LB Agar

Toxoflavin produced by *B. glumae* strains grown on LB agar was quantified following a previous method (Chen et al., 2012) with miscellaneous modifications. Briefly, overnight bacterial cultures were concentrated to OD_{600} = 100 ($\sim 10^{11}$ cfu/ml) and one loopful of each inoculum was streaked on the entire area of a LB agar plate. After 24 h incubation at 37°C, the bacterial culture was scraped off from the surface of the LB agar. Five grams of the LB agar where bacteria were grown on was then cut into small pieces with a razor blade. The chopped agar pieces were mixed with 5 ml chloroform and left to sit at room temperature for 30 min. The chloroform fraction was then transferred to a new tube and air-dried under a fume hood. The remaining materials in the dried tube were dissolved in 1 ml of 80% methanol. The OD_{393nm} values were measured to determine the quantity of toxoflavin, using a spectrophotometer. The sample from uncultured LB agar was used to set the zero of OD_{393nm}.

Virulence Assay with Onion Bulb Scales

Virulence tests for the mutant strains of *B. glumae* were conducted using onion bulbs as a surrogate host, following a previously described method (Karki et al., 2012b).

Results

Fifteen Protein-Coding Genes Were Identified as Potential Genetic Components for the Tofi-Independent Production of Toxoflavin

Both LSUPB178 (a Δ*tofI* derivative carrying the P_{toxA}::*gusA* fusion in the genome) and LSUPB324 (a Δ*tofR* derivative carrying the P_{toxA}::*gusA* fusion in the genome) showed blue coloration on LB agar containing X-gluc, indicating that the introduced *gusA* fusion to the *toxA* promoter is functional (data not shown). After the random mutagenesis of LSUPB178 with mini-Tn*5 Cm*, the mutant colonies that did not exhibit blue color were picked from the selection plates (LB agar amended with Gm, Cm, Nit, and X-gluc). It was considered that mini-Tn*5 Cm* is inserted in a potential genetic component of the *tofI*-independent toxoflavin production pathway in the mutants screened. Among the 4400 random mini-Tn*5 Cm* derivatives of LSUPB178 observed, 16 mutant derivatives were initially screened and their mutated genes were identified through BLAST searches of the flanking sequences (**Table 3**). All the 16 mutant strains screened were confirmed to be toxoflavin-deficient on LB agar at 37°C (data not shown). Fifteen out of the 16 mutants screened turned out to have mini-Tn*5 Cm* insertion in protein-coding regions and one mutant had the insertion in the middle of a 23S ribosomal RNA sequence. The 15 protein-coding genes identified include *toxR* and *toxA*, which are known to be required for toxoflavin production (Herrmann and Weaver, 1999), indicating the validity of this experiment (**Table 3**). Among the 15 genes identified, three genes (*flhD* encoding a transcriptional activator for flagellar biogenesis, *dgcB* encoding a putative diaguanylate cyclase, and *wzyB* encoding a putative O-antigen polymerase family protein) were selected for the extended functional studies described below.

TABLE 3 | The list of potential genes contributing to the *tofI*-independent toxoflavin production.

Name of random mutants	Locus of inserted genes	Function of disrupted genes
LSUPB186	bglu_1g10100	Succinylornithine transaminase
LSUPB187	bglu_2g10840*	putative LysM domain-containing protein
LSUPB182	bglu_1g02180	Diguanylate cyclase
LSUPB183	bglu_1g01780*	Flagellar transcriptional activator FlhD
LSUPB184	bglu_2g07160*	Catechol 1,2-dioxygenase
LSUPB185	bglu_1g00380	General secretory pathway protein D
LSUPB188	bglu_2g22000*	Hypothetical protein
LSUPB189	bglu_1g07800	Hypothetical protein
LSUPB192	bglu_2g06390	LysR family transcriptional regulator (*toxR*)
LSUPB193	bglu_2g06400*	putative ubiquinone/menaquinone biosynthesis methyltransferase (*toxA*)
LSUPB194	bglu_2g18120	Amylo-alpha-1,6-glucosidase family protein
LSUPB209	bglu_1g33070	Flagellar hook-associated protein FlgK
LSUPB210		rRNA-23S ribosomal RNA
LSUPB211	bglu_1g29900	O-antigen polymerase family protein
LSUPB212	bglu_1g00440	Glutamate–cysteine ligase
LSUPB213	bglu_1g05190	Short chain dehydrogenase

**Mini-Tn5Cm is inserted in the promoter region.*

Three Protein-Coding Genes Were Identified as Potential Genetic Components for the tofR-Independent Production of Toxoflavin

Similar to LSUPB178, LSUPB324 (Δ*tofR*:: $P_{toxABCDE-gusA}$) exhibited blue color on LB agar X-gluc (data not shown). Three mini-Tn*5 Cm* derivatives of LSUPB324 that did not produce blue color were characterized for the mutated genes (**Table 4**), and loss of the function in toxoflavin production was observed with all the three mutants (data not shown). Particularly, one toxoflavin-deficient mini-Tn*5 Cm* mutant, LSUPB191, turned out to be disrupted in the ORF, bglu_2g06320 (**Table 4**). This ORF is located upstream of and divergently transcribed from *toxJ*, a known regulatory gene for toxoflavin biosynthesis encoding an orphan LuxR protein. Due to its apparent function in toxoflavin production, the ORF was newly named as *toxK* in this study. Among the three identified genes, *qsmR* and *toxK* were chosen for the further functional studies described below.

Targeted Mutation of *flhD*, *dgcB*, *wzyB*, *qsmR*, or *toxK* Resulted in Impaired Toxoflavin Production

To confirm the initially observed functions of *flhD*,*dgcB,* and *wzyB* in *tofI*-independent toxoflavin production, independent mutants of these genes were generated via homologous recombination. For this, internal regions of *flhD*,*dgcB,* and *wzyB* were cloned in a suicide vector (pKNOCK-Km or pKNOCK-Gm) and introduced into the Δ*tofI* background, LSUPB145, generating the corresponding mutants, LSUPB503, LSUPB462, and LSUPB515,

respectively (see the Materials and Methods section for detail). The same strategy was applied for the targeted mutation of *qsmR* and *toxK* in the Δ*tofR* background, LSUPB169, generating the corresponding mutants LSUPB277 and LSUPB445, respectively. These independent mutants of the five genes were then examined for their phenotypes in toxoflavin production in comparison with their parental strains.

flhD

Compared to LSUPB145, its *flhD*$^{-}$ derivative LSUPB503 showed a substantial reduction in toxoflavin production (**Figure 1**). There was little difference between LSUPB145 and LSUPB503 during the first 24 h of incubation period, but during the additional 24 h incubation period, reduction of toxoflavin amount was observed with LSUPB503, while more than 5 times increase of toxoflavin was observed with LSUPB145 during the same incubation period (**Figure 1**). Null mutation of *flhC*, which is located downstream of *flhD*, in the Δ*tofI* background through homologous recombination of an internal coding region also caused loss of toxoflavin production (data not shown).

TABLE 4 | The list of *tofR*-independent genes contributing to *tofR*-independent toxoflavin production.

Name of random mutants	Locus of inserted genes	Function of disrupted genes
LSUPB190	bglu_1g10250	IclR family regulatory protein gene (*qsmR*)
LSUPB191	bglu_2g06320	Hypothetical protein in the upstream of *toxJ* encoding an orphan LuxR family transcriptional regulator (named as *toxK* in this study)
LSUPB195	bglu_2g22590	Putative PAS/PAC sensor protein

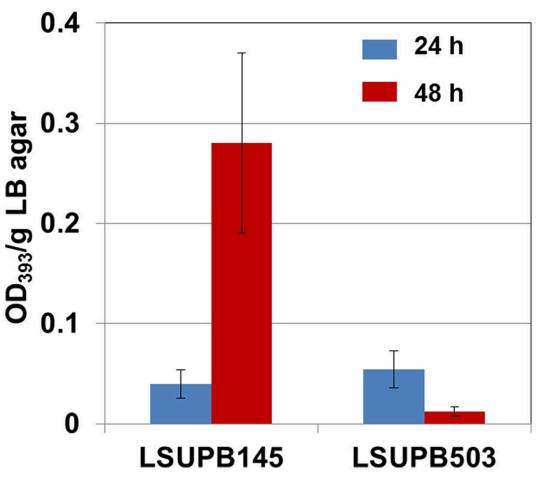

FIGURE 1 | Toxoflavin production of LSUPB503 (*flhD*$^{-}$/Δ*tofI*) compared to its parental strain, LSUPB145 (Δ*tofI*), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD_{393}/g LB agar values.

dgcB

Both of the two independent *dgcB* null mutants, LSUPB462 (a *dgcB*::pKGmdgcB derivative of the Δ*tofI* strain LSUPB145) and LSUPB182 (a *dgcB*::mini-Tn*5 Cm* derivative of LSUPB145), were toxoflavin-deficient on LB agar medium in common (data not shown). This indicates the important role of the DgcB diguanylate cyclase in the *tofI*-independent pathway for toxoflavin production. To know if *dgcB* is also critical for toxoflavin production when the *tofI*/*tofR* QS system is intact, LSUPB460 (a *dgcB*$^{-}$ derivative of the wild type strain 336gr-1) was generated and examined for toxoflavin production. As shown in **Figure 2**, LSUPB460 produced a similar level of toxoflavin production compared to 336gr-1. This suggests that *dgcB* plays a positive role in toxoflavin production but this function is dispensable in the presence of the intact *tofI*/*tofR* QS system. To explore the relationship between *dgcB* and other QS elements *tofR* and *tofM* (Chen et al., 2012), pKGmdgcB was introduced into LSUPB169 (a Δ*tofR* mutant) and LSUPB286 (a Δ*tofM* mutant) for generating *dgcB*$^{-}$ derivatives of these QS mutant strains. But *dgcB*$^{-}$ derivatives could be obtained from only LSUPB286 but not LSUPB169, and the *dgcB*$^{-}$/Δ*tofM* strain, LSUPB464, also showed a toxoflavin-deficient phenotype like its parent, LSUPB286 (**Figure 2**).

wzyB

LSUPB515, the *wzyB* disruptive mutant in LSUPB145 background grew very slowly on both LB agar (data not shown) and LB broth (**Figure 3A**). Because of the extremely slow growth of the bacterial cells, it was difficult to conduct reliable toxoflavin quantification for LSUPB515. To determine if *wzyB* is also required for the *tofR*-independent toxofalvin production, a disruptive mutant in LSUPB169 background, LSUPB516, was generated and tested for its ability to produce toxoflavin. LSUPB516 showed normal cell growth unlike LSUPB515 (**Figure 3A**) and a toxoflavin-deficient phenotype (**Figure 3B**), indicating that *wzyB* is required for both *tofI*- and *tofR*-independent toxoflavin production. Generation of a *wzyB* null mutant derivative of 336gr-1 to determine the role of this gene in the wild type background was not successful.

qsmR

LSUPB277, *qsmR* mutated in LSUPB169 background, had the same toxoflavin loss phenotype as the random mutant LSUPB190, which confirmed the important role of *qsmR* in *tofR*-independent toxoflavin production (**Figure 4**). To determine the role of *qsmR* in toxoflavin production in the presence and absence of the intact QS system, LSUPB273 (a *qsmR* mutant in the wild type background 336gr-1) and LSUPB275 (a *qsmR* mutant in the Δ*tofI* background LSUPB145) were also generated and tested for their phenotypes in toxoflavin production. As shown in **Figure 4**, all the *qsmR* knock out mutants tested were deficient in toxoflavin production regardless of the genetic background of their parental strains, indicating the essential role of *qsmR* in the production of toxoflavin.

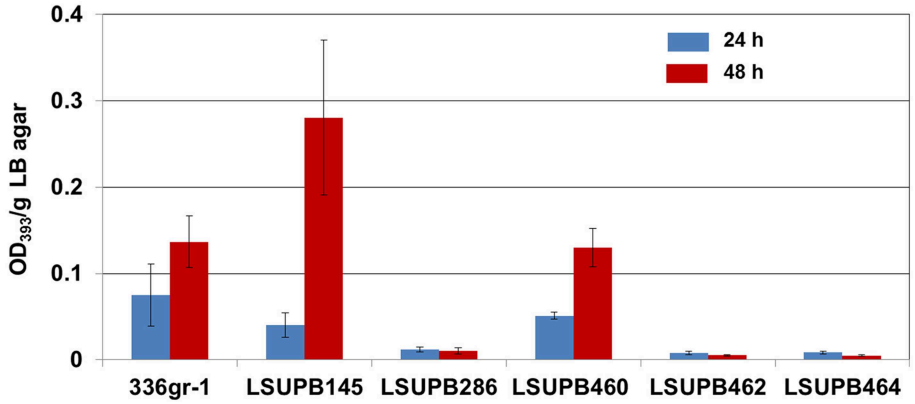

FIGURE 2 | Toxoflavin production of the *dgcB* mutants, LSUPB460 (*dgcB*$^-$), LSUPB462 (*dgcB*$^-$/Δ*tofI*) and LSUPB464 (*dgcB*$^-$/Δ*tofM*), compared to their parental strains, 336gr-1 (wild type), LSUPB145 (Δ*tofI*) and LSUPB286 (Δ*tofM*), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD_{393}/g LB agar values.

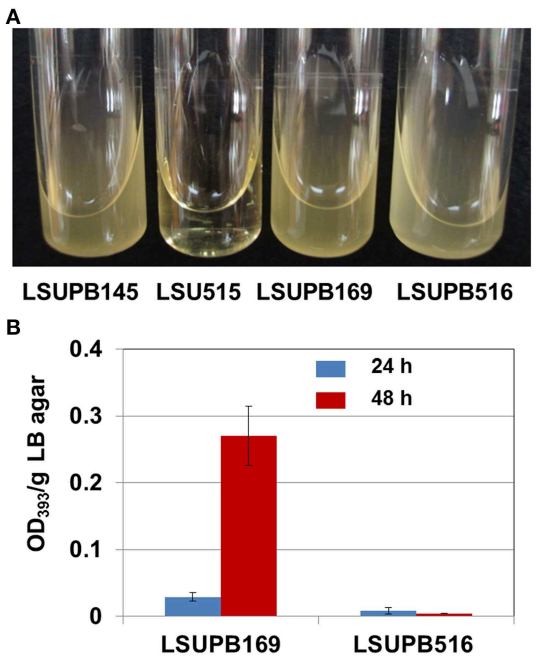

FIGURE 3 | Bacterial growth and toxoflavin production of *wzyB* mutants. (A) Bacterial growth in LB broth at 37°C: LSUPB145 (Δ*tofI*), LSUPB515 (*wzyB*$^-$/Δ*tofI*), LSUPB169 (Δ*tofR*) and LSUPB516 (*wzyB*$^-$/Δ*tofR*). Photo was taken 48 h after inoculation. **(B)** Toxoflavin production of the *wzyB* mutant, LSUPB516 (*wzyB*$^-$/Δ*tofR*), compared to its parental strains, LSUPB169 (Δ*tofR*), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD_{393}/g LB agar values.

toxK

As shown in **Figure 5**, toxoflavin production was almost abolished in the *toxK* mutant in LSUPB169 background, LSUPB445, indicating that this putative gene in front of *toxJ* encoding an orphan LuxR protein is essential for toxoflavin production of *B. glumae* in the absence of *tofR*. The function of *toxK* in the wild type and Δ*tofI* backgrounds has not been determined due to the failure of generation of *toxK*$^-$ and *toxK*$^-$/Δ*tofI* strains through homologous recombination.

Onion Maceration Caused by Mutants

The virulence functions of the five selected genes were determined with the virulence assay system using onion bulb scales as a surrogate host (Karki et al., 2012a,b). The strains included in the virulence assay were; 336gr-1 (wild type), LSUPB145 (ΔtofI), LSUPB169 (ΔtofR), LSUPB286 (ΔtofM), LSUPB503 (flhD$^-$ in ΔtofI), LSUPB445 (toxK$^-$ in ΔtofR), LSUPB273 (qsmR$^-$), LSUPB275 (qsmR$^-$ in ΔtofI), LSUPB277 (qsmR$^-$ in ΔtofR), LSUPB460 (dgcB$^-$), LSUPB462 (dgcB$^-$ in ΔtofI), LSUPB464 (dgcB$^-$ in ΔtofM), and LSUPB516 (wzyB$^-$ in ΔtofR). As shown in **Figure 6**, all the mutants tested exhibited reduced virulence compared to their parental strains even though none of them were completely avirulent, indicating the positive roles of the five genes in bacterial pathogenesis.

Discussion

Genome-Wide Screening by Random Transposon Mutagensis Revealed Novel Regulators of Toxoflavin in *B. glumae*

The *toxA* promoter and *gusA* transcriptional fusion was introduced to Δ*tofI* and Δ*tofR* mutants and random mutagenesis with mini-Tn*5 Cm* was implemented to search for the genes that could affect the expression of the *toxABCDE* operon, thus the toxoflavin biosynthesis, in *tofI*- or *tofR*-independent ways. Through this approach, fifteen and three protein-coding genes were identified as potential regulators for the *tofI*- and *tofR*-independent toxoflavin biosynthesis of *B. glumae* 336gr-1, respectively. Identification of *toxA* and *toxR*, which are known genes required for toxoflavin biosynthesis, verifies the reliability of this experiment. The reason for the abolishment of the $P_{toxA::gusA}$ expression by disruption of *toxA* may be due to the fact that ToxR, which is an essential regulatory factor for toxoflavin biosynthesis, requires a

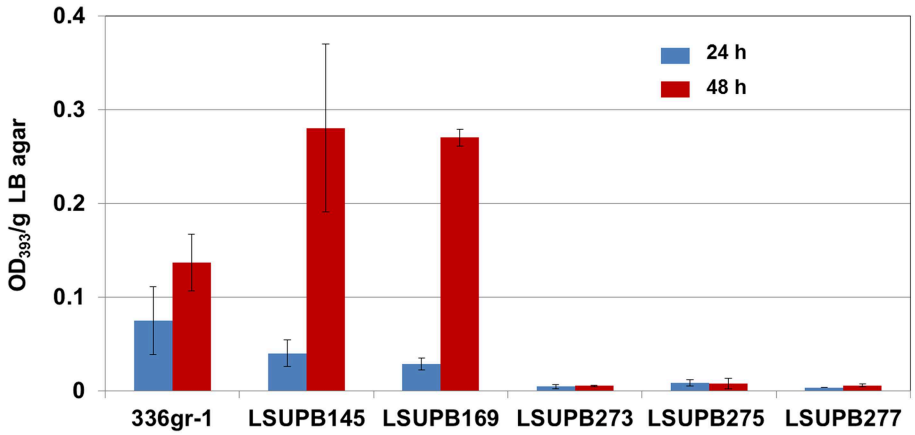

FIGURE 4 | Toxoflavin production of the *qsmR* mutants, LSUPB273 (*qsmR*$^-$), LSUPB275 (*qsmR*$^-$/Δ*tofI*) and LSUPB277 (*qsmR*$^-$/Δ*tofR*), compared to their parental strains, 336gr-1 (wild type), LSUPB145 (Δ*tofI*) and LSUPB169 (Δ*tofR*), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD_{393}/g LB agar values.

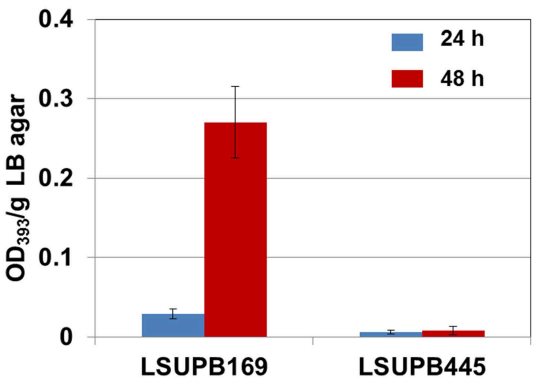

FIGURE 5 | Toxoflavin production of the *toxK* mutant LSUPB445 (*toxK*$^-$/Δ*tofR*) compared to its parental strain, LSUPB169 (Δ*tofR*), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD_{393}/g LB agar values.

residual amount of toxoflavin as a co-inducer for its functionality (Kim, 2004). Among the potential genetic elements for *tofI*- or *tofR*-independent toxoflavin production identified from screening of random mini-Tn*5 Cm* mutants, five genes (*flhD* encoding a flagella transcriptional activator, *dgcB* encoding a diguanylate cyclase, *wzyB* encoding an O-antigen polymerase family protein, *qsmR* encoding an IclR-type transcriptional regulator, and *toxK* encoding a hypothetical protein and divergently transcribed from *toxJ* encoding an orphan LuxR homolog essential for toxoflavin production) were further characterized in this study for their functions in toxoflavin production in the presence or absence of the intact TofI/TofR QS system.

Based on the annotated genome *B. glumae* BGR1 (Lim et al., 2009), it is unlikely that possible polar effects from the transposon or directional mutations can cause misinterpretation of the observed mutant phenotypes for the five genes investigated in this study. *flhD* (bglu_1g01780) and *flhC* (bglu_1g01790) comprise an operon, but their products are known to work together as one functional unit (Kim et al., 2007). In this study, both *flhD*$^-$ and *flhC*$^-$ mutants showed the toxoflavin-deficient phenotype in the Δ*tofI* background. In case of the other genes, *dgcB* (bglu_1g02180), *qsmR* (bglu_1g10250), and *toxK* (bglu_2g06320) are apparently monocistronic, while *wzyB* (bglu_1g29900) is located at the end of a putative operon structure containing three genes. Thus, polarity may not be a major concern with the five genes investigated in this study.

Flhd is Required for the Toxoflavin Production by *B. glumae* 336gr-1 in the Δ*tofI* Background

The *flhDC* operon encoding the FlhDC complex was characterized as a flagellar transcriptional activator, which was controlled by QS in *B. glumae* BGR1 (Kim et al., 2007). It was reported that a *flhD* deficient mutant of BGR1 still produced toxoflavin but lost pathogenicity as well as flagellum-mediated motility (Kim et al., 2007). In this study with another strain of *B. glumae*, 336gr-1, it was observed that *flhD* as well as *flhC* were required for the production of toxoflavin in the absence of *tofI*. At this point, it has not been determined if *flhD*$^-$ or *flhC*$^-$ derivatives of 336gr-1 still produce toxoflavin like BGR1. Mutation of these genes in the wild type as well as in the Δ*tofR* backgrounds is currently under way. Due to the significant difference between BGR1 and 336gr-1 in the QS-mediated regulation of toxoflavin, it is possible that *flhDC* is not involved in toxoflavin production in BGR1. Nevertheless, this study indicates that *flhDC* has a regulatory function in toxoflavin production in addition to flagellum-mediated motilities in at least some strains of *B. glumae.*

dgcB is Required for Toxoflavin Production in the Absence *tofI* but Not in the Wild Type Background Having the Intact *tofI*/*tofR* QS system

The results in this study indicate that *dgcB* is required for toxoflavin production in the absence of *tofI* but not in the presence

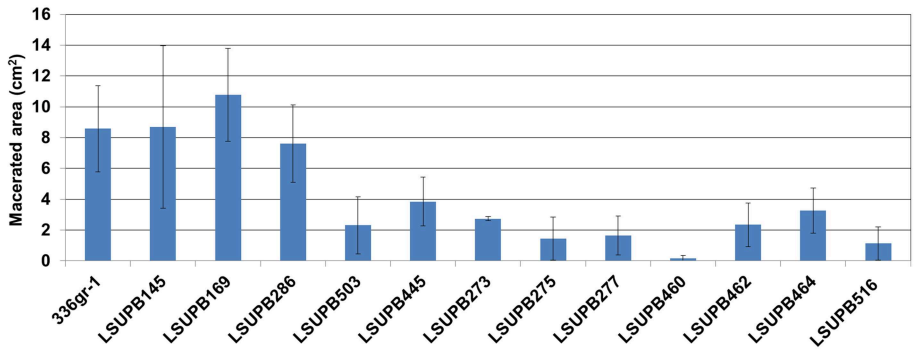

FIGURE 6 | Virulence of *Burkholderia glumae* 336gr-1 and its various mutant derivatives determined by the maceration areas they produced on onion bulb scales. The two diameters of each maceration area (*a* and *b*) were measured to calculate the size of the area, using the formula: Area (cm^2) = πab. Each error bar indicates the standard deviation from four replications.

of the intact TofI/TofR QS system. *dgcB* codes for a diguanylate cyclase, which synthesizes cyclic di-guanosine monophosphate (c-di-GMP) from two guanosine triphosphate (GTP) molecules. c-di-GMP is a small diffusible signal molecule that influences a wide range of cellular functions including bacterial virulence (Romling, 2012). The phenotype of the *dgcB* mutant, LSUPB462, observed in this study strongly implies that the c-di-GMP signaling plays a pivotal role in the regulation of virulence factors in *B. glumae*. Involvement of c-di-GMP signaling pathways for the pathogenesis by plant pathogenic bacteria has been studied mainly with *Xanthomonas* spp. Ham (2013). Regulatory functions of c-di-GMP for the expression of EPS and biofilm were recently reported with *B. cenocepacia* (Fazli et al., 2011, 2013; Deng et al., 2012). Even though this study adds to our knowledge about the function of c-di-GMP signaling in plant pathogenic bacteria, more studies including identification and functional characterization of other c-di-GMP signaling components, such as phosphodiesterases and c-di-GMP-binding protein, should be followed to clearly understand the role of this signaling system for the bacterial pathogenesis by *B. glumae*.

wzyB Encoding an O-Antigen Polymerase May Play an Important Role in the *tofI*-Independent Intercellular Signaling for Toxoflavin Production

wzyB was initially identified as a genetic element for the *tofI*-independent toxoflavin production through screening of random mini-Tn*5Cm* mutant derivatives of the Δ*tofI* strain, LSUPB145. LSUPB515, an independent *wzyB* mutant derivative of LSUPB145 generated through homologous recombination, grew slowly due to an unknown reason and produced no observable toxoflavin. LSUPB516, a *wzyB* mutant derivative of the Δ*tofR* strain LSUPB169 through homologous recombination, also lost the ability to produce toxoflavin on a solid medium. These observations indicate that *wzyB* is required for both *tofI*- and *tofR*-independent toxoflavin production. However, it remains to be determined whether or not *wzyB* is also required for the toxoflavin production in the presence of the intact TofI/TofR QS system because *wzyB* mutation in the wild type background has been failed due to an unknown reason.

O-antigen is one of the three major components (the other two are lipid A and a core oligosaccharide) of lipopolysaccharides (LPS) in the outer membrane of Gram negative bacteria; and is structurally very diverse and highly immunogenic (Raetz and Whitfield, 2002; Stone et al., 2012). Study of O-antigen and its synthesis pathway can provide important antibiotic targets for potential clinical use. O-antigen polymerase family proteins (encoded by *wzy* homologs) are responsible for polymerizing the O-antigen units to O-antigen chain (Daniels et al., 1998; Chin et al., 2010). It is also well known that LPS is a common virulence determinant (Thomsen et al., 2003; Ellis and Kuehn, 2010). As an important synthesis enzyme for O-antigen and LPS, O-antigen polymerase should be important for maintaining the virulence of pathogenic bacteria. Even though not much study has been focused on *wzy* homologs, it was recently reported that non-polar mutation of the *wzy* gene in *Salmonella enterica* serovar *Typhimurium* caused attenuated virulence in mice (Kong et al., 2011). It would be an appealing hypothesis that the outer membrane-bound O-antigen produced by *wzy* genes mediates signal transduction dependent on "physical contact" of bacterial cells populated on a solid surface.

Furthermore, it would be worthy to make more investigation on the relationship between motility genes, especially *flhDC*, and *wzyB* as well as other LPS synthesis genes. There have been reports about how the subunits of and enzymes involved in assembling LPS influence the motility function of bacteria. The mutation of an O-antigen gene in *Salmonella enterica* serovar Typhimurium resulted in defective bacterial swarming and, in the same study, it was also suggested that the role of O-antigen is to improve the wettability of the bacterial colony (Toguchi et al., 2000). The mutation of *wzxE* gene, which is responsible for transporting O-antigen units across the inner membrane to the LPS assembling site (Chin et al., 2010), caused *E. coli* to lose both swimming and swarming motility (Girgis et al., 2007). The mutation of *waaL*, an O-antigen ligase gene, in *Vibrio fischeri* resulted in loss of motility and ability to survive with the wild type in the co-colony assay (Buttner, 2012). In another study, mutation of *waaL* blocked the expression of *flhDC* genes and aborted the swarming motility of human pathogen *Proteus mirabilis* in soft

agar, but overexpression of *flhDC* genes in *trans* overcame this defect (Morgenstein et al., 2010). Therefore, motility assays are under way in our laboratory to determine the functional relationship between *wzyB* and the QS-regulated motility system in *B. glumae.*

qsmR is an Essential Regulatory Gene for the Toxoflavin Production of *B. glumae* 336gr-1

In this study, null mutation of *qsmR* resulted in lost function of toxoflavin production in the wild type (336gr-1), Δ*tofI* (LSUPB145) and Δ*tofR* (LSUPB169) backgrounds, indicating that *qsmR* is another essential regulatory factor for toxoflavin production in *B. glumae* 336gr-1. QsmR was previously reported as a positive regulator for *flhDC*, which was activated by C8-HSL and TofR complex in another strain of *B. glumae*, BGR1 (Kim et al., 2007). In the same study, disruption of *qsmR* resulted in loss of motility and pathogenicity in rice, but no observable difference in toxoflavin production in the solid medium condition (Kim et al., 2007). However, the *qsmR*$^{-}$ derivative of BGR1 showed decreased production and degradation of toxoflavin in the liquid medium condition from 12 h of incubation (Kim et al., 2007). In this study with *B. glumae* 336gr-1, the *qsmR* mutant (LSUPB273) did not produce observable toxoflavin on LB agar and still retained partial virulence on onion, which was somewhat different from the results of Kim et al. with *B. glumae* BGR1. These results from this and previous studies suggest that toxoflavin biosynthesis is regulated by *qsmR* more tightly in 336gr-1 than in BGR1. *qsmR* was also shown to regulate the *katG* catalase gene, which is required for the full virulence of *B. glumae* BGR1(Chun et al., 2009).

Additionally, recent studies with *B. glumae* BGR1 demonstrated that *qsmR* is a key player for both bacterial pathogenesis and survival in stressful conditions. From a proteomics search, the type II secretion system of *B. glumae* BGR1 encoded by *gsp* genes was found to be regulated by QS, and QsmR was shown to be a major positive regulator for *gsp* genes (Goo et al., 2010). In a study about how QS systems help representative *Burkholderia* species survive stationary phases, the sub-QS regulator QsmR was determined to be critical for activating the production of oxalate and maintaining normal pH levels of bacterial culture media (Goo et al., 2012). Without functional QsmR, there was a survival defect in *B. glumae* and *B. thailandensis* populations, which correlated with the high pH and lack of oxalate (Goo et al., 2012). Taken this and the previous studies on *qsmR* together, it is thought that *qsmR* plays an important role in general in the expression of various virulence factors in *B. glumae* despite some functional variations among different strains.

Discovery of the *toxK* Gene Adds More Complexity to the Known Regulatory Cascade for Toxoflavin Biosynthesis Involving the Orphan LuxR Protein, ToxJ

The ORF (gene ID of the reference sequence: bglu_2g06320) located upstream of and divergently transcribed from *toxJ,* the QS-dependent toxoflavin regulator encoding an orphan LuxR protein, was identified as a new genetic element required for the *tofR*-independent toxoflavin production of *B. glumae* 336gr-1 and named as *toxK*. There is one *lux* box like sequence (*tof* box) between *toxJ* and *toxK*, and it was previously proven that TofR and C8-HSL complex can bind on that site and activate the expression of *toxJ* (Kim et al., 2004). It may be the case that the *tof* box between *toxJ* and *toxK* promotes the expression of both genes, even though this notion should be proved experimentally. The deduced protein encoded by *toxK* contains 144 residues with 15.5 kDa of estimated molecular mass without an obvious homolog. Instead, the deduced protein (ID: ACR31068) has a conserved domain found in heterogeneous ribonucleoproteins (hnRNPs), implying that binding to target RNAs is its major functional mechanism.

It is interesting that the orphan *lux* homolog, *toxJ*, is next to an RNA-binding protein-coding gene instead of a *luxI* homolog. Regarding its location, size and predicted RNA-binding activity, *toxK* may be considered as a "chaperone" that modulates the expression and function of *toxJ*. More information about the function of *toxK* will be obtained when *toxK* mutants in the wild type and Δ*tofR* backgrounds are available and more phenotypic assays with *toxK* mutants in various genetic backgrounds are performed. In addition, future molecular studies to determine if mRNA of *toxJ* is a target of ToxK and how ToxK affect the expression of *toxJ* as well as other potential target genes will provide valuable insights into the functional basis of ToxJ and, further, into a type of functional mechanism adopted by other LuxR solos.

In conclusion, potential genetic components involved in the deviant quorum-sensing signaling pathways of *B. glumae* for the production of the major virulence factor, toxoflavin, were identified in this study. In particular, the genes known for other functions, *flhDC*, *dgcB*, *wzyB,* and *qsmR*, as well as a previously unknown gene, *toxK*, were newly found to function in the *tofI*- and *tofR*-independent toxofalvin production via directional mutation of each gene. The information obtained from this study provides not only great insights into the complex cell-to-cell signaling network of this bacterium but a good foundation of future studies for comprehensive functional analyses of the individual genes identified in this study.

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