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Development of tools for genetic analysis of phenanthrene degradation and nanopod production by *Delftia* sp. Cs1-4

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The bacterium *Delftia* sp. Cs1-4 produces novel extracellular structures (nanopods) in conjunction with its growth on phenanthrene. While a full genome sequence is available for strain Cs1-4, genetic tools that could be applied to study phenanthrene degradation/nanopod production have not been reported. Thus, the objectives of this study were to establish such tools, and apply them for molecular analysis of nanopod formation or phenanthrene degradation. Three types of tools were developed or validated. First, we developed a new expression system based on a strong promoter controlling expression of a surface layer protein (NpdA) from *Delftia* sp. Cs1-4, which was *ca.* 2,500-fold stronger than the widely used lactose promoter. Second, the Cre-*loxP* system was validated for generation of markerless, in-frame, gene deletions, and for in-frame gene insertions. The gene deletion function was applied to examine potential roles in nanopod formation of three genes (*omp32*, *lasI*, and *hcp*), while the gene insertion function was used for reporter gene tagging of *npdA*. Lastly, pMiniHimar was modified to enhance gene recovery and mutant analysis in genome-wide transposon mutagenesis. Application of the latter to strain Cs1-4, revealed several new genes with potential roles in phenanthrene degradation or *npdA* expression. Collectively, the availability of these tools has opened new avenues of investigation in *Delftia* sp. Cs1-4 and other related genera/species with importance in environmental toxicology.

Keywords: genetic manipulation, *Delftia* sp. Cs1-4, nanopods, phenanthrene, polynuclear aromatic hydrocarbons, biodegradation, surface layer protein

INTRODUCTION

Bacteria of the genus *Delftia* mediate a diversity of processes important in environmental toxicology, including xenobiotic biodegradation and biotransformation of heavy metals (Vacca et al., 2005; De Gusseme et al., 2010; Juarez-Jimenez et al., 2010; Leibeling et al., 2010; Paulin et al., 2010; Zhang et al., 2010; Morel et al., 2011; Yang et al., 2011). Additionally, *Delftia* spp. have been identified as endobionts in a variety of organisms including humans and, in the latter case, some are emerging as opportunistic pathogens (Hail et al., 2011; Preiswerk et al., 2011). Genome sequence data will be an essential resource for identification of functions in *Delftia* spp. that are key to these activities, and one recently completed genome is that of the phenanthrene degrader *Delftia* sp. Cs1-4.

In addition to its abilities as a phenanthrene degrader, strain Cs1-4 is noteworthy as the organism in which new extracellular structures, termed nanopods, were discovered (Shetty et al., 2011). Nanopods are tubular elements that contain outer membrane vesicles (OMV) within a sheath composed of a surface layer protein (SLP). The latter was termed Nanopod protein A (NpdA), and mutants lacking this protein were unable to form nanopods. Proteomic analyses of nanopods revealed a variety of proteins that were associated with these structures, two being outer membrane protein 32 (Omp32) and hemolysin co-regulated protein (Hcp). These proteins were of interest as we hypothesized that they, along with NpdA, could have key roles in nanopod structure. For Omp32, this hypothesis was based on its occurrence of OMV in nanopods, and Omp32 being the major protein in the outer membrane of strain Cs1-4 (Shetty et al., 2011). The protein Hcp, which is part of the recently discovered type 6 secretion system (T6SS), can self-assemble into *ca.* 10 nm diameter rings, which subsequently stack into *ca.* 100 nm tubes (Mougous et al., 2006; Ballister et al., 2008). The functions of such tubes are unknown, but in the case of nanopods, we hypothesized that they could have a structural role in nanopod formation, perhaps forming an inner core. One other gene/protein of interest in nanopod formation was *lasI*, which is involved in quorum sensing *via* the acyl homoserine lactone (AHL) synthase it encodes. Its potential connection to nanopod formation was based on two observations: (1) the increased abundance of nanopods in late-growth phase of phenanthrene-grown cultures (Shetty et al., 2011), and (2) the close association of the lone genomic copy of *lasI* with the phenanthrene degradation gene cluster. Thus, we hypothesized that nanopod production may be regulated by quorum sensing.

Testing of the above-described hypotheses has been hindered by a lack of genetic tools that have been developed for use in *Delftia* spp. The objectives of this study were thus to develop such tools, and apply them for molecular analysis of nanopod formation or phenanthrene degradation. Three types of tools were developed

and/or validated. First, a new expression system was developed based on a strong promoter (controlling *npdA* expression) from *Delftia* sp. Cs1-4. Second, the Cre-*loxP* gene deletion system was validated for generation of markerless, in-frame, gene deletions. Third, pMiniHimar was modified to enhance gene recovery and mutant analysis in genome-wide transposon mutagenesis.

MATERIALS AND METHODS

BACTERIAL STRAINS, PLASMIDS, AND GROWTH CONDITIONS

Bacterial strains and plasmids used in this work are listed in **Table 1**. *E. coli* JM109 was used for cloning. For conjugation, donor strains were either *E. coli* BW19851 (λ *pir*) or *E. coli* S17 (λ *pir*) and recipient strains were either *E. coli* TransforMax EC100+ (for propagation of constructs) or *Delftia* sp. Cs1-4. *E. coli* strains were routinely grown in Luria-Bertani (LB) broth at 37°C. Mineral salt medium (MSM; Hickey and Focht, 1990) containing phenanthrene as the sole carbon source (1 mg/mL) was routinely used for *Delftia* sp. Cs1-4 culture. Liquid cultures were grown with shaking (ca. 200 rpm) at either 25°C (strain Cs1-4) or 37°C (*E. coli*). For solid LB media, Bacto-Agar (Difco, Detroit, MI, USA) was added to a final concentration of 15 g/L. For *E. coli*, antibiotics were added when required at 100 μg/mL (ampicillin, Ap), 50 μg/mL (kanamycin, Km), or 10 μg/mL (tetracycline, Tc). Kanamycin and tetracycline were used in some *Delftia* sp. Cs1-4 cultures, and in these cases were added at 300 and 40 μg/mL, respectively.

DNA MANIPULATIONS

Genomic DNA was prepared using a genomic DNA extraction kit (Promega, Madison, WI, USA), and plasmid DNA was purified with the QIAprep spin miniprep kit (QIAGEN, Germantown, MD, USA). Restriction and modification enzymes were purchased from Promega (Madison, WI, USA) or New England Biolabs (Beverly, MA, USA). Klenow fragment or T4 DNA polymerase (Promega) was used to fill in recessed 3′ ends and to trim protruding 3′ ends of incompatible restriction sites. All PCR amplifications were done with the Failsafe PCR system (Epicenter Technology, Madison, WI, USA). Amplicons were separated in 0.7–1.0% (w/v) agarose gels, and DNA fragments were purified with the QIAquick gel extraction system (QIAGEN). Ligation mixtures were transformed into *E. coli* JM109 (Promega), and transformants were plated onto LB plates with appropriate antibiotic selection. Resistant colonies were isolated, and then screened for the acquisition of plasmids. All constructs were sequenced to verify structure. For conjugal transfer of plasmids from *E. coli* to *Delftia* sp. Cs1-4, LB-grown cultures of both cells were harvested (mid-log phase) by centrifugation, washed with LB and then equal amounts (*ca.* 10^{12} cells of each strain) were mixed, and spotted onto LB plates containing 5 mM $CaCl_2$. Following overnight incubation at 22°C, cells were then scraped off of the plates, diluted, and plated on LB plates containing the appropriate antibiotics.

TRANSCRIPTION START SITE DETERMINATION

Total RNA was isolated from phenanthrene-grown strain Cs1-4 cells, and purified of genomic DNA by DNase I digestion. Analysis by 5′-RACE was done using TaKaRa 5′-full RACE Core set under conditions recommended by the supplier (TaKaRa). Reverse transcription (RT) was done with a 5′-phosphorylated RT primer (Delf1; **Table 2**). After RT, mRNA was digested with RNaseH, and then cDNA was concatenated using T4 RNA ligase. The region of interest was then amplified *via* nested PCR using two sets of primers to regions of *npdA*. In the first PCR, RT products were used as template, and amplified with primers Delf2 and Delf3 (**Table 2**). In the second PCR, template was a 10-fold dilution of the round one PCR product, and amplification was done using primers Delf4 and Delf5 (**Table 2**). The 5′-RACE products were isolated, purified, ligated into pGEM-T easy and then sequenced.

The *npdA* fragment including the non-coding and partial structural gene regions was amplified with primers Delf6 and Delf7 (**Table 2**) using strain Cs1-4 genomic DNA as template. The *Renilla* luciferase (*rluc*) gene was amplified from pRL-SV40 using primers Delf8 and Delf9 (**Table 2**). These fragments were fused *via* overlap PCR. To analyze the structure of the putative *npdA* promoter, deletion derivatives of non-coding fragments upstream of *npdA* were amplified by employing the same PCR strategy as described above, except using different N-terminal primers, namely Delf10, Delf11, Delf12, Delf13, Delf14, and Delf15 (**Table 2**). The above amplicons were inserted in pGEM-T easy, released from this vector by *Sac*I and *Sac*II digestion, and inserted into the same sites of pBBR1MCS-3 to create the deletion series. The reporter vector was then conjugated into strain Cs1-4.

CONSTRUCTION OF STRONG EXPRESSION SYSTEM AND FLUORESCENT PROTEIN REPORTER VECTORS

Genes encoding green fluorescent protein and red fluorescent protein were amplified from pKEN2 and pmStrawberry using the primers Delf16/Delf17 and Delf18/Delf19, respectively, and engineered *via* PCR to contain an *E. coli* ribosome binding site on the 5′-end (**Table 2**). The amplicons were cloned into pGEM-T easy (pSCH374 and pSCH378, respectively), *gfpmut3* was then released by *Apa*I and *Sac*II digestion, and inserted into the same sites on pBBR1MCS3 (pSCH397). The *mStrawberry* gene was cut from pSC378 by digestion with *Kpn*I and *Sac*II, and inserted into *Kpn*I/*Sac*II sites on pBBR1MCS3 (pSCH395).

A strong expression system controlled by P*npdA* was constructed as follows. The PnpdA region (genome position 5862152–5862685) was amplified from strain Cs1-4 genomic DNA using primers Delf20 and Delf21 (**Table 2**). The amplicon was then cloned into pGEM-T easy (pSCH426), released by digestion with *Apa*I and *Sma*I, and inserted into the same sites on pBBR1MCS3 (pSCH442). Green fluorescent protein (GFP, *gfpmut3*) and red fluorescent protein (RFP, *mStrawberry*) marker genes were released from pSCH374 and pSCH378 by digestion with *Sac*II, cloned into pSCH442 and transformed into *E. coli* JM109. Colonies with strong green (pSCH476) and red (pSCH473) fluorescence were recovered, and orientation of reporter genes was confirmed by sequencing. These plasmids were next conjugated into *Delftia* sp. Cs1-4, leading to strains SCH481 (pSCH476) and SCH482 (pSCH473).

CONSTRUCTION OF *GFP* REPORTER VECTOR FOR CHROMOSAOMAL TAGGING OF *NPDA*

To transcriptionally tag *npdA*, *gfp* was inserted immediately downstream of *npdA* using the Cr*e-loxP* recombination method of Denef et al. (2005). An *npdA* fragment with the stop codon

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

Strain or plasmid	Relevant characteristics and/or plasmid construction	Source
BACTERIA		
E. coli		
BW19851 (λ *pir*)	RP4-2*tet*::Mu-1*kan*::Tn*7* integrant; Δ*uidA*::*pir*$^+$ *recA1 hsdR17 creB510 endA1 zbf-5 thi*	Metcalf et al. (1994)
TransforMax EC100+	*F*$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC) Φ80dlacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK* λ$^-$ *rpsL (Str*^{R}*) nupG*	Epicenter
S17-1	*hsdR17* (rK$^-$ mK$^-$) *recA* RP4-2 (Tcr::Mu-Kmr::Tn*7* Str^r)	Simon et al. (1983)
JM109	F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]/*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1 mcrA* (*lac-proAB*)	Promega
Delftia sp. Cs1-4		Vacca et al. (2005)
Wild type	Growth on phenanthrene, nanopod production	Shetty et al. (2011)
SCH482	Wild type carrying expression cassette *PnpdA* + *mStrawberry*	This study
SCH481	Wild type carrying expression cassette *PnpdA* + *gfpmut*3	This study
SCH369	Δ*lasI::Km*	This study
SCH340	Δ*hcp::Km*	This study
SCH411	Δ*omp32*	This study
SCH456	*npdA:gfp* on the chromosome	This study
SCH514	*npdA:gfp; (omp32::Km*	This study
PLASMIDS		
pGEM-T easy	Cloning vector; Amp^r	Promega
pRL-SV40	*Renilla* luciferase gene (*rluc*)	Promega
pKEN2	Source of *gfpmut3*; Ap^r	Comack et al. (1996)
pmStrawberry	*mStrawberry* gene template; Ap^r	Shaner et al. (2004)
pBBR1MCS3	Broad-host-range plasmid; Tc^r	Kovach et al. (1994)
pJK100	Allelic exchange vector; Tc^r and Km^r	Denef et al. (2006)
pCM157	Cre expression vector; Tc^r	Denef et al. (2006)
pHimarEm1	Plasmid carrying mini-*Himar* RB1; Km^r	Braun et al. (2005)
pSCH29	Derivative of pMiniHimar RB1; Km^r	This study
pSCH160	*gfpmut*3 on pSCH29; Km^r	This study
pSCH402	*mStrawberry* on pSCH29; Km^r	This study
pSCH375	*Gfpmut3* on pGEM-T easy; Ap^r	This study
pSCH378	*mStrawberry* on pGEM-T easy; Ap^r	This study
pSCH394	*mStrawberry* on pBBR1MCS3; Tc^r	This study
pSCH397	*Gfpmut3* on pBBR1MCS3; Tc^r	This study
pSCH426	P*npdA* on pGEM-T easy; Ap^r	This study
pSCH442	P*npdA* on pBBR1MCS3; Tc^r	This study
pSCH473	*mStrawberry* reporter under P*npdA* on pSCH442; Tc^r	This study
pSCH476	*gfpmut3* reporter under P*npdA* on pSCH442; Tc^r	This study
pSCH447	*npd* gene fragment on pGEM-T easy; Ap^r	This study
pSCH430	Downstream *npdA* gene fragment on pGEM-T easy; Ap^r	This study
pSCH431	Insert from pSCH430 cloned into pJK100; Tc^r	This study
pSCH485	Insert from pSCH447 cloned into pSCH431; Tc^r	This study
pSCH451	*gfpmut3* from pSCH375 was inserted into pSCH485; Tc^r	This study
pSCH487	Upstream fragment of *hcp* on pGEM-T easy; Ap^r	This study
pSCH486	Downstream fragment of *hcp* on pGEM-T easy; Ap^r	This study
pSCH339	*hcp* knock out plasmid; Tc^r and Km^r	This study
pSCH490	Upstream fragment of *omp32* on pGEM-T easy; Ap^r	This study
pSCH418	Downstream fragment of *omp32* on pGEM-T easy; Ap^r	This study
pSCH371	*omp32* knock out plasmid; Tc^r and Km^r	This study
pSCH356	Downstream fragment of *lasI* on pGEM-T easy; Ap^r	This study
pSCH488	Upstream fragment of *lasI* on pGEM-T easy; Ap^r	This study
pSCH363	*lasI* knock out plasmid; Tc^r and Km^r	This study

Table 2 | Primers used in this study.

Primer	Sequence (5′–3′)	Modification[a]
Delf1	(P) ctttgagcaacgttc	
Delf2	Ccgcctgaaccccaagctgtc	None
Delf3	Cgtgttgtcagctgcagcagagc	None
Delf4	Cacaactcgtctggcgttgcagc	None
Delf5	Gccacgctggcgaagcccag	None
Delf6	GCGAGCTCGgggcagtggtggctgtacatggag	*Sac*I
Delf7	gactggcttaggtcgtctcttacgacgggtgtagcggtggggcc	None
Delf8	aagagacgacctaagccagtcatgacttcgaaagtt tatgatccagaacaaagg	None
Delf9	GCCGCGGCttattgttcatttttgagaactcgctcaacgaacg	*Sac*II
Delf10	GCGAGCTCGctgtacatggagtaagttcctctacacctg	*Sac*I
Delf11	GCGAGCTCGgtaagttcctctacacctgtgtgcaaatgtc	*Sac*I
Delf12	GCGAGCTCGtgtgcaaatgtctgggataattcgccgtc	*Sac*I
Delf13	GCGAGCTCGtctgggataattcgccgtccctgtac	*Sac*I
Delf14	GCGAGCTCGacgattccccggcgatcaatcgtgg	*Sac*I
Delf15	GCGAGCTCGcgatcaatcgtggtggttgcaactacc	*Sac*I
Delf16	GGTACCGGATCCtttaagaaAGGAGAtatacatatg agtaaaggagaagaagaac	*Kpn*I–*Bam*HI
Delf17	CCGCGGgaattcttatttgtatagttcatccatgcc atgtgtaatccc	*Sac*II
Delf18	GGTACCGGATCCtttaagaAGGAGAtatacatatgg tgagcaagggcgag	*Kpn*I–*Bam*HI
Delf19	CCGCGGttacttgtacagctcgtccatg	*Sac*II
Delf20	GGGCCCagggcagtggtggctgtacatggag	*Apa*I
Delf21	CCCGGGaggtcgtcagagttggcagcgcc	*Sma*I
Delf22	AGATCTcgtgcactggcagttgacag	*Bgl*II
Delf23	GGTACCcaaattaacgacgggtgtagcg	*Kpn*I
Delf24	CCGCGGactgctaccgcgaacggcgctg	*Sac*II
Delf25	GAGCTCgcctgtgtcttggcttcggggg	*Sac*I
Delf26	AGATCTgctcgtttggagcgacggtgtgttc	*Bgl*II
Delf27	CATATGtcgacgatctccaattcggcctccag	*Nde*I
Delf28	GTTAACacgccatgggaacacgcgtgagcgg	HpaI
Delf29	GAGCTCcacccggcagcgaacaggctgagc	*Sac*I
Delf30	AGATCTgagctttgccgcccggccagg	*Bgl*II
Delf31	CATATGggctgggtagcaccccaggtg	*Nde*I
Delf32	GGGCCCggcaagggcgccgagagccag	*Apa*I
Delf33	GAGCTCgccaggacccggctcaaggcag	*Sac*I
Delf34	AGATCTtcgtcatccttcatgcgggccaccacg	*Bgl*II
Delf35	CATATGTattgcaccagaccaccgctgcccatgc	*Nde*I
Delf36	CCGCGGggacgcgttcccggcggcaaccgcagg	*Sac*II
Delf37	GAGCTCgcccgtggtgcccaggccctcatggatc	*Sac*I
Delf38	CGCGGATCCGCGTCCCCGCGGgaccccttaattaac cccgaaaagtgccacctgacg	*Bam*HI–*Sac*II
Delf39	CGCGGATCCGCGCGGGGTACCccggacgcgtcgaat taattccgctagc	*Bam*HI–*Kpn*I

[a]*Delf1 introduces 5′-phosphorylation. Nucelotide sequences in uppercase were used to introduce the indicated modifications in PCR products. The engineered E.coli RBS sequences were capitalized and underlined.*

(genome position 5860670–5861289) was amplified using primers Delf22 and Delf23 (**Table 2**). The downstream fragment of *npdA* (genome positions 5860066–5860809) was amplified using primers Delf24 and Delf25 (**Table 2**). These fragments were then cloned into pGEM-T easy (pSCH447 and pSCH430). The downstream fragment from pSCH430 was released by digestion with *Sac*II and *Sac*I and inserted into the same sites on pJK100 (pSCH431). The *npdA* fragment from pSCH447 was released by *Nde*I and *Kpn*I digestion, and then inserted into the same sites on pSCH431 (pSCH485). The *gfpmut3* gene was released from pSCH375 by *Kpn*I and *Not*I digestion, and assembled into the same sites on pSCH485 (pSCH451). Conjugation of pSCH451 into strain Cs1-4 gave Km^r/Tc^s colonies, which were recovered for further analysis. The Cre-expressing vector, pCM157, was next introduced into a selected colony (SCH483) in order to remove K_m resistance, leading to strain SCH484 (Km^s/Tc^r). Curing of pCM157 from SCH484 was done by serial transfers in LB medium. A selected colony (Km^s/Tc^s) with green fluorescence was then confirmed for the correct construct by PCR and sequencing (SCH456).

MUTANT CONSTRUCTION

To knock out *lasI*, its upstream (strain Cs1-4 genome positions 1950815–1951882) and downstream (strain Cs1-4 genome positions 1952504–1953573) fragments were amplified with primers Delf26/Delf27 and Delf28/Delf29, respectively (**Table 2**). The amplicons were gel purified and cloned into pGEM-T easy (pSCH488 and pSCH356). The upstream fragments were released by *Bgl*II/*Nde*I digestion, and downstream fragments were released by *Apa*I/*Sac*I from pGEM-T easy and then sequentially assembled on the same sites on pJK100 (pSCH363). To knock out *hcp*, upstream (strain Cs1-4 genome position 3366999–3367911) and downstream fragments (strain Cs1-4 genome position 3368229–3369041) were amplified using PCR primers Delf30/Delf31 and Delf32/Delf33, respectively (**Table 2**). The amplicons were gel purified and cloned into pGEM-T easy (pSCH487 and pSCH486). These fragments were sequentially assembled on the same sites on pJK100 (pSCH339) using the same strategy as described above. To knock out *omp32*, upstream (Cs1-4 genome positions 1041477–1042202) and downstream (Cs1-4 genome position 1044310–1045032) fragments were amplified with primers Delf34/Delf35 and Delf36/Delf37 (**Table 2**). The amplicons were gel purified and cloned into pGEM-T easy vector (pSCH490 and pSCH418). These fragments were sequentially assembled on the same sites on pJK100 (pSCH371). Each of the three constructs (pSCH363, pSCH339, pSCH371) was introduced into strain Cs1-4 by conjugation, and Tc^s/Km^r transconjugants were selected, leading to strains SCH369, SCH340, and SCH389, respectively.

GENOME-WIDE TRANSPOSON MUTAGENESIS

Modification of pHimarEm1 was done to introduce additional unique *Kpn*I–*Bam*HI–*Sac*II restriction sites, to remove the erythromycin resistance gene and to insert genes encoding GFP and RFP. To do so, PCR was done with pHimarEm1 DNA as template, and using forward primer Delf38 and reverse primer Delf39 (**Table 2**). The amplicon was digested with *Bam*HI, self-ligated and transformed into *E. coli* S17 λ*pir*. The *gfpmut3* fragment was digested with *Kpn*I and *Sac*II from pSCH375 and inserted into pSC29 at the same restriction sites (pSCH160). The promoterless *mStrawberry* fragment was then released from pSCH378 by *Kpn*I

and *Sac*II digestion, inserted into pSCH29 at the same restriction sites (pSCH402), and then introduced into strain SCH456 by conjugation. The Km-resistant colonies were randomly picked and replicated in 96-well plates containing MSM with either pyruvate and or phenanthrene as the carbon source. After incubation with shaking (24 h), the OD_{600} and GFP fluorescence were determined (see below).

REPORTER ASSAYS

Renilla luciferase assays were done as described in our prior work (Chen et al., 2009) using a commercially available kit (Promega) according to the manufacturer's protocol. Quantitative analysis of fluorescent protein production was done using a Synergy 2 plate reader with the following conditions (all 0.2-s interval, 22°C): GFP, excitation at 485 nm, emission 510 nm; RFP, excitation at 574 nm, emission at 596 nm. All measurements were corrected for background with wild type (WT) *Delftia* sp. Cs1-4 cells.

DNA SEQUENCE AND SEQUENCE ANALYSIS

The complete genome sequence of *Delftia* sp. Cs1-4 was deposited in Genbank as accession NC(015563.1. All constructs were sequenced by the dideoxy termination method using an Applied Biosystems (Foster City, CA, USA) 3730 × l DNA Analyzer available at the University of Wisconsin-Madison, Biotechnology Center. GenBank database searches were carried out using the National Center for Biotechnology Information BLAST-N web server.

RESULTS

ANALYSIS OF *NPDA* PROMOTERS IN *DELFTIA* SP. CS1-4 AND DEVELOPMENT OF STRONG EXPRESSION SYSTEM

Three TSS were identified for *npdA*, and were located at (nucleotide) −34-bp (A), −56-bp(G), and −172-bp (A), respectively upstream of the *npdA* start codon (**Figure 1A**). Three putative promoter motifs, P*npdA*$_1$ (TCCTCT-N$_{15}$-TGTCTG), P*npdA*$_2$ (TAGGGG-N$_{15}$-TACGAT), and P*npdA*$_3$ (TACGAT-N$_{17}$-TGGTGG) situated at −38, −61, and −180-bp, respectively were identified (**Figure 1A**). Serial deletion of non-coding regions upstream of *npdA* was done to establish involvement in *npdA* regulation of one or more of the three putative promoters. There was no significant difference in levels of gene expression between the WT and D1 (*npdA* −220 bp; **Figure 1B**). However, further deletion of an 11-bp fragment from D1 (D2, *npdA* −209 bp) yielded a *ca.* 20% decrease in Rluc activity relative to the WT (**Figure 1B**). Since the D2 construct carried the putative −35 motif in P*npdA*$_1$, we inferred the fragment (−220 to −209 bp) was also important for *npdA* expression. Deletion of the −35 region of P*npdA*$_1$ (D3, *npdA* −190 bp) decreased Rluc activity by >40% compared to the WT. Construct D4 (*npdA* −180 bp) had only *ca.* 20% Rluc activity. The latter contained a deletion that originated at −180 bp, and thus had the entire P*npdA*$_1$ region disrupted, indicating that P*npdA*$_1$ was the most important promoter for driving *npdA* expression. A further deletion (D5, *npdA* −67 bp) that removed the –35 bp motif in P*npdA*$_2$ retained *ca.* 5% of WT level. Removing the P*npdA*$_2$ region (D6, *npdA* −54) reduced Rluc activity to background levels.

To test the utility of the P*npdA* expression system, the genes encoding a GFP and RFP were inserted downstream of the P*npdA* cassette, which contained the 220-bp fragment described above. Transformants appeared green or red under ambient light, indicating strong expression of *gfp* and *mstrawberry*, respectively. The apparent high-level expression of these proteins was non-toxic to *Delftia* sp. Cs1-4, as growth of cultures expressing GFP or RFP was not distinguishable from that of the WT (**Figure 2A**). Production of GFP and RFP followed similar patterns, with levels increasing with culture growth, achieving stable accumulations upon reaching stationary phase (**Figure 2B**). In the absence of antibiotic selection, the expression vector was stable in *Delftia* sp. Cs1-4 for at least 56 generations (**Figure 2C**).

GENE DELETION AND GENOME-WIDE MUTAGENESIS

For generation of gene knockouts, the vector was used to target *omp32*, *hcp*, and *lasI*. Deletion of all three genes was successful, and confirmed by PCR and/or Southern hybridization. However, none of the gene deletions resulted in a loss of nanopod production, and only the Δ*omp32* mutant exhibited phenotypes different from that of the WT. In whole cell protein profiles, the latter mutant showed a loss of the predominant band corresponding to Omp32 (Shetty et al., 2011) and appearance of two other proteins, also identified as porins (**Figure 3A**). The Δ*omp32* mutant had an irregular cell shape (**Figure 3B**), and its growth was impaired on both pyruvate and phenanthrene, but the impact of Omp32 loss appeared to be greater with the latter substrate (**Figures 3C,D**).

Following conjugal delivery to *Delftia* sp. Cs1-4, the transposition frequency of pMiniHimar was *ca.* 2×10^{-5} to 5×10^{-6} per recipient, a frequency comparable to those reported for *Shewanella oneidensis*, *Geobacter sulfurreducens*, and *B. pseudomallei* (Choi et al., 2008; Rollefson et al., 2009). From the 13,000 colonies screened, seven mutants were recovered that were impaired in either growth on phenanthrene (Mutants 1–6; **Table 3**) or in *npdA* expression (Mutant 7; **Table 3**). For the former, three mutants had insertions in the gene cluster encoding the phenanthrene catabolic pathway. Of these, Mutant 3 was intriguing as the gene bearing the insertion was predicted to encode an Ycf48 homolog. For Mutants 5 and 7, insertions were in genes outside of the phenanthene degradation cluster, and were predicted to encode a SpoT/RelA-type (p)ppGpp synthetase, and a HylD Family, type I secretion membrane fusion protein, respectively.

DISCUSSION

Promoters proceeding SLP genes are among the most potent in many bacteria. For example, in *Lactobacillus acidophilus*, the strength of the SLP gene promoter is roughly twice that controlling the lactate dehydrogenase gene (Boot et al., 1996). Strong promoters may be needed for genes encoding SLP, as SLP are typically among the most abundant cellular proteins, as is the case with NpdA in strain Cs1-4 (Shetty et al., 2011). Thus, to develop a strong expression system, we focused on identification of the *npdA* promoter.

Collectively, the serial deletion analyses indicated that at least 220 bp upstream of *npdA* were required for maximal, log phase expression of *npdA* in strain Cs1-4 growing on phenanthrene. The presence within this region of multiple putative promoters is a feature that appears to be common for genes encoding SLP. For example, the SLP-encoding genes of *Lactobacillus brevis* ATCC 8287 (Hynönen et al., 2010), *Aeromonas salmonicida* (Chu et al.,

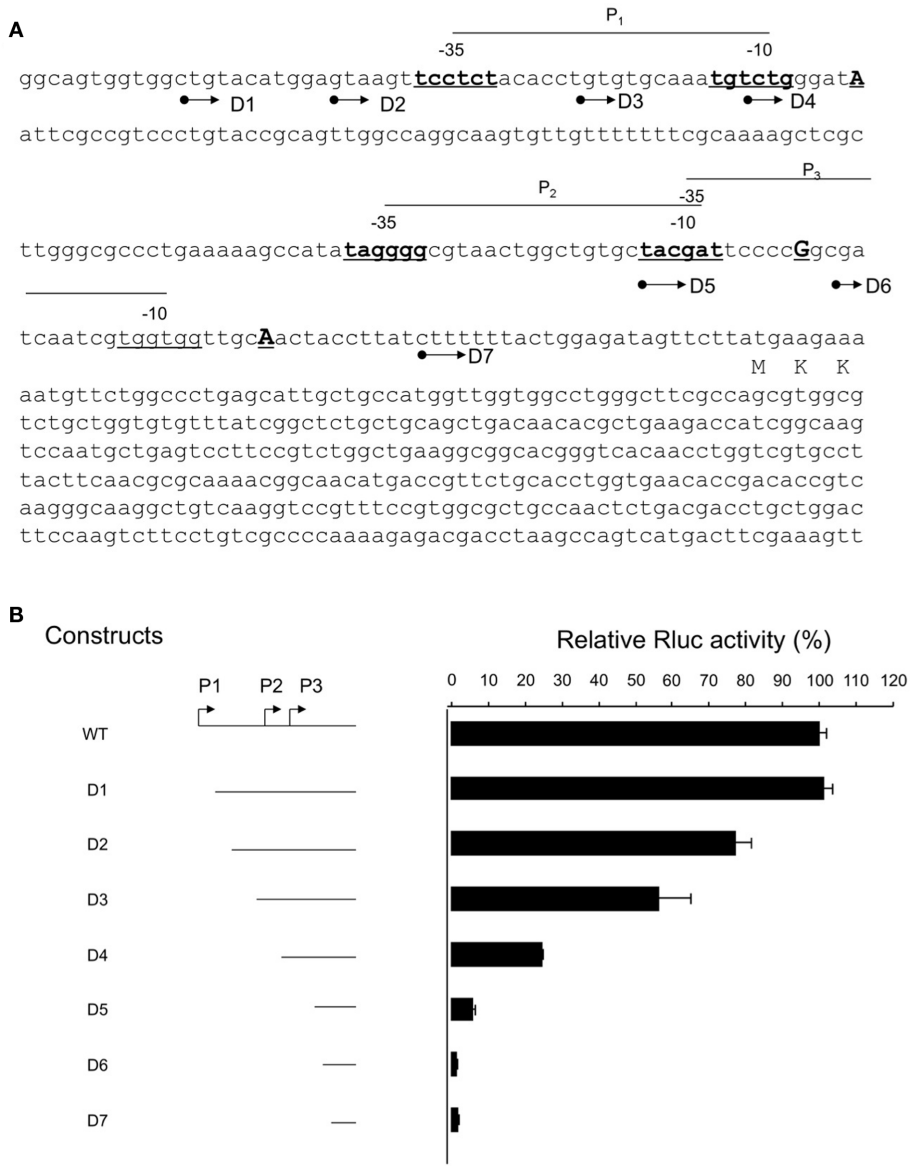

FIGURE 1 | Analysis of *npdA* promoter regions. (A) Putative −10- and −35-bp motifs are indicated with P1, P2 and P3. Transcription start points are capitalized and underlined. Arrows indicate positions of deletions (D1-7). **(B)** Effect of serial deletion on *rluc* expression. Results were normalized to Rluc activity of the wild-type. Reactions were done in triplicate, and standard deviations are indicated by error bars.

1993), and *Bacillus stearothermophilus* ATCC 12980 (Jarosch et al., 2000) had at least two promoters, while in *Bacillus brevis* three promoters were arranged tandemly upstream of the *cwp* operon (Adachi et al., 1989). The reason(s) why SLP genes have multiple promoters are unknown. Possibly, these could be needed to respond to a variety of stimuli that could affect the expression of SLP genes (Sleytr and Messner, 1983; Adachi et al., 1989; Soual-Hoebeke et al., 1999). As yet, specific functions for the S-layer in *Delftia* sp. strain Cs1-4 are unknown, however, some involvement in phenanthrene degradation is a possibility as mutants lacking NpdA (and consequently the S-layer) are impaired in their ability to grow on this compound (unpublished data).

Expression systems based on well-characterized promoters such as P*lac* or P*tac* are widely used (Dykxhoorn et al., 1996), but have had limited success in the *Burkholderiales* (Lefebre and Valvano, 2002). Likewise, for strain Cs1-4, Rluc was weakly expressed under control of *Plac*, as Rluc activity was *ca.* 2,500-fold lower than that from P*npdA*:*rluc*. An alternative approach is to use promoters that originate from the *Burkholderiales*, and one example is the promoter regulating expression of small ribosomal protein S12 (P*rsp*). The latter promoter has been successfully utilized in *Burkholderia xenovorans* LB400 (Yu and Tsang, 2006) and in *B. cepacia* (Lefebre and Valvano, 2002). However, in strain Cs1-4, gene expression under P*rsp* was poor, and not significantly

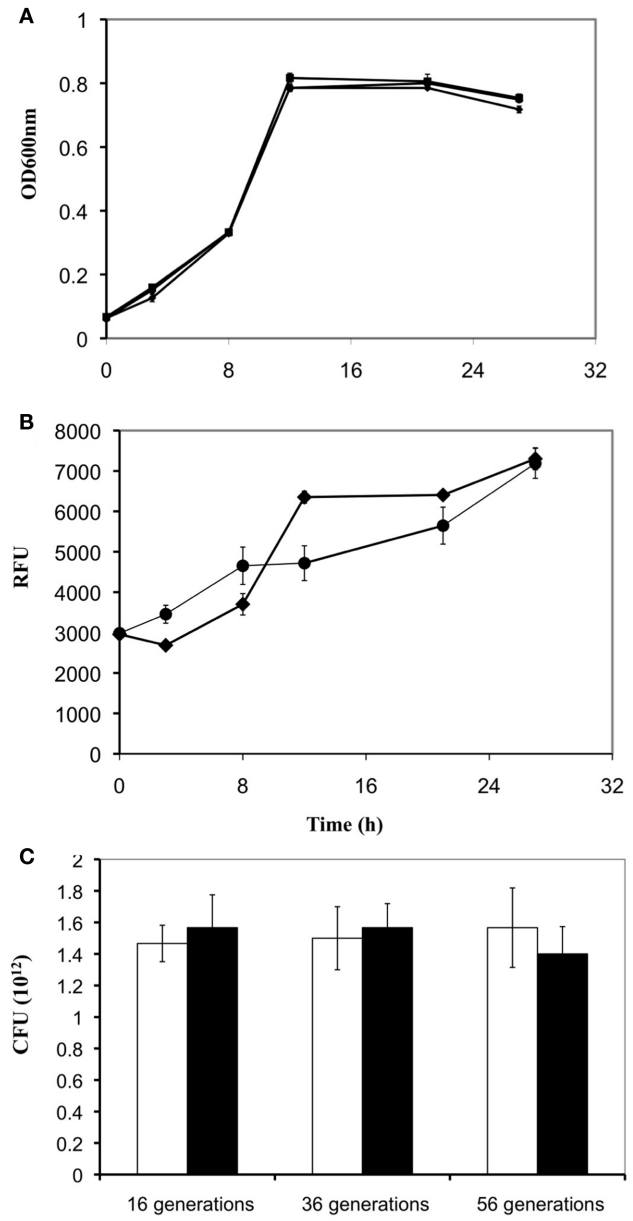

FIGURE 2 | Growth and fluorescence characteristics of the GFP and RFP reporter strains. (A) SCH481 (P*npdA:gfp*, circle), SCH482 (P*npdA:mStrawberry*, diamond), and WT (square) were adjusted to the same cell density and cultured for 30 h. **(B)** Fluorescence determination for GFP (circle) and RFP (diamond) reporter strains. Values in **(A)** and **(B)** are means of measurements made from triplicate cultures, and error bars indicate standard deviation) **(C)** Stability test of the expression vector (pSCH477, P*npdA:gfp*) in *Delftia* sp. Cs1-4. The strain SCH481 (P*npdA:gfp*) was serially transferred in LB medium and the CFU were determined at generations of 16, 36 and 56. The blank bar is without tetracycline addition and the black one is supplemented with tetracycline.

different from that of *Plac* (data not shown). Thus, demonstration of P*npdA* as a strong promoter functional in *Delftia* sp. Cs1-4 has provided a much-needed tool for genetic analyses of this organism, and potentially other related bacteria.

The Δ*omp32* mutant had an irregular cell shape (**Figure 3B**), suggesting that Omp32 may have a key role in establishment of cell envelope structure, as shown for other outer membrane proteins (Lazar and Kolter, 1996; Watts and Hunstad, 2008). Analysis of the Δ*hcp* mutant demonstrated that, as opposed to our hypothesis, Hcp did not have a structural function essential for nanopod formation. However, Western blot data indicated that Hcp was associated in some manner with nanopods as the majority of this protein accumulated in the >50-nm diameter fraction along with nanopods (data not shown). It is possible that Hcp was secreted separately from nanopods, and formed extracellular structures that were co-purified with nanopods. If so, such structures were not discernable in samples imaged by transmission electron microscope. Alternatively, Hcp may be associated with nanopods as cargo carried by OMV. In this case, Hcp may function as a virulence factor that may be employed by strain Cs1-4 in interactions with competing bacteria, as has been shown for T6SS in other bacteria (Schwarz et al., 2010; Leung et al., 2011; Records, 2011). Lastly, for the Δ*lasI* mutant, the absence of any detectable change in the formation of nanopods suggested that the process was not affected by quorum sensing, at least in the sense that it was regulated by AHL produced by a canonical AHL synthetase. This finding is noteworthy as it helps to narrow the spectrum of possible mechanisms that may control nanopod production.

Efficient targeting for gene inactivation is critical for functional genomic studies and, in bacteria, two widely used systems for generating in-frame, unmarked deletions are those based on *sacB* counter selection (Jäger et al., 1995; Chen et al., 2010), and Cre-*loxP* system (Denef et al., 2006; Choi et al., 2008). For strain Cs1-4, the *sacB* system proved unsuccessful; merodiploids (first recombination) were recovered at a high frequency, but these were not effectively resolved as *Delftia* sp. Cs1-4 grew in YT agar medium containing 5–15% (wt/vol) sucrose (data not shown). Similar observations have been reported for *Streptomyces lividans* and some *Burkholderia* strains, which carry an intrinsic *sacBC* operon. Alternatively, Cre-*loxP* system was successfully adapted for gene deletion or insertion, and was an efficient way for recycling antibiotic markers in *Delftia* sp. Cs1-4. To our knowledge, this is the first report of the Cre-*loxP* system being used for gene deletion analysis in *Delftia* spp.

Of the mutants recovered from genome-wide mutagenesis, three were of particular interest as they may encode new functions associated with nanopod production and/or phenanthrene degradation. One of these putatively encoded an Ycf48-like protein. In phototrophs, Ycf48 functions in the assembly and repair of Photosystem II (Komenda et al., 2008; Rengstl et al., 2011). Activities of an Ycf48-like protein that may be related to phenanthrene degradation are unknown, but, given the significant reduction (*ca.* 64%) in nanopod produced by this mutant, it's interesting to speculate that it may have a role in the assembly of these structures. The putative *spoT*/*relA* mutant, had an insertion in a (p)ppGpp synthetase. The alarmone (p)ppGpp primarily governs the stringent response to amino acid starvation (Martinez-Costa et al., 1998; Åberg et al., 2006; Gomez-Escribano et al., 2008; Abranches et al., 2009) and, since growth of the *spoT*/*relA* mutant on pyruvate was not impaired, the effect of the mutation appeared related to use of phenanthrene as a carbon source. The third gene of

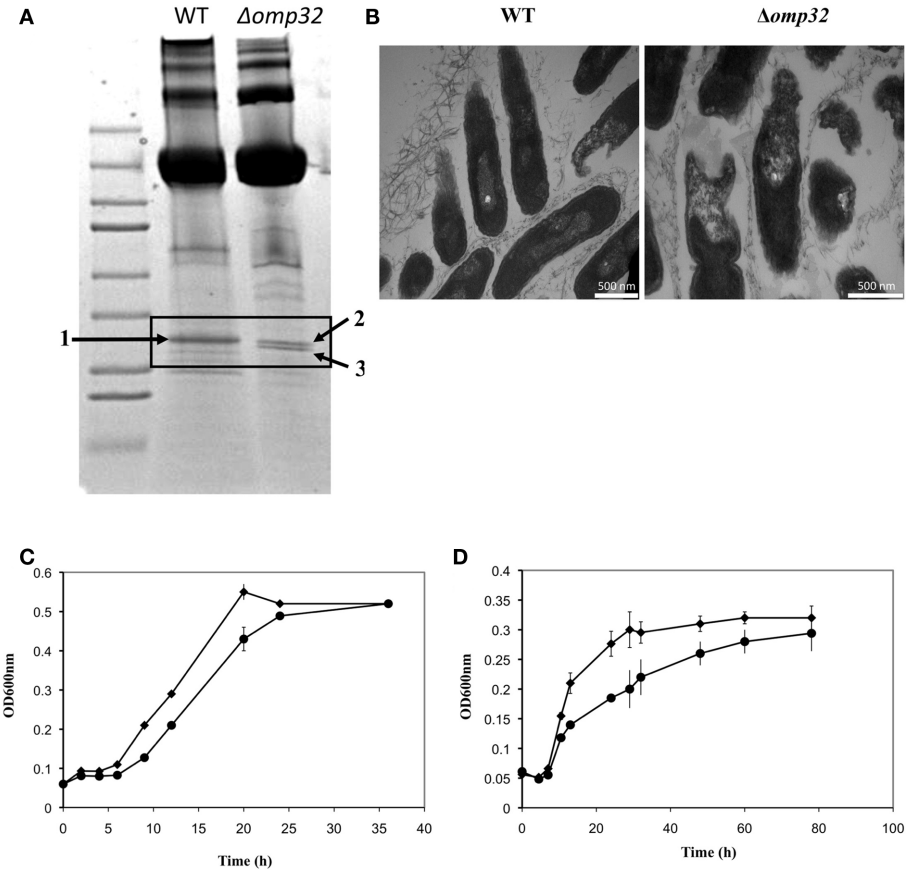

FIGURE 3 | Characterization of the *Delftia* sp. Cs1-4 Δ*omp32* mutant. **(A)** Protein profiles of the wild type (WT) and mutant. Boxed area indicates the region of the Omp32 band in the WT. In the mutant, arrows indicate two bands identified as different porins, which were not detected in the WT. **(B)** Transmission electron micrographs of strain Cs1-4 biofilm cells grown on phenanthrene illustrating the mutant's cellular deformities. **(C)** and **(D)** Growth of the WT (diamonds) and mutant (circles) on the indicated substrate.

Table 3 | Mutants recovered from miniHimar transposon mutagenesis.

Mutant	Insertion locus[a]	OD_{600}[b]	Nanopod production[c]	GFP[d]	Gene product
1	1741	N/A	N/A	N/A	Phenanthrene dioxygenase component; Ferredoxin-NAD(+) reductase (PhnAa)
2	1742 and 1743	0.09 ± 0.01	0.18 ± 0.02	0.59 ± 0.02	Non-coding region between *phnB* (*cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase) *and phnAc* (phenanthrene 1,2-dioxygenase, large subunit)
3	1760	0.13 ± 0.01	0.32 ± 0.16	0.31 ± 0.05	2-Carboxybenzaldehyde dehydrogenase (PhnI)
4	3891	0.15 ± 0.01	0.81 ± 0.01	0.74 ± 0.03	(p)ppGpp synthetase (SpoT/RelA)
5	4612	0.15 ± 0.01	0.99 ± 0.09	0.93 ± 0.03	Type I secretion membrane fusion protein, HlyD family
6	1758	0.20 ± 0.02	0.36 ± 0.04	0.74 ± 0.03	Ycf48-like protein
7	3984	0.24 ± 0.02	0.84 ± 0.05	0.68 ± 0.11	Heavy metal translocating P-type ATPase
Wild type	N/A	0.23 ± 0.01	1.00	1.00	N/A

[a] *Locus in Delftia sp. Cs1-4 genome, all locus numbers are proceeded by "DelCs14("*

[b] *Optical density measured after 7 d incubation in MSM supplemented with phenathrene. Values are averages (±SD) of single measures from triplicate cultures.*

[c] *Determined as described by Shetty et al. (2011). Values are averages (±SD) of single measures from triplicate cultures, and are normalized to those of WT.*

[d] *GFP Fluorescence. Values are averages (±SD) of single measures from triplicate cultures, and are normalized to those of WT.*

interest, encoding an HlyD-like protein, was clustered with other genes predicted to encode pili formation. But, it remains to be determined how amino acid starvation and pili formation may be connected to phenanthrene degradation. Mutant 7 was not impaired in growth on phenanthrene, but did show decreased expression of *npdA*, and a depressed level of nanopod production. The protein predicted for the locus bearing the insertion contained a heavy-metal-associated domain that is also found in a number of proteins that transport or detoxify heavy metals; the relation of such a protein to *npdA* expression and nanopod formation remains to be determined.

Minitransposons are widely used for genome-wide mutagenesis in Gram-negative and Gram-positive bacteria (Lampe et al., 1999; Youderian et al., 2003; Maier et al., 2006; Choi et al., 2008) and, compared to other minitransposons, pMiniHimar is advantageous as it does not require host-specific factors for transposition, it lacks site specificity and the transposase is not introduced into the chromosome, thus enhancing insertion stability. The transposition frequency of pMiniHimar was sufficient ($>5 \times 10^{-6}$ per recipient) for saturation mutagenesis of the strain Cs1-4 genome. In the present study, pMiniHimar RB1 was modified by adding unique restriction sites for insertion of additional genetic elements. In our tests, these elements were promoterless *gfpmut3* and *mStrawberry*, and the resultant vectors can be utilized for random generation of genomic transcriptional fusions. Such vectors can provide a convenient way to conduct genome-wide investigations of gene expression levels under selected conditions (de Lorenzo et al., 1990; Hahn et al., 1991; Boyle-Vavra and Seifert, 1995; Velayudhan et al., 2007).

CONCLUSION

The present report outlined the development of tools needed for genetic manipulation of *Delftia* sp. Cs1-4. These tools included a new expression cassette (P*npdA*-based) that can be used for tagging of chromosomal genes as well as for complementation of knockout mutants, and a pMiniHimar transposon modified to enhance gene recovery and mutant analysis. The effectiveness in *Delftia* sp. of the Cre-*loxP* for gene deletion was also demonstrated. These tools were developed and validated for manipulation of *Delftia* sp. Cs1-4, but could also be applied to other related genera and species with importance in environmental toxicology.

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