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Comparative genomics of the *Staphylococcus intermedius* group of animal pathogens

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The *Staphylococcus intermedius* group consists of three closely related coagulase-positive bacterial species including *S. intermedius*, *Staphylococcus pseudintermedius*, and *Staphylococcus delphini*. *S. pseudintermedius* is a major skin pathogen of dogs, which occasionally causes severe zoonotic infections of humans. *S. delphini* has been isolated from an array of different animals including horses, mink, and pigeons, whereas *S. intermedius* has been isolated only from pigeons to date. Here we provide a detailed analysis of the *S. pseudintermedius* whole genome sequence in comparison to high quality draft *S. intermedius* and *S. delphini* genomes, and to other sequenced staphylococcal species. The core genome of the SIG was highly conserved with average nucleotide identity (ANI) between the three species of 93.61%, which is very close to the threshold of species delineation (95% ANI), highlighting the close-relatedness of the SIG species. However, considerable variation was identified in the content of mobile genetic elements, cell wall-associated proteins, and iron and sugar transporters, reflecting the distinct ecological niches inhabited. Of note, *S. pseudintermedius* ED99 contained a clustered regularly interspaced short palindromic repeat locus of the Nmeni subtype and *S. intermedius* contained both Nmeni and Mtube subtypes. In contrast to *S. intermedius* and *S. delphini* and most other staphylococci examined to date, *S. pseudintermedius* contained at least nine predicted reverse transcriptase Group II introns. Furthermore, *S. pseudintermedius* ED99 encoded several transposons which were largely responsible for its multi-resistant phenotype. Overall, the study highlights extensive differences in accessory genome content between closely related staphylococcal species inhabiting distinct host niches, providing new avenues for research into pathogenesis and bacterial host-adaptation.

Keywords: *Staphylococcus*, genomics, pathogenesis, host-adaptation, animal, antibiotic resistance

INTRODUCTION

Population genetic analysis has revealed the existence of a group of closely related coagulase-positive staphylococcal species associated with different host-species, collectively known as the *Staphylococcus intermedius* group (SIG; Varaldo et al., 1988; Bannhoer et al., 2007; Sasaki et al., 2007b). The SIG consists of *Staphylococcus pseudintermedius*, an opportunistic pathogen which predominantly colonizes the skin and mucosal surfaces of dogs (Kloos, 1980; Greene and Lammler, 1993), *Staphylococcus delphini* which has been isolated from a wide array of animals, including minks, horses, cows, and pigeons (Sasaki et al., 2007b), and *S. intermedius*, which has only been isolated from pigeons to date (Bannoehr et al., 2007; Sasaki et al., 2007b). Although *S. pseudintermedius* is a component of the canine normal flora, disruption of the normal skin flora or an underlying condition such as atopic dermatitis, can lead to *S. pseudintermedius* infections such as superficial and deep canine pyoderma, and otitis media or externa (Cole et al., 1998). Recently, strains of *S. pseudintermedius*, which are refractory to treatment by most commonly used classes of antibiotic including methicillin have emerged and disseminated widely (Perreten et al., 2010; Ruscher et al., 2010). *S. pseudintermedius* is rarely isolated from humans but can occasionally cause severe zoonotic infections, typically through dog bite wounds (Mahoudeau et al., 1997; Tanner et al., 2000; Pottumarthy et al., 2004). Furthermore, *S. pseudintermedius* has the capacity to produce enterotoxins related to those made by *Staphylococcus aureus* and has been implicated in several food poisoning outbreaks (Khambaty et al., 1994; Becker et al., 2001). Our understanding of the molecular pathogenesis of *S. pseudintermedius* canine pyoderma is very limited (Fitzgerald, 2009). However, the recent announcement of the first genome sequences for *S. pseudintermedius* strains has revealed the complement of genes encoding putative virulence factors (Ben Zakour et al., 2011; Tse et al., 2011), leading to proteomic studies which have identified novel host–pathogen interactions (Bannoehr et al., 2011a,b). An enhanced understanding of the pathogenesis of *S. pseudintermedius* is required in order to facilitate the design of novel therapeutics for the control of bacterial pyoderma infection caused by multi-resistant *S. pseudintermedius*. Furthermore,

the distinct host-tropisms of the three members of the SIG suggest that these closely related species would represent an excellent system for investigating evolutionary events underlying bacterial host-adaptation, a fundamental aspect of bacteriology which has not been well examined to date.

Recently, we published an announcement of the *S. pseudintermedius* ED99 genome, briefly listing several noteworthy features of the genome (Ben Zakour et al., 2011). Here we provide a comprehensive analysis of the ED99 genome in comparison to high quality draft genomes of the closely related species *S. delphini*, and *S. intermedius* generated in the current study, and to publicly available genomes for other staphylococcal species. The resulting data represent an excellent framework for investigations into the pathogenesis of canine pyoderma, and the molecular basis for staphylococcal host-specificity.

MATERIALS AND METHODS

BACTERIAL STRAINS

The previously sequenced *S. pseudintermedius* ED99 (formerly M732/99) was isolated from a clinical case of canine bacterial pyoderma in 1999 in Scotland and was selected to represent one of the common clones identified in a previous population genetic study of *S. pseudintermedius* (Bannoehr et al., 2007; Ben Zakour et al., 2011). *S. delphini* 8086 was isolated from the trachea of a horse in the UK (Bannoehr et al., 2007), and the type strain *S. intermedius* NCTC11048, from the anterior nares of a pigeon in the Czech Republic (Hajek, 1976).

GENOMIC DNA PREPARATION

Genomic DNA was isolated from 1 ml of overnight culture of *S. pseudintermedius* in BHI (Oxoid) at 37°C with shaking at 200 rpm. Genomic DNA extraction was carried out with a bacterial genomic DNA purification kit (Edge Biosystems) according to the manufacturer's instructions, except that prior to incubation at 37°C for 10 min, 125 μg/ml lysostaphin (AMBI L) was included.

GENOME SEQUENCING

Whole genome sequencing of *S. pseudintermedius* ED99 was carried out as previously described (Ben Zakour et al., 2011). Genome sequencing of *S. delphini* 8086 and *S. intermedius* NCTC11048 was carried out using the Illumina 3G Genome Analyzer. For each strain, we generated a total of 4,087,613 and 3,879,139 paired-end reads, respectively, with a fixed length of 36 bp and an average insert size of 200 bp, corresponding to more than 58× and 50× genome coverage, respectively. *De novo* assembly was performed by using the Velvet short reads assembler program (Zerbino and Birney, 2008). For each genome, contigs were mapped against the completed whole genome of *S. pseudintermedius* ED99 using MauveAligner (Rissman et al., 2009) and manually inspected for potential mis-assemblies. To confirm the reliability of the sequences obtained by this *de novo* sequencing approach based only on very short reads, re-sequencing of *S. pseudintermedius* ED99 as an internal control was also performed in parallel to *S. delphini* 8086 and *S. intermedius* NCTC11048. An automatic annotation was then performed by the RAST annotation server to predict CDS and their putative functions (Aziz et al., 2008). Functional categories were assigned by searching all predicted proteins against the COG database (www.ncbi.nlm.nih.gov/COG). The software AlienHunter (Vernikos and Parkhill, 2006) was used to detect atypical genome regions corresponding to putative horizontal gene transfer, insertion sequence (IS) elements were identified by interrogation of the IS database (Siguier et al., 2006), and clustered regularly interspaced short palindromic repeat (CRISPR) elements were characterized by the CRISPRFinder web software (Grissa et al., 2007a). The draft genome sequences of *S. delphini* 8086 and *S. intermedius* NCTC11048 have been deposited in the GenBank WGS database and have Genome Bioproject ID numbers PRJEA87011 and PRJEA87009, respectively.

COMPARATIVE GENOMIC ANALYSIS

Orthologous CDS between *S. pseudintermedius* ED99 (accession number CP002478), *S. aureus* Mu50 (accession number BA000017; Kuroda et al., 2001), *Staphylococcus epidermidis* RP62A (accession number CP000029; Gill et al., 2005), *Staphylococcus haemolyticus* JCSC1435 (accession number AP006716; Takeuchi et al., 2005), *Staphylococcus saprophyticus* ATCC15305 (accession number AP008934; Kuroda et al., 2005), *Staphylococcus carnosus* TM300 (accession number AM295250; Rosenstein et al., 2009), *S. delphini* 8086, and *S. intermedius* NCTC11048, were identified by reciprocal best hits using BLASTP, with a maximum *e*-value of 0.01, a minimum percentage identity of 40% and a minimum percentage sequence coverage of 80%. The average nucleotide identity (ANI) between the complete and draft genome sequences available for all staphylococcal species and the outgroup *Macrococcus caseolyticus* JCSC5402 (accession number AP009484; Baba et al., 2009), was determined by using the *in silico* DNA–DNA hybridization method of the JSpecies software using default parameters (Richter and Rossello-Mora, 2009). The distance matrix based on the ANI values obtained was then used in Splitstree to construct a Neighbor-Joining tree (Huson and Bryant, 2006).

RESULTS AND DISCUSSION

GENERAL FEATURES OF THE SIG GENOMES

We previously sequenced and annotated the complete genome of *S. pseudintermedius* ED99, used in this study as a reference for comparative genomic analysis with other SIG species (Ben Zakour et al., 2011). As previously reported, the ED99 genome was composed of a single circular chromosome of 2,572,216 bp (**Figure 1**) with an average G + C content of 37.6%, contained five ribosomal operons, 58 tRNA loci and encoded for 2401 predicted protein-coding sequences (CDSs; Bannoehr et al., 2011a). Wider analysis for the current study revealed that 44 putative CDS are pseudogenes, and functional information could be assigned to 77.6% of all CDS (1863 CDS), with 13.1% encoding conserved hypothetical proteins (315 CDS), and 9.3% encoding hypothetical proteins without significant homology to proteins of known function.

The draft genome sequence of *S. delphini* 8086 was assembled into 133 contigs and had an approximate size of 2.53 Mb including 2369 predicted CDS. The draft genome sequence of *S. intermedius* NCTC11048 was assembled into 228 contigs, and had an approximate size of 2.78 Mb, significantly larger than the two other members of the SIG, and was predicted to encode 2589

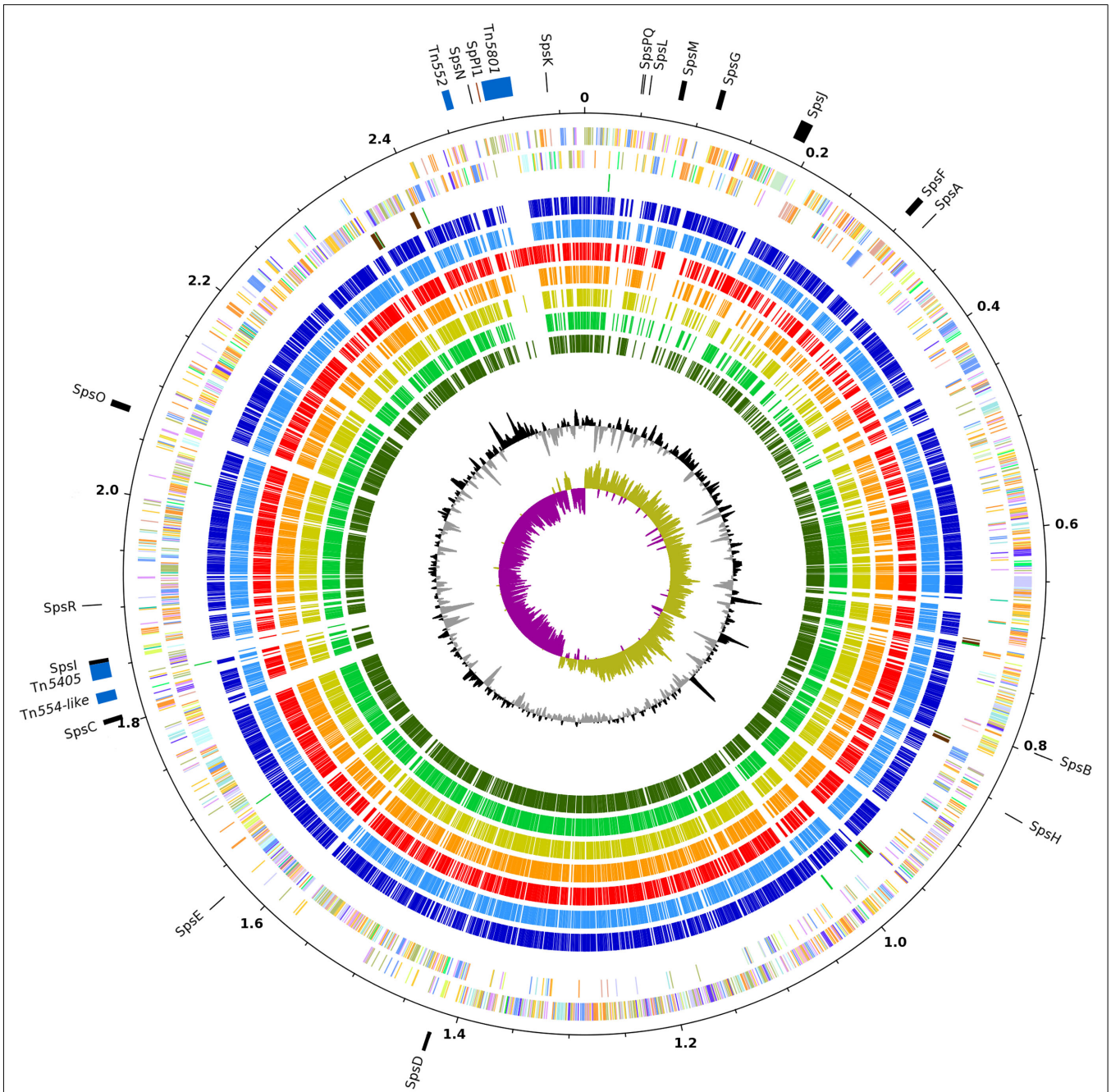

FIGURE 1 | Genome atlas of *S. pseudintermedius* ED99 compared to other staphylococci. Orthologs depicted in other species are identified by reciprocal best hits using BLASTP. Major features of the *S. pseudintermedius* ED99 are indicated on the outermost circle, including MGE (blue), CWA proteins (black), and SpPI1 (maroon) with positions on the reference genome indicated in Mb. From the outer to inner circles, circle 2 and 3, represent *S. pseudintermedius* ED99 genes on plus and minus strands, respectively (colored according to their COG category); circle 4, tRNA (green), rRNA (brown); circle 5, *S. delphini* 8086 (dark blue); circle 6, *S. intermedius* NCTC11048 (blue); circle 7, *S. aureus* Mu50 (red); circle 8, *S. epidermidis* RP62A (orange); circle 9, *S. haemolyticus* JCSC1435 (yellow); circle 10, *S. saprophyticus* ATCC15305 (light green); circle 11, *S. carnosus* TM300 (dark green); circle 12, G + C content plot (black and gray); and circle 13, GC skew plot (purple and khaki).

CDS (**Table 1**). Of note, the average GC content of the three SIG genomes ranges from 37.4 to 38.3%, which is considerably higher than any other staphylococcal species sequenced to date suggesting the existence of distinct selective pressures influencing the SIG genome nucleotide composition.

COMPARATIVE GENOMIC ANALYSIS OF THE *STAPHYLOCOCCUS* GENUS

The genomes of the three SIG species were compared with those of *S. aureus* Mu50, *S. epidermidis* RP62A, *S. haemolyticus* JCSC1435, *S. saprophyticus* ATCC15305, and *S. carnosus* TM300 (**Table 1**). Comparison of the eight selected staphylococcal chromosomes by

Table 1 | General features of the SIG genomes in comparison with other staphylococci.

Feature	*S. pseudintermedius* ED99	*S. delphini* 8086	*S. intermedius* NCTC11048	*S. aureus* Mu50	*S. epidermidis* RP62A	*S. haemolyticus* JCSC1435	*S. saprophyticus* ATCC15305	*S. carnosus* TM300
Length of sequence (bp)	2,572,216	∼2,528,000	∼2,780,000	2,878,529	2,616,530	2,685,015	2,516,575	2,566,424
G + C content (%)								
Total	37.6	38.3	37.4	32.9	32.2	32.8	33.2	34.6
Open reading frames								
Number	2401	∼2369	∼2589	2697	2494	2676	2446	2462
Average size (bp)	894	∼881	∼860	894	867	863	861	894
Percentage	83.4	∼82.6	∼80.1	83.7	82.6	86.0	83.7	85.8
Ribosomal RNAs								
16S	5	NA	NA	5	6	5	6	5
23S	5	NA	NA	5	6	5	6	5
5S	7	NA	NA	6	7	6	8	5
Transfer RNAs	58	NA	NA	60	61	59	60	60
Genomic islands								
IS	21 (12)	1#	12#	26 (13)	64 (18)	82 (60)	9 (2)	0
Transposons	4	0	0	3	5	2	0	0
SCC	0	0	0	1	1	1	2	0
Prophages	0	1#	3#	2	1	2	0	1
Plasmids	1*	NA	0	1	1*	3	2	0
Other islands	2	NA	NA	5	2	5	1	1

*NA, not available; #estimated number. *Cryptic plasmid. Number of intact IS element genes is indicated in parenthesis.*

reciprocal BLASTP revealed a high level of conservation and synteny (**Figure 1**). However, a large chromosomal region (∼465 kb) of low similarity was identified which corresponds to the *oriC* environ, a region of staphylococcal genomes containing many species-specific CDS (Takeuchi et al., 2005). Similar to *S. saprophyticus* ATCC15305 and *S. haemolyticus* JCSC1435, the genome of *S. pseudintermedius* ED99 contains a large chromosomal inversion at the beginning of the *oriC* environ, from the position 33,035 to 2,568,216, and which spans the entire chromosome. Consistent with their close-relatedness, the *oriC* environ exhibits a high level of gene content conservation among the closely related SIG members.

By performing reciprocal best blast hits analysis, we have determined a set of 1214 genes defining the core genome shared by *S. pseudintermedius* ED99, *S. aureus* Mu50, *S. epidermidis* RP62A, *S. haemolyticus* JCSC1435, *S. saprophyticus* ATCC15305, *S. carnosus* TM300, *S. delphini* 8086, and *S. intermedius* NCTC11048 (Table S2 in Supplementary Material). Of the predicted proteins, 12.4% have a general predicted function, 9.1% are of unknown function and 4.3% have no homolog found in the COG database, which is consistent with previous studies (Takeuchi et al., 2005; Rosenstein et al., 2009). Based on the core genome only, the average percentage similarity of *S. pseudintermedius* ED99 proteins compared to other staphylococcal species ranges from 68.8% with *S. carnosus* TM300 to 95.2 and 97.7% with *S. delphini* 8086 and *S. intermedius* NCTC11048, respectively. The evolutionary relatedness of the staphylococcal species was examined by calculation of their ANI. The phylogenetic tree based on pairwise comparison of the ANI confirms the close-relatedness of the SIG compared to the other staphylococcal species and is consistent with previously determined phylogenies (**Figure 2**). Of note, despite sharing distinct ecological niches, *S. pseudintermedius* ED99 and *S. delphini* 8086 share an ANI of 93.61%, which is very close to the threshold of species delineation of 95% ANI, equivalent to the DNA–DNA re-association threshold of 70% (Goris et al., 2007).

DISTRIBUTION OF MOBILE GENETIC ELEMENTS AMONG THE SIG

Although the *S. pseudintermedius* ED99 genome demonstrated a large degree of conservation and synteny with the other staphylococcal genomes, numerous regions of differences (RDs) of greater than 5 kb in size were identified including IS elements, genomic islands, and prophage- and plasmid-related sequences (**Figure 1**). A total of 21 predicted IS elements were identified including 7 identical copies of IS*Sp1* which had 47% nucleotide identity with IS*Sha1* of *S. haemolyticus* and IS*1182* integrated in Tn*5405* which had 100% nucleotide homology with the same element in *S. aureus*. In several cases the closest homologs of *S. pseudintermedius* IS elements were in other genera such as *Clostridium tetani* E88 (46% identity), and *Geobacillus thermodenitrificans* NG80-2 (52% identity). In addition, transposase-related sequences indicating the presence of at least one IS element in *S. delphini* 8086 and 12 IS elements in *S. intermedius* NCTC11048 were identified.

Reverse transcriptase (RT) Group II introns are self-splicing retro-elements composed of an intron RNA domain and a RT gene which are present in 25% of sequenced bacterial genomes, including members of the Firmicutes such as *Bacillus*, *Streptococcus*, and

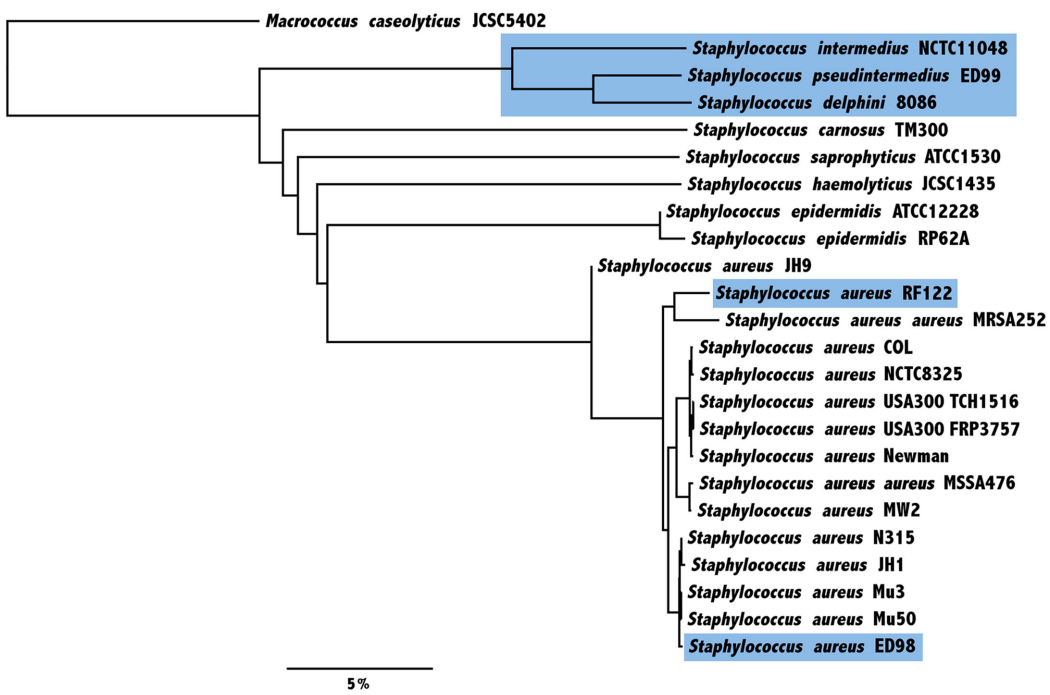

FIGURE 2 | Average nucleotide identity (ANI)-based Neighbor-Joining tree of 23 sequenced strains from eight staphylococcal species in addition to an outgroup *Macrococcus caseolyticus*. The evolutionary relatedness was examined by calculation of ANI, based on core genes only. Species and strains of animal origin are highlighted in blue (scale indicates the % difference ANI).

Lactococcus (Matsuura et al., 1997; Granlund et al., 2001; Tourasse and Kolsto, 2008). RT Group II introns are extremely rare in staphylococcal genomes, and generally present in low number with one or two copies, such as in *S. aureus* JKD6159 (Chua et al., 2011) and *S. pseudintermedius* HKU-10 (Tse et al., 2011). A total of nine intact copies and one pseudogene of the RT gene were identified in the genome of *S. pseudintermedius* ED99. All copies had a predicted intergenic insertion site, and no particular trend in their location or the nature of the genes adjacent to these elements was observed. However, it is possible that the integration of RT Group II introns may influence the expression of downstream genes or promote recombinational events (Tourasse and Kolsto, 2008). Of note, RT Group II introns were not identified in the genomes of *S. delphini* 8086 and *S. intermedius* NCTC11048.

The *S. pseudintermedius* ED99 genome does not contain any predicted complete prophages but contains three RDs containing phage-related genes, and a putative integrated 3.5 kb plasmid with 38% overall identity to plasmid pC221 of *S. aureus* was detected within the *oriC* environ. The genome also contains a novel member of the staphylococcal pathogenicity island family (SpPI1) adjacent to Tn*5801*, which is 4.1 kb in size, and includes attachment sites and genes with 55% identity to the terminase *orf5*, 67% identity to the repressor *orf20*, and 90% identity to the integrase *int* of SaPIbov of *S. aureus* (**Figure 3**). Of note, these three genes belong to the core minimal set of genes required for a functional mobile SaPI (Ubeda et al., 2008). In theory, SpPI1 may represent an ancestral minimal form of SaPI but *orf20* and *int* contain premature stop codons implying that SpPI1 is no longer mobile. Predicted virulence genes were not identified in SpPI1 but two genes encoding hypothetical proteins of unknown function were present. We examined the distribution of SpPI1 by PCR screening of 13 *S. pseudintermedius* strains, which represented diverse sequence types, with various geographical, host and clinical origins, and found that it was present in 11 strains (data not shown).

Staphylococcus pseudintermedius ED99 contains a novel 14 kb genomic island located adjacent to a *tRNA* locus, which is also present in *S. delphini* 8086 (**Figure A1** in Appendix). The island contains two genes with 51 and 32% homology respectively to *orf32* and *orf33* of ΦMu50B, two genes encoding the bi-component leukotoxin Luk-I identified previously *in S. pseudintermedius* SD91 (Prevost et al., 1995), and seven genes related to L-ascorbate transport and utilization not found in other staphylococci sequenced to date. The L-ascorbate utilization operon is organized in a novel combination of the *ula* operon of *Escherichia coli* (Yew and Gerlt, 2002) and shares similarity ranging from 55 to 68% with the ascorbate PTS system IIABC of *Streptococcus* sp. and the other members of the operon (*ulaG*, *ulaD*, *ulaE*, and *ulaF*) found in *Enterococcus* sp. The operon was identified in all 25 representative strains of *S. pseudintermedius*, *S. intermedius*, and *S. delphini*, with the exception of *S. intermedius* NCTC11048, which only contains the bi-component leukotoxin Luk-I (data not shown). In addition, despite being located downstream of a *t*RNA locus, which is a well-known integration site for mobile elements, mobility genes and flanking repeats regions were not identified in the sequence of both islands. Taken together, these characteristics imply an ancient acquisition in a progenitor of the SIG group and active maintenance of the acquired function among the SIG species.

S. PSEUDINTERMEDIUS ED99 AND *S. INTERMEDIUS* NCTC11048 CONTAIN CRISPR LOCI

Clustered regularly interspaced short palindromic repeats, recently described as an adaptative bacterial immune system, were shown to provide protection against viruses in *Streptococcus thermophilus*

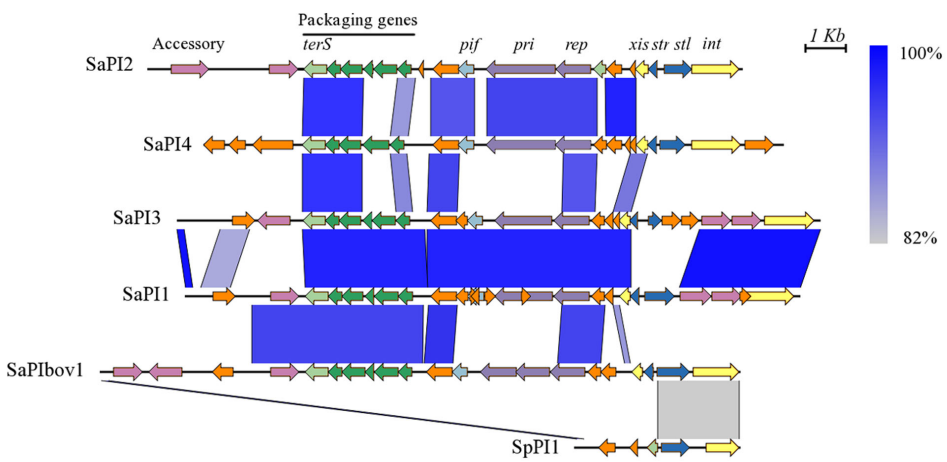

FIGURE 3 | Comparison of SpPI1 with the major staphylococcal pathogenicity islands. The pathogenicity island SpPI1 from *S. pseudintermedius* ED99 is compared to SaPI2 from *S. aureus* RN3984, SaPI4 from *S. aureus* MRSA252, SaPI3 from *S. aureus* COL, SaPI1 from *S. aureus* RN4282, and SaPIbov1 from *S. aureus* RF122. The similarity between regions is indicated by a spectrum of blue to gray, from 100 to 82% similarity. Genes are colored according to their sequence and function, as described previously (Novick et al., 2010): *int* (integrase) and *xis* (excisionase) in yellow; transcription regulators in dark blue; replication genes including the primase gene, *pri*, and the replication initiator gene, *rep* in purple; encapsidation genes in green; terminase small subunit gene (*terS*) in light green; superantigen and other accessory genes in pink; *pif* (related to phage interference) in light blue; and genes encoding hypothetical proteins in orange. Figure produced with EasyFig (Sullivan et al., 2011).

(Barrangou et al., 2007) and prevent conjugative transfer of plasmids in *S. epidermidis* (Marraffini and Sontheimer, 2008). CRISPR are widespread and have been identified in ∼40 and 90% of bacterial and archeal genomes sequenced (Grissa et al., 2007b). Occurrences of CRISPR are rare in staphylococcal species and are so far represented by the Mtube subtype (Haft et al., 2005) in the genome of *S. epidermidis* RP62A (Marraffini and Sontheimer, 2008), *S. lugdunensis* HKU09-01 (Tse et al., 2010) and on a novel SCC*mec*V element carried by livestock-associated methicillin-resistant *S. aureus* (Golding et al., 2010). Both *S. pseudintermedius* and *S. intermedius* genomes harbor a CRISPR locus of the Nmeni subtype, exclusively associated with vertebrate pathogens and commensals (Gill et al., 2005), and for which the closest homologs identified were found in *Streptococcus* M1 GAS and *Ruminococcus lactaris* ATCC 29176 (**Figure 4**). The identity between *S. pseudintermedius* and *S. intermedius* CRISPR-associated genes ranged from 78.7 to 86.7% while the 36-bp-repeat units were almost identical (97%), suggesting that the locus may have been acquired before speciation by a common ancestor to the SIG and then lost by *S. delphini*, although independent acquisition by both species cannot be ruled out. The repeat array of the *S. pseudintermedius* CRISPR locus contains 22 different spacers, with a size ranging from 29 to 31 bp, for which 6 share similarity with sequences of prophages and plasmids such as Φ2638A, pMG1, and pH308197 associated with staphylococcal, enterococcal, and bacillus species, respectively. Of note, eight additional spacers were found to share similarity with prophage-related regions from the *S. intermedius* genome. In addition, a CRISPR locus of the Mtube subtype was also identified in the genome of *S. intermedius*, for which the closest homologs were the CRISPR loci found in *S. epidermidis* RP62A and *S. lugdunensis* HKU09-01 (**Figure 4**). The presence of CRISPR loci in *S. pseudintermedius* and *S. intermedius* correlates with the absence of plasmids, and prophages as previously described (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). However, the role of CRISPRs in immunity to non-phage genomic islands is unclear, as illustrated by the presence of several recently acquired transposons in the *S. pseudintermedius* ED99 genome. Furthermore, other mechanisms such as restriction–modification can limit the transfer of mobile genetic elements (MGE) between bacteria (Kobayashi, 2001). Although, we did not mention it specifically in the paper,

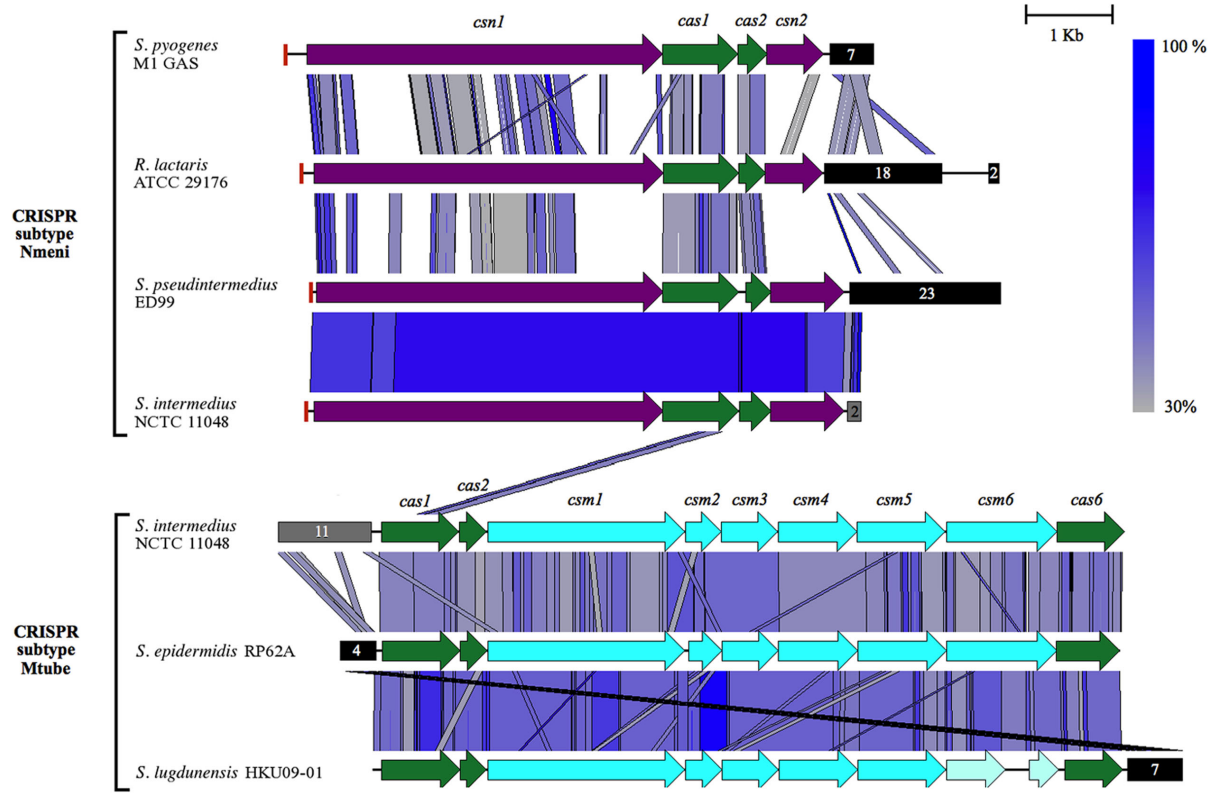

FIGURE 4 | Comparison of the CRISPR loci of *S. pseudintermedius* ED99 and *S. intermedius* NCTC 11048 with related Nmeni and Mtube types. The CRISPR locus subtype Nmeni of *S. pseudintermedius* ED99 and *S. intermedius* NCTC 11048 are compared to those of *Streptococcus* M1 GAS and *Ruminococcus lactaris* ATCC 29176. The CRISPR locus subtype Mtube of *S. intermedius* NCTC 11048 is compared to those of *S. epidermidis* RP62A and *S. lugdunensis* HKU09-01. The similarity between regions is indicated by a spectrum of blue to gray colors depicting a range of 100–30% similarities. Genes of the *csn* family are shown in purple, the *cas* family in green, and the *csm* family in light blue. Of note, the *csm6* gene in *S. lugdunensis* HKU09-01 appears to be a pseudogene. Black boxes represent clusters of direct repeats regions with the number of spacers between the direct repeats indicated. The red box at the beginning of each Nmeni subtype CRISPR locus corresponds to a single copy of the repeats identified in the cluster of downstream direct repeats. The gray boxes indicate the approximate number of direct repeats at the locus in *S. intermedius* NCTC 11048 (due to the unfinished status of the genome). Figure produced with EasyFig (Sullivan et al., 2011).

S. delphini contains several genes related to these functions, which could partly explain the lack of genomic islands in *S. delphini*. In addition, little is known about the bacterial composition of the environmental niche occupied by *S. delphini*. Therefore, hypotheses explaining the lack of genomic islands in *S. delphini* include (i) that less diversity is encountered by this species in its niche, or (ii) most of the other species encountered are distantly related, resulting in less frequent successful horizontal gene transfers.

GENOMIC INSIGHTS INTO THE PATHOGENESIS OF *S. PSEUDINTERMEDIUS* AND SIG INFECTIONS

All SIG species genomes encode a number of predicted toxins including several previously identified such as the enterotoxin SeI-nt (Becker et al., 2001; Hendricks et al., 2002; Futagawa-Saito et al., 2004), hemolysin III, the β-hemolysin (Dziewanowska et al., 1996), the bi-component leukotoxin Luk-I (Prevost et al., 1995) and several exfoliative toxins (Terauchi et al., 2003; Futagawa-Saito et al., 2009; Iyori et al., 2010; **Table 2**). We also identified a putative novel exfoliative toxin specific to *S. pseudintermedius ED99* designated SPETA which has 78 and 76% amino acid identity with the exfoliative toxin A SHETA from *Staphylococcus hyicus* and ETA from *S. aureus*, respectively. The existence of several exfoliative toxin variants made by different *S. pseudintermedius* strains is consistent with the skin tropism of *S. pseudintermedius* and the characteristic skin pathology associated with pyoderma. A wide range of exoenzymes is encoded by all three SIG species, such as the serine protease HtrA, two lipases encoded by the genes *lip* and *geh*, a thermonuclease, a protease ClpX, and a thermolysin (Wladyka et al., 2008). In contrast to other staphylococcal genome sequences, *S. pseudintermedius* ED99 and *S. delphini* 8086 genomes both contain genes encoding a putative sialidase or neuraminidase. The neuraminidase modification of host sugars may contribute to host colonization by providing a carbon source for growth, contributing to biofilm formation, or by enhancing adherence by exposing receptors on the host cell (Sakarya et al., 2010). Overall, the SIG species share a large number of toxins and exoenzymes reflecting their recent common ancestry and possibly their common skin niche.

All three SIG species contained genes encoding proteins with ∼42% identity to the Von Willebrand-binding protein of *S. aureus* which is involved in the formation of abscesses (Cheng et al., 2010). *S. pseudintermedius* ED99 contains a cluster of eight genes encoding predicted glutamyl-endopeptidases which share 56–75% identity with each other, and 29–42% identity with the probable glutamyl-endopeptidase ORF2 encoded by the *etd* pathogenicity island of *S. aureus* TY114 (Yamaguchi et al., 2002). This glutamyl-endopeptidases cluster is also present in *S. intermedius* NCTC 11048 and *S. delphini* 8086 and is not associated with a predicted MGE implying it is part of the core genome of the SIG species.

A total of 18 genes encoding putative cell wall-associated (CWA) proteins, designated SpsA to SpsR, were previously identified in the genome of *S. pseudintermedius* ED99 (Bannoehr et al., 2011a). Of these, nine were also encoded in the *S. intermedius* and *S. delphini* genomes, revealing a considerable number of *S. pseudintermedius*-specific CWA proteins which may be important for its canine host tropism. Recently, it was demonstrated that SpsD and SpsL, mediated binding to several extracellular matrix proteins. Of note, there was enhanced affinity of SpsL for canine in comparison to human fibrinogen implying a role in host-specific interactions (Bannoehr et al., 2011a).

Finally, the control of *S. aureus* virulence gene expression is coordinated by an array of global regulators (Novick, 2003). With the exception of the *S. aureus* regulators (*saeSR*) and the staphylococcal accessory regulators encoded by *sarSTU*, homologs of numerous well-characterized regulators of virulence previously identified in sequenced staphylococcal species were detected in the genomes of the SIG species (**Table 2**).

NICHE ADAPTATION BY THE SIG

Compared to human-specific bacterial species, the SIG species encounter distinct environmental conditions dependent on their host-species with several biophysical parameters influenced, including skin hydration and pH (Montagna, 1967). For instance, in contrast to humans, sweat glands in the skin of animals such as dogs are not involved in thermoregulation (Affolter and Moore, 1994) resulting in lower relative skin hydration (Boelsma et al., 2003; Shimada et al., 2009). In addition, skin pH in dogs has been reported to differ from humans and to vary greatly depending on the anatomical site and breed of dog (Matousek and Campbell, 2002). Furthermore, there are known host-dependent differences in the availability of iron which influence the mechanisms of iron acquisition employed by staphylococci (Pishchany et al., 2010). In relation to these differing environments, the SIG species have a diverse complement of genes encoding proteins potentially involved in osmo-protection and resistance to oxidative stress. For example, *S. pseudintermedius* ED99 has four putative nitroreductase genes, one more than *S. intermedius* NCTC11048 and *S. delphini* 8086. All SIG species harbor several sodium/salt transporters, with the exception of the high-affinity potassium system Kdp which is absent from *S. intermedius* NCTC11048. *S. pseudintermedius* ED99 and *S. delphini* 8086 also contain a second catalase gene known as *katB* which has to date only been identified among staphylococcal species that inhabit high osmotic and oxidative stress niches including, *S. xylosus*, *S. saprophyticus*, and *S. equorum* (Blaiotta et al., 2010). Another source of genetic variation among the SIG species which could be related to their host niche is the transport and metabolism of small molecules such as carbohydrates. *S. pseudintermedius* ED99 contains genes encoding proteins predicted to mediate the transport and utilization of lactose/galactose in contrast to *S. intermedius* NCTC11048 and *S. delphini* 8086 which encode variant sugar transporters likely to have distinct substrates. *S. intermedius* NCTC11048 also encodes a novel ribose ABC transporter while *S. delphini* 8086 harbors two additional PTS systems, predicted to be involved in transport of cellobiose and glucitol/sorbitol, respectively.

In common with other staphylococci, the SIG species have developed several mechanisms for acquisition of iron (**Table 3**). One such mechanism is the production of low-molecular-weight chelating agents called siderophores. For example, the SIG genomes contain the genes *sfaABCD* and *htsABC* involved in the biosynthesis and transport of staphyloferrin A and transport of heme (Hammer and Skaar, 2011). In contrast to other non-*S. aureus* species sequenced, the SIG species can also produce

Table 2 | Distribution of virulence factors identified in eight staphylococcal species.

Product	Gene name	Location in SP	SA	SP	SD	SI	SE	SS	SH	SC
EXOENZYMES										
1-Phosphatidylinositol phosphodiesterase	*plc*	*oriC* environ	+	−	−	−	−	−	−	−
Staphylocoagulase	*coa*		+	+	+	+	−	−	−	−
Triacylglycerol lipase	*lip*		+	+	+	−	+	+	+	+
Lipase	*geh*		+	+	−	−	+	+	−	+
Serine protease	*htrA*		+	+	+	+	+	+	+	+
Cysteine protease	*sspB, C*		+	−	−	−	+	−	−	−
Serine V8 protease	*sspA*		+	−	−	−	+	+	−	−
Glutamyl-endopeptidase			+	+ (8)	+ (13)	+(10)	−	−	−	−
Thermonuclease	*nuc*		+	+ (3)	+ (3)	+ (2)	+	+	+	+
Serine proteases	*spl(s)*		+	−	−	−	−	−	−	−
Staphylokinase	*sak*		+	−	−	−	−	−	−	−
Hyaluronidase	*hysA*		+	−	−	−	−	−	−	−
Zinc metalloproteinase aureolysin	*aur*	*oriC* environ	+	+	+	+	+	+	−	−
Cell wall hydrolase	*lytN*		+	−	−	−	−	−	−	−
Sialidase	*nanB*		−	+	+	−	−	−	−	−
proteases ClpX	*clpX*		+	+	+	+	+	+	+	+
TOXINS										
Exotoxins/superantigen-like proteins	*set(s)*		+	−	−	−	−	−	−	−
α-Hemolysin	*hly*		+d	−	−	−	−	−	−	−
β-Hemolysin	*hlb*		+d	+	+ (2)	+	+	−	−	−
δ-Hemolysin	*hld*		+	+	+	+	+	+	+	+
Hemolysin III			+	+	+	+	+	+	+	+
Leukotoxins	*lukDE*		+d	−	−	−	−	−	−	−
Leukocidins	*lukF, M*		+d	−	−	−	−	−	−	−
	lukF-I, S-I		−	+	+	+	−	−	−	−
Panton–Valentine leukocidin	*lukS, F-PV*		+d	−	−	−	−	−	−	−
Toxic shock syndrome toxin 1	*tst*		+d	−	−	−	−	−	−	−
γ-Hemolysin components	*hlgA, B, C*		+	−	−	−	−	−	−	−
Enterotoxins	*SE(s)*		+	*se-int*	+	+	−	−	−	−
Exfoliative toxins	*eta, etb*	Upstream SpPI1	+	*speta*	−	−	−	−	−	−
			−	*siet*	+	+	−	+	−	−
ADHESINS										
Extracellular matrix binding proteins	*ebhA, B*		+	−	−	−	+	−	−	−
Elastin-binding protein	*ebpS*		+	+	+	+	+	+	+	+
Fibronectin-binding proteins	*fnbA, B*		+	−	−	−	−	−	−	−
Intercellular adhesion proteins	*icaABCD*		+	+	+	+	+	−	−	−
Collagen adhesin precursor	*cna*		+	−	−	−	−	−	−	−
Clumping factors	*clfA, B*		+	−	−	−	−	−	−	−
Ser-Asp rich proteins	*sdr*		+	−	−	−	+	+	+	−
Iron-responsive surface determinant	*isdA-G*		+	−	−	−	−	−	−	−
Other putative cell surface proteins identified in *S. pseudintermedius* ED99										
	spsA	*oriC* environ	−	+	+	+	−	−	−	−
	spsB		−	+	+	+	−	−	−	−
	spsC		+	+	+	+	+	+	+	+
	spsD		−	+	−	−	−	−	−	−
	spsE		+	+	+	+	+	+	+	+
	spsF	*oriC* environ	−	+	−	−	−	−	+	−
	spsG	*oriC* environ	−	+	+	−	−	−	−	−
	spsH		−	+	+	+	−	−	−	−

(Continued)

Table 2 | Continued

Product	Gene name	Location in SP	SA	SP	SD	SI	SE	SS	SH	SC
	spsI		−	+	−	−	−	−	−	−
	spsJ	*oriC* environ	+	+	−	−	−	−	−	−
	spsL		−	+	−	−	−	−	−	−
	spsM		−	+	−	−	−	−	−	−
	spsN		−	+	+	+	−	−	−	−
	spsO		−	+	−	−	−	+	−	−
	spsR		−	+	+	+	−	−	−	−
OTHERS										
Immunoglobulin G (IgG)-binding protein A	*spa*	*oriC* environ	+	*spsP*	−	−	−	−	−	−
		oriC environ	−	*spsQ*	−	+	−	−	−	−
Capsular polysaccharide synthesis proteins	*capA-G*		+	−	−	−	−	+	+	−
Lipoproteins	*lpl(s)*		+	−	−	−	−	−	−	−
IgG-binding protein SBI	*sbi*	*oriC* environ	+	*spsK*	+	+	−	−	−	−
TWO-COMPONENT REGULATORY SYSTEMS										
Accessory gene regulator	*agrA, B, C, D*		+	+	+	+	+	+	+	+
S. aureus exoprotein expression regulator	*saeS, R*	*oriC* environ	+	+	+	+	+	−	−	−
Staphylococcal respiratory response protein	*srrA, B*		+	+	+	+	+	+	+	+
Autolysis-related locus	*arlS, R*		+	+	+	+	+	+	+	+
	lytS, R	*oriC* environ	+	+	+	+	+	+	+	+
SarA PROTEIN FAMILY										
Staphylococcal accessory regulator A	*sarA*		+	+	+	+	+	+	+	+
Staphylococcal accessory regulator R	*sarR*		+	+	+	+	+	+	+	+
Staphylococcal accessory regulator Z	*sarZ*	*oriC* environ	+	+	+	+	+	+	+	+
Staphylococcal accessory regulator T, U	*sarT, U*		+	−	−	−	−	−	−	−
Repressor of toxins	*rot*		+	+	+	+	+	+	+	−

SP, S. pseudintermedius ED99; SD, S. delphini 8086; SI, S. intermedius NCTC11048; SA, S. aureus Mu50; SE, S. epidermidis RP62A; SH, S. haemolyticus JCSC1435; SS, S. saprophyticus ATCC15305; SC, S. carnosus TM300. Number of copies is indicated in parentheses. d: Strain-dependent.

and transport staphylobactin A mediated by the operons *sbnA-I* and *sirABC*. Uptake of ferrous iron is also enabled through the presence of the FeoAB system, which is not shared with *S. saprophyticus* ATCC15305 or *S. haemolyticus* JCSC1435. Further differences with other staphylococcal species include the absence of the siderophore–Fe transport system SstABC, the lipoprotein receptor for Fe^{3+} (FhuD) and the hemoglobin–Fe transport system IsdA-H, found in *S. aureus*. In addition, variation among the three SIG species were observed with the absence of the ferrous iron transporter EfeUOB in *S. pseudintermedius* ED99 and the presence of an inactivating mutation in the iron–manganese *mntABC* operon in *S. delphini* 8086. Overall, in common with other staphylococci, the SIG species have an extensive array of systems for acquiring iron from the host, which may be important in the context of the skin environment where iron sources are particularly limited.

EVOLUTION OF ANTIMICROBIAL RESISTANCE IN *S. PSEUDINTERMEDIUS*

In common with *S. aureus* and *S. haemolyticus*, multi-resistance in *S. pseudintermedius* strains is frequent and includes resistance to tetracycline (Schwarz et al., 1998), macrolides, lincosamides and streptogramins (Eady et al., 1993; Boerlin et al., 2001), aminoglycosides and aminocyclitols (Noble et al., 1996; Boerlin et al., 2001), fluoroquinolones (Intorre et al., 2007), and methicillin (Piriz et al., 1995; Kania et al., 2004; Sasaki et al., 2007a). We determined that *S. pseudintermedius* ED99 is resistant to ampicillin, erythromycin, tetracycline, and trimethoprim whereas *S. delphini* 8086 and *S. intermedius* NCTC11048 are sensitive to all antibiotics cited above (data not shown). Four transposons containing one or more antibiotic resistance genes were identified in the genome of *S. pseudintermedius* ED99 (**Table 4**). Of these, 2 (Tn*552* and Tn*554*-like) found also in *S. haemolyticus* JCSC1435, and in *S. epidermidis* and *S. aureus* strains respectively, encode the *bla* operon, which confers β-lactam resistance. Tn*5405* encodes the aminoglycoside–streptothricin resistance genes *aad6–sat4–aphA-3* and is associated with the macrolide–lincosamide–streptogramin resistance gene *ermB* previously described in *Enterococcus faecium* (Werner et al., 2003) and *S. intermedius* (Boerlin et al., 2001), and the putative conjugative transposon Tn*5801* (25.8 kb) encodes the *tetM* gene responsible for resistance to tetracycline. Tn*5801* was previously identified in *S. aureus* Mu50 and is related to the conjugative transposon Tn*916* of *Enterococcus faecalis* (Flannagan et al., 1994). The existence of transposons which are nearly identical to those found in human-associated staphylococcal species indicates a recent inter-species horizontal transfer of antibiotic resistance. In contrast to the closely related species *S. delphini* and *S. intermedius*, which have limited clinical importance,

Table 3 | Distribution of iron transport related genes identified in eight staphylococcal species.

Product	Gene names	SA	SP	SD	SI	SE	SS	SH	SC
Siderophore staphylobactin production	*sbnABCDEFGHI*	+	+	+	+	−	−	−	−
Siderophore ABC transporter	*sirABC*	+	+	+	+	−	−	−	−
Siderophore production (staphyloferrin A)	*sfaABCD*	+	+	+	+	+	+	+	+
Siderophore ABC transporter (staphyloferrin A)	*htsABC*	+	+	+	+	+	+	+	+
Transcriptional repressor of iron uptake	*fur*	+	+	+	+	+	+	+	+
Iron-regulated ABC transporter (siderophore?)	*sstABCD*	+	−	−	−	+	+	+	+
Ferrichrome ABC transporter	*fhuCBG*	+	+	+	+	−	+	+	+
Lipoprotein receptor for Fe^{3+}	*fhuD*	+	−	−	−	−	+	+	+
Iron-responsive surface determinant (iron uptake)	*isdA-G, srtB*	+	−	−	−	−	−	−	−
Iron–manganese ABC transporter	*mntABC (sitABC)*[c]	+	+	−[a]	+	+	+	−[b]	+
Heme-regulated ABC transporter (heme detoxification)	*hrtAB*	+	+	+	+	+	+	+	+
Ferrous iron transporter	*efeUOB*	+	−	+	+	−	−	+	+
Ferrous iron uptake homolog	*feoAB*	+	+	+	+	+	−	−	+

SP, S. pseudintermedius ED99; SD, S. delphini 8086; SI, S. intermedius NCTC11048; SA, S. aureus Mu50; SE, S. epidermidis RP62A; SH, S. haemolyticus JCSC1435; SS, S. saprophyticus ATCC15305; SC, S. carnosus TM300.

[a] sitA is a pseudogene in S. delphini. [b] sitABC regulator sitR is missing in S. haemolyticus. [c] in S. epidermidis.

Table 4 | Mobile genetic elements associated with antibiotic resistance identified in *S. pseudintermedius* ED99.

Name	Resistance factor(s) encoded	Closest homologs
*Tn*5801	*tetM*	*S. aureus*
*Tn*552	*bla* operon	*S. haemolyticus, S. epidermidis*
*Tn*554-like	*bla* operon	*S. aureus*
*Tn*5405	*aad6–sat4–aphA-3, ermB*	*Streptococcus* sp., *Enterococcus faecium*

S. pseudintermedius encounters considerable antibiotic selective pressures, which have contributed to the spread of MGE encoding antibiotic resistance. Since the 1990s, methicillin-resistant *S. pseudintermedius* strains have emerged through several independent SCCmec acquisition events and disseminated widely (Piriz et al., 1995; Kania et al., 2004; Bannhoer et al., 2007; Sasaki et al., 2007a). Importantly, *S. pseudintermedius* strains containing MGE encoding antibiotic resistance could represent a reservoir for the spread of resistance genes to human commensal skin flora (Guardabassi et al., 2004a,b).

CONCLUSION

The spread of antibiotic-resistant strains of *S. pseudintermedius* and the lack of an effective vaccine means that alternative approaches for controlling canine pyoderma are required. The identification of novel virulence determinants in the genome of *S. pseudintermedius* has provided candidate new targets for therapeutic interventions. In particular, the identification of CWA-associated proteins and toxins which contribute to the pathology associated with pyoderma infection should provide the impetus for detailed characterization of these critical host–pathogen interactions and for investigations into their potential as vaccine components. Furthermore, the discovery that transposons are largely responsible for the multi-resistant phenotype of *S. pseudintermedius* provides important insights into the evolution of antibiotic resistance within the species. Future genome sequencing projects should include representatives of the widespread methicillin-resistant *S. pseudintermedius* clones (Bannoehr et al., 2007). Furthermore, comparative genomic analysis also revealed the presence of CRISPR loci, and an unusually high %GC content of the SIG species, which provides intriguing avenues for basic research into bacterial genome evolution. Finally, several additional species-specific features which likely reflect the distinct ecological niches occupied, were identified such as genes involved in carbohydrate metabolism iron acquisition and resistance to oxidative stress. The identification of the genetic events which led to differentiation of the SIG species and the determinants which correlate with their distinct host-tropisms provide avenues for fundamental studies into the molecular basis of bacterial host-adaptation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cellular_and_Infection_Microbiology/10.3389/fcimb.2012.00044/abstract

Table S1 | Genes specific to *S. pseudintermedius* ED99.

Table S2 | Core genes identified in *S. pseudintermedius* ED99.

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APPENDIX

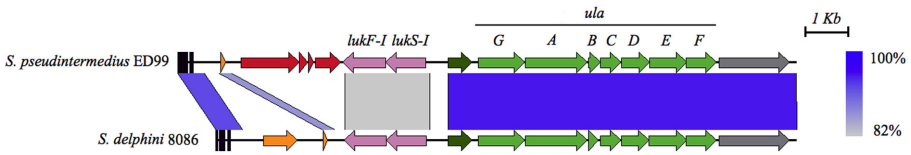

FIGURE A1 | Comparison of the genomic island containing the bi-component leukotoxin Luk-I and the L-ascorbate transport and utilization operon in *S. pseudintermedius* ED99 and *S. delphini* 8086. The similarity between regions is indicated by a spectrum of blue to gray, ranging from 100 to 82% similarity. Genes are colored according to their sequence and function as follow: phage-related genes in red; leukotoxin genes in pink; putative phosphoglucomutase in dark green; L-ascorbate transport and utilization genes in green; putative transcriptional regulator in gray; *tRNA* genes are represented by black boxes; and genes encoding hypothetical proteins in orange.