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Novel insights into the role of the mobilome in ecological diversification and success of *Staphylococcus haemolyticus* as an opportunistic pathogen

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Abstract

Staphylococcus haemolyticus is a species of coagulase-negative staphylococci that has primarily been studied as a human skin microbiome member and an emerging nosocomial pathogen. Here, we present the first complete genome of *S. haemolyticus* strains SE3.9, SE3.8 and SE2.14 reported as an endophyte of rice seed. Detailed investigation of the genome dynamics of strains from diverse origins revealed an expanded genome size in clinical isolates, and a role of many insertion sequence (IS) elements in strain diversification. Interestingly, several of the IS elements are also unique or enriched in a particular habitat. Comparative studies also revealed the potential movement of mobile elements from rice endophytic *S. haemolyticus* to strains from other pathogenic species such as *Staphylococcus aureus*. The study highlights the importance of ecological studies in the systematic understanding of genome plasticity and management of medically important *Staphylococcus* species.

DATA SUMMARY

All the sequencing data have been deposited in GenBank under BioProject ID nos. PRJNA263225, PRJNA263226 and PRJNA263227. All the supporting data have been provided through supplementary data files.

INTRODUCTION

Staphylococcus haemolyticus is a gram-positive, coagulase-negative bacterium inhabiting human skin and mucous as a commensal organism. It occurs mainly associated with bloodstream infection and medical device-associated infection [1]. After *Staphylococcus epidermidis*, it is the most frequent isolate from human bloodstream infections, particularly from sepsis [2–4]. Recent studies have shown that 75% of analysed *S. haemolyticus* isolates are multidrug-resistant [5]. Its ability to form biofilms [6, 7], to produce phenol-soluble modulins [8] and the presence of a large number of insertion sequences leading to frequent genomic rearrangements [9] have been proposed as factors contributing to its multidrug resistance phenotype. Additionally, it contributes to the transfer of resistant genes to virulent pathogen *Staphylococcus aureus*, leading to the emergence of extremely rampant clones of *S. aureus* [10–12].

Apart from inhabiting humans, *S. haemolyticus* is also known to be associated with a multitude of hosts such as plants and the environment. In a previous study, we provided insights into genome-based taxonomy and evolution of *S. haemolyticus* adapted

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Abbreviations: ANI, average nucleotide identity; CDS, coding sequence; COG, Clusters of Orthologous Groups; CRISPRs, clustered regularly interspaced short palindromic repeats; GI, genomic island; IS, insertion sequence; LCB, local collinear block; MFS, major facilitator superfamily; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system.
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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. One supplementary table is available with the online version of this article.

Impact Statement

S. haemolyticus is a commensal and inhabitant of diverse hosts such as humans, animals and plants. As plants are not mobile, bacteria associated with plants face a variety of intense biotic and abiotic stresses. Our systematic, complete genome-based study revealed that ecologically diverse strains can act as gene reservoirs for stress-resistant genes and can transfer them to other *Staphylococcus* species such as *S. aureus*. Plasmids are known to evolve at a faster rate and can also change the selective effect of chromosomal mutations through epistatic interactions. Apart from mobile elements, our study indicates the role of several plasmids in a plant isolate in adaptation as well as evolution of ecologically diverse *S. haemolyticus* strains and in other medically important *Staphylococcus* species.

to different habitats, particularly as a rice endophyte [13]. As the study was based on draft genome sequences, it is challenging to study repetitive elements like insertion sequence (IS) elements and other mobile elements such as plasmids integrons, CRISPRs, conjugative transposons, and phages. With the advent of third-generation sequencing, it is possible to obtain complete genome sequences to understand intragenomic heterogeneity and inter-strain variations in an effective manner [14]. In the present study, we provide evidence for genome expansion and the role of specific IS elements along with plasmids in the ecological diversification of *S. haemolyticus*. Interestingly, plasmids from the rice isolate harbour toxin genes, antibiotic resistance genes, lantibiotic genes, insertion elements, and metal resistance genes. Comparative genomic analysis revealed the distinct difference in genome size and role of mobile elements in diversification of *S. haemolyticus* with diverse lifestyles. We also found evidence of the role of plasmids in acquisition of novel genes and also transfer to medically important species such as *S. aureus*. The study highlights the importance of ecological genomics studies in *S. haemolyticus* with particular emphasis on the mobilome of rice or plant isolates.

METHODS

DNA isolation and genome sequencing

The strains were grown in nutrient broth media at 28 °C for 24 h. Genomic DNA was isolated using a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The quantity and quality of DNA were assessed using a Nanodrop and Qubit 2.0 fluorometrics. For Nanopore sequencing, 3.5 μg of initial DNA was used for DNA end prep using NEBNext Ultra II End repair/dA-Tailing modules (NEB). The library was prepared using Ligation Sequencing Kit 1 D (SQK-LSK109) and Native Barcoding Kit 1 D (EXP-NBD104). The barcode and adapter ligation steps were performed using the NEB ligase master mix module and the NEB T4 DNA ligase module. All bead washing steps in the protocol were performed using AMPure beads (Beckman Coulter). Finally, 12 μl of prepared DNA library was loaded onto the flow cell according to the manufacturer's instructions and sequenced using a MinION (FLO-MIN-106 version R9.4) flow cell using MinKNOW software (http://community.nanoporetech.com) (Oxford Nanopore Technologies) (v.1.13.1) for 48 h. Raw reads were base called using Guppy v3.4.3 software (http://community.nanoporetech.com).

Genome assembly and annotation

Hybrid genome assembly was first performed using Unicycler v0.4.8-beta [15] with ONT long reads with bold mode. The complete genome obtained using ONT reads was then polished for multiple rounds of Pilon v1.22 [16] using Illumina raw reads [13]. The genomes were assembled in a closed circular single chromosome. Assembly quality in terms of completeness and contamination was assessed using CheckM v1.0.13 [17]. Genome coverage was calculated using BBMap v38.42 [18]. Annotation was performed using the National Center for Biotechnology Information-Prokaryotic Genome Annotation Pipeline (NCBI-PGAP) [19].

Genome comparison and identification of mobile elements

Genome comparison between strains SE3.9, SE3.8, SE2.14, S167, SGAir0252, ATCC 29970^T and JCSC1435 was performed using the progressive Mauve tool with default parameters [20]. The pictorial representation of plasmids of SE3.9 was drawn using DNA plotter [21]. IS elements were identified using the ISsaga tool [22]. Circular genome representation of SE3.9 was done using the CGview comparison tool [23]. Genomic islands of SE3.9 were predicted using the Islandviewer 4 tool [24] with atypical GC (%) content of <29% and >35% in reference to GC content of the *S. haemolyticus* genome (32%).

Data deposition

The projects have been deposited at the National Center for Biotechnology Information (NCBI) under accession numbers CP049091–CP049096, CP084229–CP084234 and CP084235–CP084240.

Table 1. Genomic features of *S. haemolyticus* strains SE3.9, SE3.8 and SE2.14

	SE3.9	SE3.8	SE2.14
Size (bp)			
Chromosome	2323296	2323407	2323230
Plasmid1	25444	25444	25444
Plasmid2	21031	21031	21031
Plasmid3	18550	18550	18550
Plasmid4	4101	4101	4101
Plasmid5	3674	3674	3685
Genome coverage	57×	46×	52×
CDS	2191	2279	2283
tRNA	59	61	58
IS elements	15	14	15
Completeness	99.62	99.62	99.62
Contamination	0.71	0.71	0.71
NCBI accession numbers	CP049091–CP049096	CP084229–CP084234	CP084235–CP084240

RESULTS AND DISCUSSION

Complete genome sequencing of rice endophytic strains SE3.9, SE3.8, and SE2.14 were performed using Oxford Nanopore MinION (see Methods). Strains were taxonomically classified as *S. haemolyticus* by the average nucleotide identity (ANI) method and showed an identity range of 96.78–97.02% with the available reference genome of the type strain ATCC 29970^T. The GC content for the chromosome was 32.8% and for plasmids was 29.5, 26.2, 30.0, 33.2, and 30.4% respectively. The genomic features of strains are detailed in Table 1 and a circular genome representation for strain SE3.9 is given in Fig. 1a). The protein-coding genes of SE3.9 were classified into 21 Clusters of Orthologous Groups (COGs). COG categories were assigned to a total of 1768 coding sequences (CDS), of which class E (amino acid metabolism and transport) constitute maximum proportion, i.e., 8.96% (174 out of 1,941), followed by class J (translation, ribosomal structure and biogenesis) with 7.93% (Fig. 1a, Table 2).

We carried out comparative genome analysis using Progressive Mauve to understand the genome dynamics of *S. haemolyticus* variants. The high degree of synteny among genomes indicated the conservation of arrangements of local collinear blocks (LCBs) (Fig. 2a). LCBs represent homologous backbone sequences, and differences in locations of LCBs indicate genomic rearrangements such as inversions and translocations. Additionally, there is a considerable expansion in genome size from rice to clinical isolates by 361719 bp. In this context, the major driving force in bacterial evolution and adaptation is the horizontal acquisition of accessory genes through mobile genetic elements such as plasmids, genomic islands (GIs), and IS elements. GIs are discrete segments of DNA, varying from 10 to 100 kb in size. Based on the type of accessory genes they carry, GIs are classified as pathogenicity, resistance, metabolic and symbiosis islands [25]. The Islandviewer4 tool predicted three GIs in rice strain SE3.9 (Fig. 3, Table S1, available in the online version of this article). GI 1 (10kb) is an acquired resistance island with beta-lactamase genes providing potential resistance to antibiotics having beta- lactam rings such as ampicillin and penicillin. GI 2 (9kb) has virulence genes such as pathogenicity islands and terminase small subunit which has a role in phage packaging. GI 3 (14.7kb) is basically a metabolic island with genes such as PTS galactitol transporter, type 1 glutamine amidotransferase, LacI family transcriptional regulator, and 3-hexulose-6-phosphate synthase involved in carbohydrate metabolism and the pentose phosphate pathway. Overall, new genes acquired by horizontal transfer are largely for adaptation to a plant.

To understand the role of IS elements in genome plasticity, we studied IS element content among diverse *S. haemolyticus* strains. As shown in Fig. 2b, there is variation in the number and type of IS elements in *S. haemolyticus* isolates of diverse origins. About 14 or 15 IS elements were observed for rice strains SE3.9, SE3.8 and SE2.14 classified into six families; a total of 65 IS elements were found for strain S167 isolated from a leafy vegetable, classified into six families, a total of 73 IS elements were found for environmental strain SGAir0252 classified into six families; a total of 41 IS elements were found for commensal strain ATCC 29970^T, classified into four families; and a total of 99 elements were found for clinical strain JCSC1435, classified into six families. Three IS groups (ISL3, ISNCY, IS1182) comprised 80% of the IS elements. ISL3-SH of *S. haemolyticus* exhibited 94.67% nucleotide identity to ISL3-SA of *S. aureus* and 94.58% identity to ISL3-SE of *S. epidermidis*. The IS30 and IS3_ssgr_IS150 families are exclusively present in strains isolated from rice seeds. They are associated with MFS transporter and toxic anion resistance genes potentially

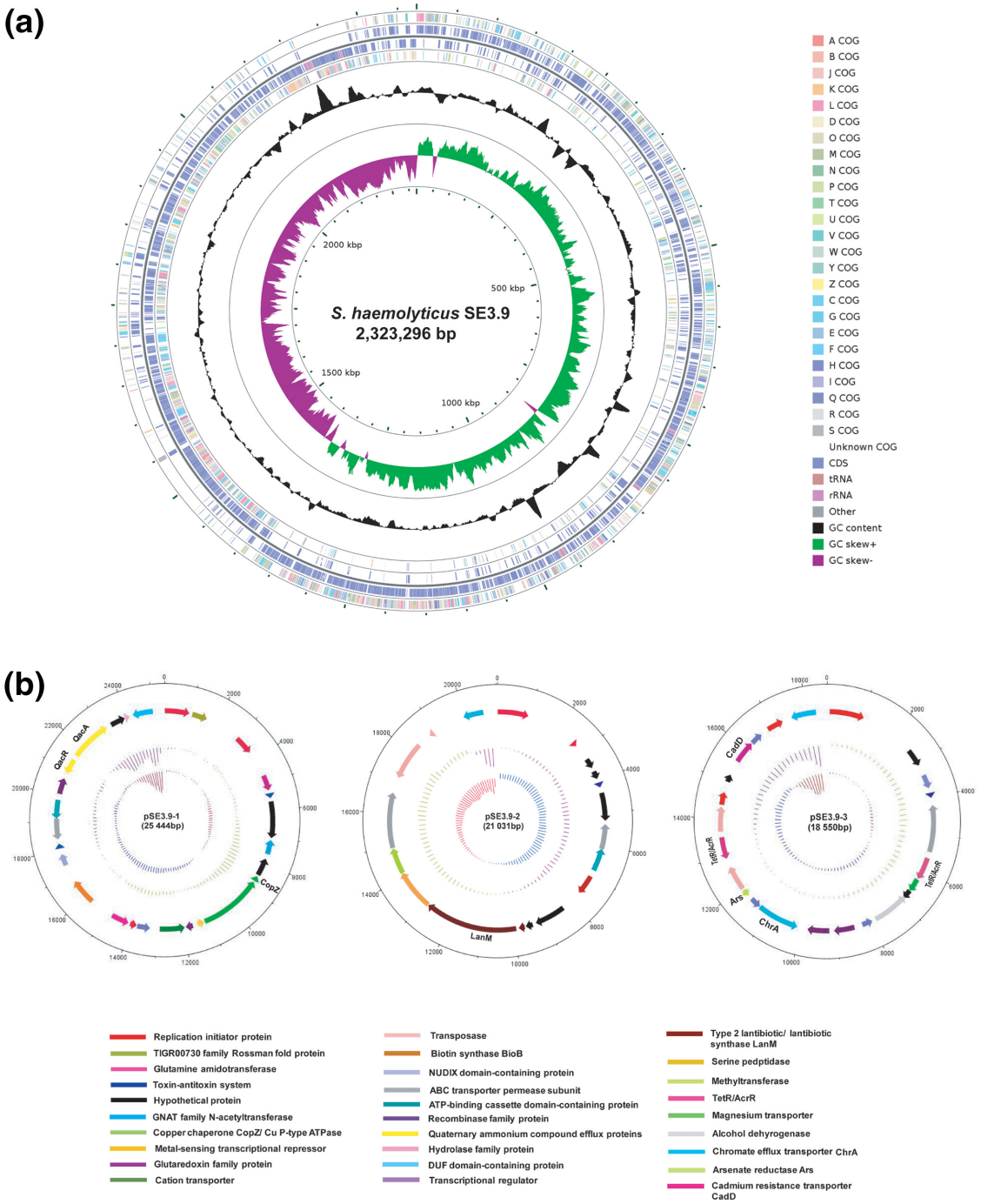

Fig. 1. (a) Chromosomal map of the *S. haemolyticus* SE3.9 chromosome. From outer to inner: the first circle represents forward strand genes, coloured according to COG classification; the second and third circles represent CDS and non-coding RNAs on the forward and reverse strand, respectively; the fourth circle represents reverse strand genes, coloured according to COG classification. The inner area represents the GC content, GC skew, and genome size. (b) Circular map of plasmids showing pSE3.9-1, pSE3.9-2 and pSE3.9-3 of *S. haemolyticus* strain SE3.9 with antibiotic, antimicrobial and metal resistance genes highlighted.

related to stress tolerance in rice seeds. The IS6 family is associated with resistance genes such as tetracycline resistance, arsenate reductase, quaternary ammonium compound efflux (*qacA*), and magnesium and cadmium transporter genes. They are known to disseminate antibiotic resistance genes and are present in plant, environmental, and clinical isolates. IS200_IS605_ssgr_IS200 is present in all isolates studied except those from rice seed. It is an ancestral IS of bacteria inhabiting a human host [26, 27]. IS256 is present in a large number in clinical strains and is a well-known molecular marker for clinical *Staphylococcus epidermidis*

Table 2. Clusters of orthologous groups of *S. haemolyticus* SE3.9

COG classes	Description	Gene count	Percentage
B	Chromatin structure and dynamics	1	0.05%
C	Energy production and conversion	111	5.71%
D	Cell cycle control, cell division, chromosome partitioning	21	1.08%
E	Amino acid transport and metabolism	174	8.96%
F	Nucleotide transport and metabolism	69	3.55%
G	Carbohydrate transport and metabolism	118	6.07%
H	Coenzyme transport and metabolism	100	5.15%
I	Lipid transport and metabolism	54	2.78%
J	Translation, ribosomal structure and biogenesis	154	7.93%
K	Transcription	135	6.95%
L	Replication, recombination and repair	102	5.25%
M	Cell wall/membrane/envelope biogenesis	105	5.40%
N	Cell motility	7	0.36%
O	Post-translational modification, protein turnover and chaperones	64	3.29%
P	Inorganic ion transport and metabolism	125	6.43%
Q	Secondary metabolites biosynthesis, transport and catabolism	28	1.44%
R	General function prediction	257	13.24%
S	Function unknown	201	10.35%
T	Signal transduction mechanisms	57	2.93%
U	Intracellular trafficking, secretion and vesicular transport	28	1.44%
V	Defence mechanisms	30	1.54%

[28]. The presence of many ISs in clinical strain JCSC1435 is probably the reason for the frequent genome rearrangements in *S. haemolyticus* owing to its multidrug resistance.

Plasmids contribute to bacterial ecology and evolution by mobilization of accessory genes through horizontal gene transfer [29]. The genomes of *S. haemolyticus* strains completed in this study consist of five plasmids (Fig. 1b). Since all three genomes from rice have plasmids of similar size, we discuss the plasmids of strain SE3.9 in detail. Of these, four plasmids encode genes helping adapt to survive in harsh and stressful conditions. pSE3.9-1 harbours the gene *qacA* encoding a multidrug efflux pump reported to export toxic molecules such as quaternary ammonium compounds, some antibiotics and disinfecting agents [30, 31]. pSE3.9-1 also contains copper resistance genes, and hence there is a potential for transferring copper resistance genes to human isolates of clinical origin. Copper-containing compounds such as the Bordeaux mixture are widely used antibacterial agents to control plant diseases such as bacterial leaf blight in rice plants [32, 33]. Copper is also a promising antibacterial agent to control hospital pathogens including *S. haemolyticus* and *S. aureus*. Hence there is the worrying possibility of plant isolates being a source of copper resistance in clinical isolates. The presence of alleles of copper and other stress tolerance genes on plasmids provides further scope for diversification and selection. pSE3.9-2 encodes LanM type lantibiotic, which provides immunity against other pathogens by disrupting their membrane integrity. pSE3.9-3 encodes metal resistance genes, including cadmium, arsenic, magnesium and chromium, to overcome the metal resistance in the environment. Additionally, it contains antibiotic resistance genes for tetracycline. pSE3.9-4 mainly contains hypothetical genes. Interestingly, BLASTN analysis revealed pSE3.9-5 has 100% nucleotide identity and 85% query coverage to *S. aureus* strain USA300-SUR24 plasmid pUSA07-1-SUR24 and plasmid pUSA07-1-SUR15, isolated from a hospital outbreak [34] (Fig. 4). This result indicates the movement of plasmids across *Staphylococcus* species.

CONCLUSION

Mobile and repetitive elements are known to drive the evolution of niche-specific bacteria through horizontal gene transfer events. The complete genome sequence of an ecological variant allowed us to gain insights into distinct evolutionary routes and mobile

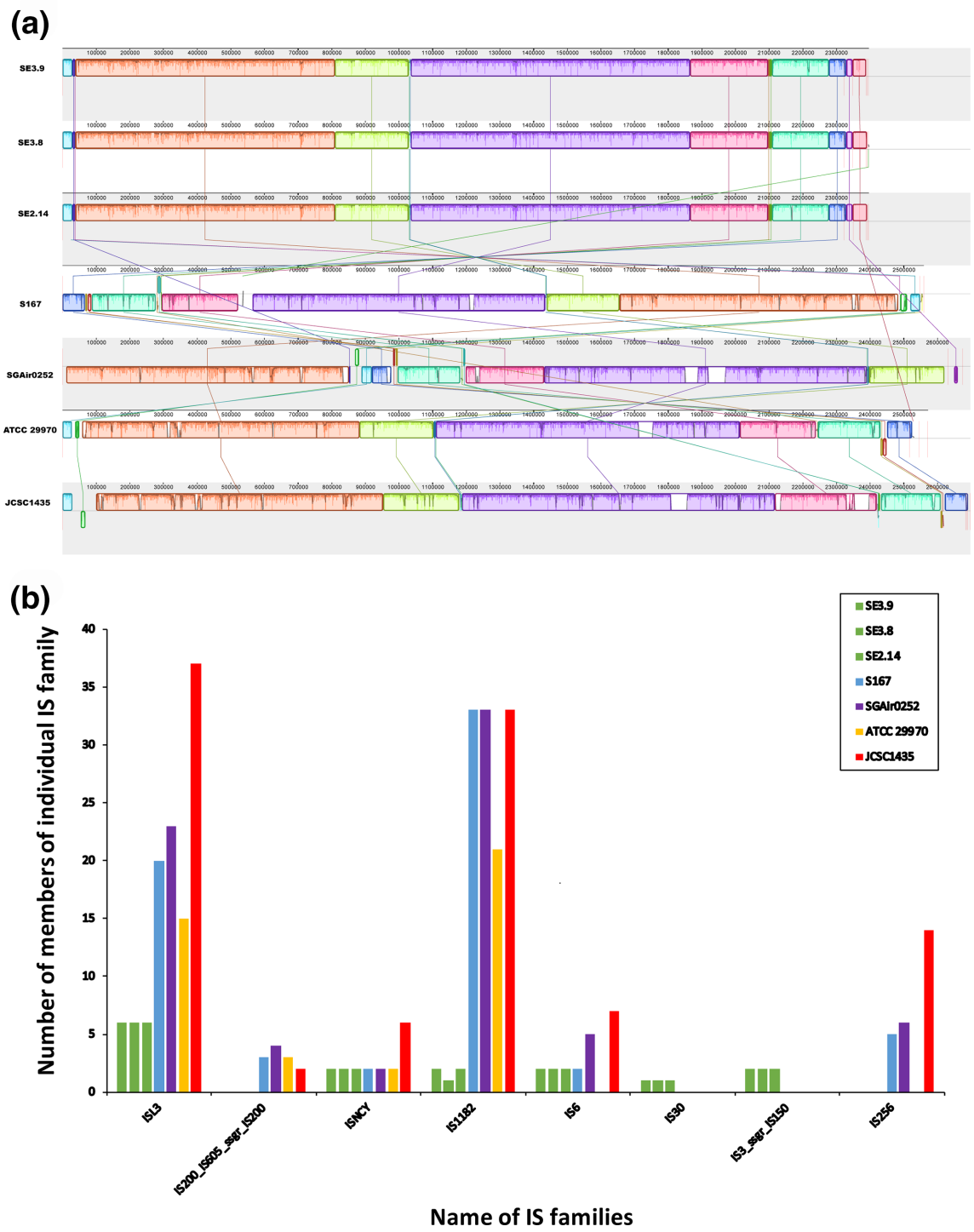

Fig. 2. (a) Complete genome alignment of strains SE3.9, SE3.8, SE2.14, S167, SGAir0252, ATCC 29970^T and JCSC1435. The scale represents coordinates of each genome. Different colour blocks represent LCBs, which are conserved segments in the genomes. (b) Distribution of IS elements into different IS families in the genomes of *S. haemolyticus* strains of diverse origin. The green colour bar represents strains SE3.9, SE3.8 and SE2.14; blue colour represents strain S167; purple colour represents strain SGAir0252; yellow colour represents strain ATCC 29970^T; and red colour represents strain JCSC1435.

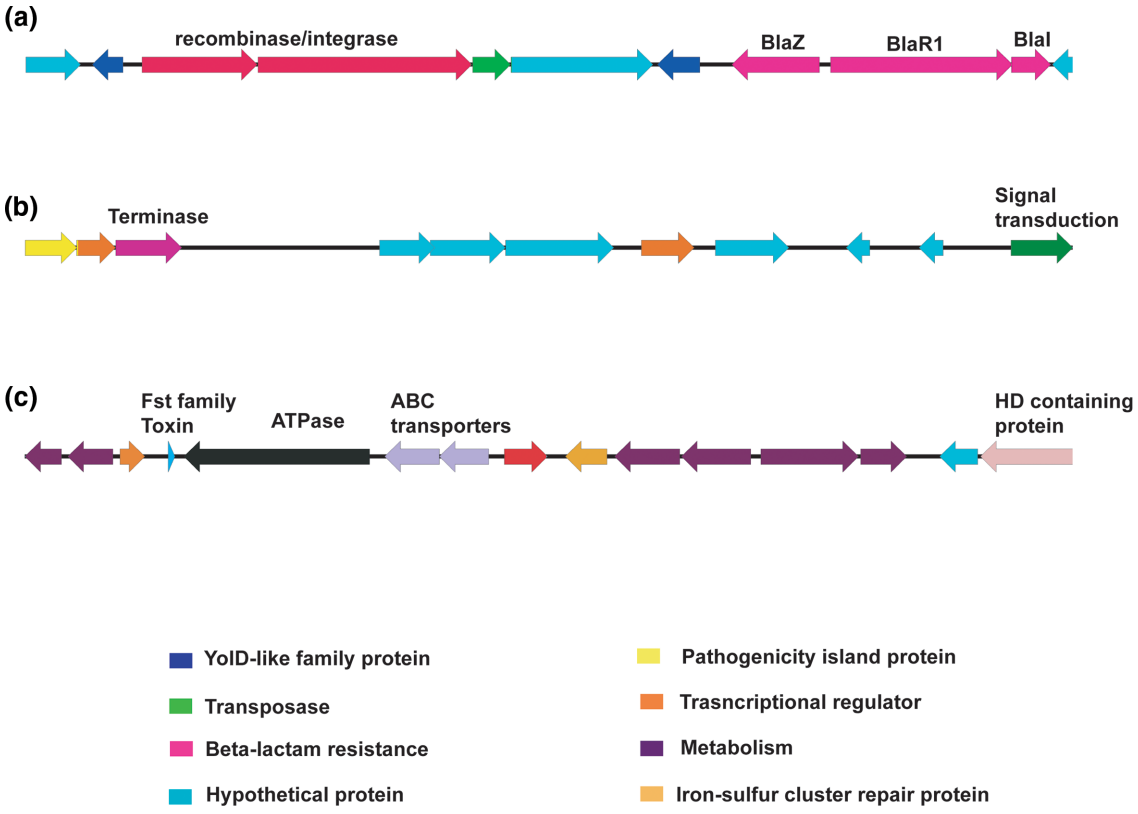

Fig. 3. Schematic representation of GIs of strain SE3.9. Panels represent three islands, GI 1, GI 2, GI 3, in strain SE3.9. Genes are depicted as filled arrows coloured according to their proposed functional category (see enclosed box).

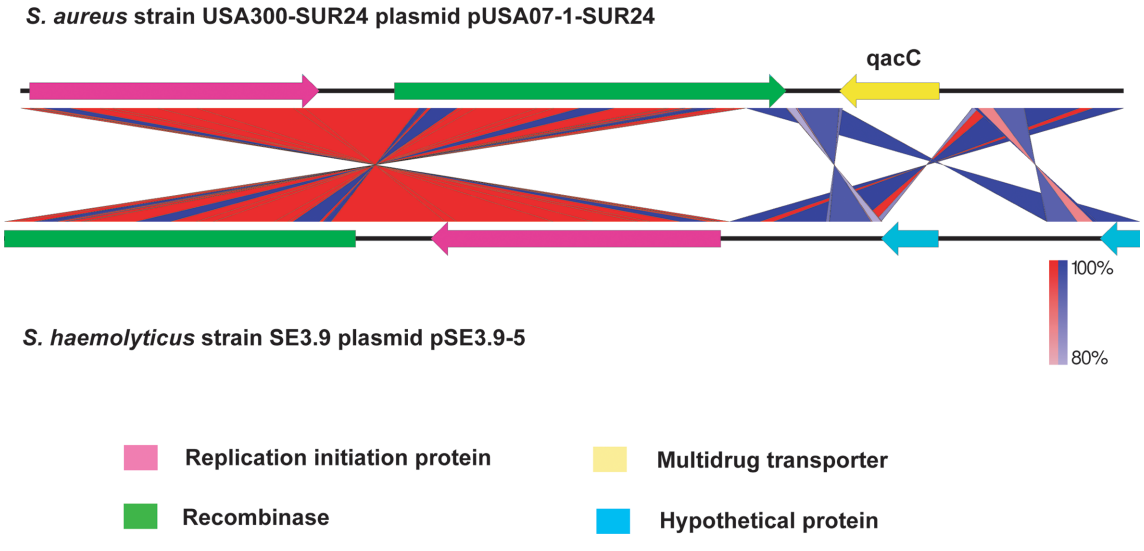

Fig. 4. Comparison of the genetic organization of *S. aureus* strain USA300-SUR24 plasmid pUSA07-1-SUR24 with *S. haemolyticus* strain SE3.9 plasmid pSE3.9-5. Arrows represent predicted CDSs. Highly conserved regions determined by pairwise BLASTN comparisons with E-values <0.001 were plotted. Regions with forward and reverse matches are indicated by red and blue shading, respectively, with colour intensity indicating nucleotide identity levels (from 80 to 100%). The absence of red and blue area denotes no homology.

elements, particularly IS elements and multispecies plasmids in the adaptation of *S. haemolyticus*. At the same time, there is the possibility of transfer of plasmids encoding antimicrobial resistance and fitness genes from plant strains into pathogenic strains of *S. haemolyticus* and also other pathogenic species such as *S. aureus*. In this context, the presence of resistance and fitness genes on plasmids that are usually present in multiple copies has implications for the further success and adaptation of clinical species and strains. Overall, these resources and systematic insights should stimulate further ecological-based molecular and functional studies of the pathogenic lifestyle of *S. haemolyticus*.

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Author contributions
T.S. was involved in complete genome sequencing and data analysis. T.S. carried out comparative genomic studies with inputs from V.C. P.B.P. was involved in funding acquisition and conceptualization of the study. T.S. drafted the manuscript with input from V.C. and P.B.P.

Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial support that could be considered as a potential conflict of interest.

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