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Comparative Genomics of *Staphylococcus* Reveals Determinants of Speciation and Diversification of Antimicrobial Defense

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The bacterial genus *Staphylococcus* comprises diverse species with most being described as colonizers of human and animal skin. A relational analysis of features that discriminate its species and contribute to niche adaptation and survival remains to be fully described. In this study, an interspecies, whole-genome comparative analysis of 21 *Staphylococcus* species was performed based on their orthologues. Three well-defined multi-species groups were identified: group A (including *aureus/epidermidis*); group B (including *saprophyticus/xylosus*) and group C (including *pseudintermedius/delphini*). The machine learning algorithm Random Forest was applied to prioritize orthologs that drive formation of the *Staphylococcus* species groups A-C. Orthologues driving staphylococcal intrageneric diversity comprised regulatory, metabolic and antimicrobial resistance proteins. Notably, the BraSR (NsaRS) two-component system (TCS) and its associated BraDE transporters that regulate antimicrobial resistance showed limited distribution in the genus and their presence was most closely associated with a subset of *Staphylococcus* species dominated by those that colonize human skin. Divergence of BraSR and GraSR antimicrobial peptide survival TCS and their associated transporters was observed across the staphylococci, likely reflecting niche specific evolution of these TCS/transporters and their specificities for AMPs. Experimental evolution, with selection for resistance to the lantibiotic nisin, revealed multiple routes to resistance and differences in the selection outcomes of the BraSR-positive species *S. hominis* and *S. aureus*. Selection supported a role for GraSR in nisin survival responses of the BraSR-negative species *S. saprophyticus*. Our study reveals diversification of antimicrobial-sensing TCS across the staphylococci and hints at differential relationships between GraSR and BraSR in those species positive for both TCS.

Keywords: *Staphylococcus*, antibiotic resistance, competition, machine learning, antimicrobial, defense, genome, resistance

BACKGROUND

Staphylococcus Species and Genomics

The existence of taxonomically distinct species groups was first proposed for *Staphylococcus* based on differential DNA–DNA hybridization methods (Kloos et al., 1991). These groups were supported by 16S rDNA sequence analysis of 38 taxa (Takahashi et al., 1999) and multilocus sequence data of around 60 species and subspecies (Lamers et al., 2012).

A comparative analysis that utilized next generation genome sequencing data of staphylococci to probe phylogenetic relationships with 491 shared orthologues across 12 *Staphylococcus* species (Suzuki et al., 2012) proposed *S. pseudintermedius* and *S. carnosus* as the most basal lineages. Moreover, with 10 species in their analysis being residents of human skin, the authors proposed that evolution selected for human adaptation after branching from *S. carnosus*. The relationships between the strains generated from shared orthologues were maintained using total gene content (Suzuki et al., 2012). However, in contrast to the conclusions of 16S rDNA and multilocus data (Takahashi et al., 1999; Lamers et al., 2012) their analysis revealed discrete clustering of *Staphylococcus* species. In contrast with this analysis, no distinct clustering of *S. hominis* with *S. haemolyticus* was observed, and *S. saprophyticus* was assigned to the *S. epidermidis* group of species (Suzuki et al., 2012). Currently, there is a knowledge gap in *Staphylococcus* species comparisons with a need to determine if this clustering of staphylococcal species is supported using whole genome data. Our findings here begin to close this gap.

Two Component Systems

Prokaryotes are receptive to environmental stimuli through diverse sensory and transducing two component systems (TCS). These TCS archetypically comprise a sensor histidine kinase (HK) that spans the cell membrane to interact with the external environment. Stimulus perception causes conditional autophosphorylation that is relayed to an interacting response regulator (RR) to enable DNA-binding directed transcription modulation (Stock et al., 2000).

While TCS are widespread and diverse across prokaryotes, the intramembrane-sensing histidine kinases (IM-HK) are specific to the Firmicutes. This family of small HKs has a short, 25 amino acid linker region between each 400 amino acid transmembrane helix. *S. aureus* GraSR uses a IM-HK to regulate a global network responsible for resistance to antimicrobial peptides (AMPs). GraSR modulates the expression of DltABCD and MprF that in concert alter the *S. aureus* surface charge to evade electrostatic interaction-mediated targeting of cationic AMPs (Falord et al., 2011).

An orthologous TCS to GraSR described in *S. aureus* was concurrently designated BraSR and NsaSR by two different groups (Blake et al., 2011; Hiron et al., 2011). Serial passage in sub-MIC concentrations of the lantibiotic nisin was shown to select increased nisin MIC due to a SNP in *nsaS* gene encoding sensor histidine kinase of NsaRS (nisin susceptibility-associated sensor regulator) (Blake et al., 2011). The TCS was

separately designated BraSR (bacitracin resistance-associated sensor regulator) from the reduced MIC of bacitracin and nisin determined for the TCS gene mutant (Hiron et al., 2011). BraR binding sites were revealed upstream of the ABC transporter genes *braDE* and *vraDEH* (Popella et al., 2016) that were not transcribed in the mutant but induced in the presence of bacitracin. The transporter BraDE contributes to the detection of nisin and bacitracin and subsequent signal transduction via BraSR, whereas VraDE is more directly involved in detoxification by efflux (Hiron et al., 2011). Transcription of *braSR* is increased following exposure to multiple antibiotics, including ampicillin, phosphomycin and nisin. Inactivation of *braS* (*nsaS*) revealed differential transcription of 245 genes (Kolar et al., 2011), revealing the TCS might report cell envelope stress to directly regulate biofilm formation, cellular transport and responses to anoxia.

In this study, a comparative genome analysis of 21 *Staphylococcus* species was performed based upon their orthologous gene content. Species groups were revealed and then interrogated using the Random Forest algorithm to identify group-contributing genes. The operon encoding the BraSR TCS was found to differentiate the *S. aureus*/*S. epidermidis* species group from other species groups determined in the study and the TCS was found to have restricted distribution across 49 species of *Staphylococcus*. Experimental evolution of representative *braSR*-positive and -negative species with nisin selection identified differential selection of BraSR and GraSR to produce resistance to this AMP.

RESULTS AND DISCUSSION

Analysis of Orthologous Gene Content Across the Staphylococci

The orthologous gene content of 21 sequenced staphylococcal species' genomes (Table 1) was determined using OrthoMCL to group orthologous genes (homologues separated by speciation) into clusters across the different species. The number of shared orthologous clusters between the different species' genomes was then represented as a heatmap (Figure 1). The output from this analysis revealed the assembly of three major groups of species, each with high numbers of shared orthologous clusters. An associated cladogram supported three groups (groups A, B, and C) when defined as containing three or more species (Figure 1). This supported previous reported groupings from 16S rDNA and multilocus analyses (Takahashi et al., 1999; Lamers et al., 2012). Additionally, three species pairs showed a high degree of shared orthologous clusters of genes and branched together in the cladogram: *S. aureus*/*S. simiae*, *S. simulans*/*S. carnosus*, and *S. lentus*/*S. vitulinus*. *S. aureus* and *S. simiae* were proposed as members of the *S. aureus* group of staphylococci from gene content (Takahashi et al., 1999).

The largest and least well-defined, species group comprises *S. epidermidis*, *S. capitis*, *S. warneri*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. pettenkoferi*, *S. aureus*, and *S. simiae* (Figure 1). Designated group A, it is dominated by species that colonize human skin (Kloos, 1980; Schleifer and Bell, 2015). The

TABLE 1 | *Staphylococcus* species and strains included in OrthoMCL analysis.

Staphylococcus species	Strain	Genome accession	Sequence status (Reference)
<i>S. arlettae</i>	CVD059	ALWK01000000 (Uid175126)	Draft (Dinakaran et al., 2012)
<i>S. aureus</i>	Newman	AP009351 (Uid58839)	Complete (Baba et al., 2008)
<i>S. capitis</i>	SK14	ACFR01000000 (Uid55415)	Draft
<i>S. carnosus</i>	TM300	NC012121 (Uid59401)	Complete (Rosenstein et al., 2009)
<i>S. cohnii</i>	ATCC29974	LT963440	Draft (This study)
<i>S. delphini</i>	8086	CAIA00000000 (Uid199664)	Draft
<i>S. epidermidis</i>	ATCC_12228	NC005008 (Uid57861)	Complete (Zhang et al., 2003)
<i>S. equorum</i>	Mu2	CAJL01000000 (Uid169178)	Draft
<i>S. haemolyticus</i>	K8	LT963441	Draft (This study)
<i>S. hominis</i>	J6	LT963442	Draft (This study)
<i>S. intermedius</i>	NCTC_11048	CAIB01000000 (Uid199665)	Draft
<i>S. lentus</i>	F1142	AJXO01000000 (Uid200144)	Draft (Nam et al., 2012b)
<i>S. pettenkoferi</i>	VCU012	AGUA00000000 (Uid180074)	Draft
<i>S. lugdunensis</i>	HKU09	CP001837 (Uid46233)	Complete (Tse et al., 2010)
<i>S. pseudintermedius</i>	HKU10	Uid62125	Complete (Tse et al., 2011)
<i>S. saprophyticus</i>	ATCC_15305	AP008934 (Uid58411)	Complete (Kuroda et al., 2005)
<i>S. simiae</i>	CCM_7213	AEUN00000000 (Uid77893)	Draft (Suzuki et al., 2012)
<i>S. simulans</i>	ATCC 27848	LT963435	Draft (This study)
<i>S. vitulinus</i>	F1028	AJTR00000000 (Uid200114)	Draft (Nam et al., 2012a)
<i>S. warneri</i>	SG1	CP003668 (Uid187059)	Complete (Cheng et al., 2013)
<i>S. xylosus</i>	ATCC29971	LT963439	Draft (This study)

Genomes were sequenced for this study, as indicated, or retrieved from NCBI for analysis; genome integrity is indicated.

likelihood of a strain-dependent effect structuring group A was investigated by substituting *S. epidermidis*, *S. hominis*, and *S. aureus* strains based on multiple available genomes (**Table 1**

and **Supplementary File S1**). Substituting these individual species with alternative strains and repeating the OrthoMCL analysis did not alter species groupings. Groups B and C were similarly unaffected by switching strains of *S. saprophyticus* and *S. pseudintermedius*, respectively.

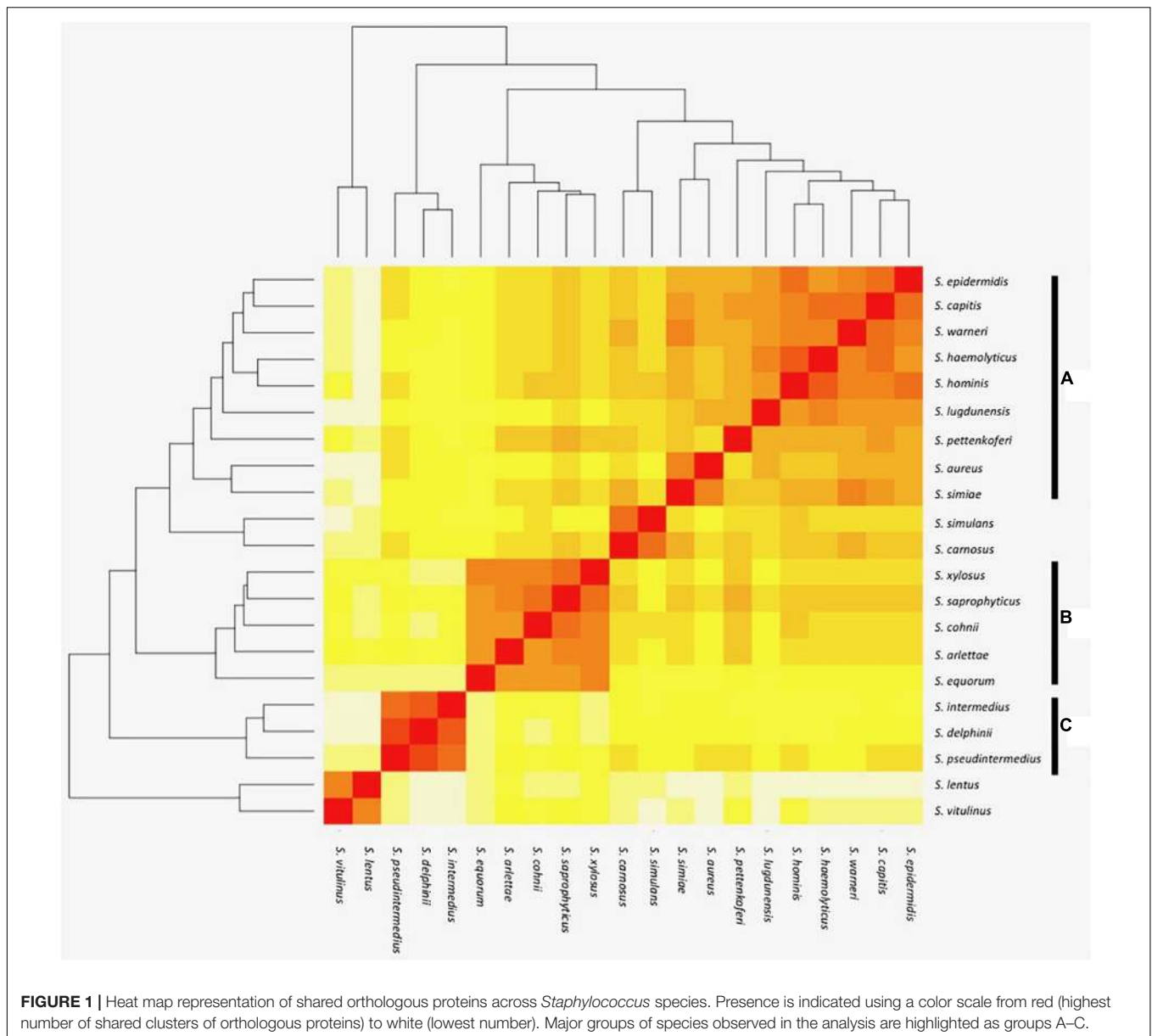
The smaller species group B comprises *S. equorum*, *S. arlettae*, *S. cohnii*, *S. saprophyticus*, and *S. xylosus* (**Figure 1**). Though not universal, a frequent lifestyle identified in the group B species is human or animal host colonization; several species are associated with meat products and novobiocin resistance (Devriese et al., 1985; Bannerman et al., 1991) with commonalities in their cell wall composition (Schleifer et al., 1984).

Species group C comprises *S. pseudintermedius*, *S. delphini*, and *S. intermedius* and this collective was previously designated the *S. intermedius* group (SIG); the species cause opportunistic infection of companion animals and equids (Devriese et al., 1985). Emerging antibiotic resistance in the SIG species group is a clinical veterinary concern (Stull et al., 2014) and their routine speciation is complicated by their high degree of 16S rRNA locus sequence identity (Sletteameås et al., 2010).

While preserving known species groupings, the whole genome analysis identified discrete species groups of staphylococci (A-C) and an explanation for their formation was sought. Genetic determinants directing the formation of species group A were tested in R using machine learning with the Random Forests algorithm for classification (Breiman, 2001). This algorithm was used to identify variables, in this case OrthoMCL clusters (data not shown), that contributed to formation of the groups, based on a forest of trees generated from these variables. A gene from each cluster was then determined and mapped back to a representative genome and the PROKKA annotation of each protein coding sequence was verified using BLAST, for group A this representative was *S. epidermidis*. Contributing variables were investigated for group A, based on the strain set described in **Table 1**, where permutations were used to verify the existence and reproducibility of species groups (**Supplementary File S1**).

Clusters Driving Formation of Group A Species

The presence of 7 and absence of 6 OrthoMCL clusters collectively contribute to defining group A, with differing levels of support (Mean Decrease in Accuracy [MDA] values) (**Table 2** and **Supplementary File S2**). Four orthologs that are sequentially encoded in the genome as an operon (epi_02134 - epi_02137; MDA 3.2, 3.0, 2.6, 2.2, respectively) were also the most strongly supported in this analysis (**Table 2**). The latter cluster pair epi_02136/epi_02137 was annotated by PROKKA as a TCS sensor/regulator (**Table 2** and **Supplementary File S2**) and shares ~100% similarity with BraSR (SA2417/SA2418 of *S. aureus* N315), a TCS associated with resistance to AMPs nisin and bacitracin (Hiron et al., 2011). The adjacent clusters encoded in the same operon (epi_02134, epi_02135) comprise the BraD/BraE ABC transporter subunits with 98 and 99% similarity with SA2415/SA2416 of *S. aureus* N315, respectively (Hiron et al., 2011). We demonstrate as a key finding of our analysis that BraSR and BraDE



are associated with genomes of group A *Staphylococcus* species.

The presence of orthologue epi_00542 (MDA 2.2; **Table 2** and **Supplementary File S2**) contributes to species group A, with support that the protein functions as a putative cell wall hydrolase from the Nlp-P60 family hydrolase domain that is associated with hydrolysis of peptidoglycan. Also, contributing to defining group A are the absences of two orthologue clusters (sap_00398; MDA 3.3 and sap_00399; MDA 1.4; **Table 2** and **Supplementary File S2**) that are annotated as multidrug ABC transporters. A range of cytotoxic molecules are mobilized across the cell membrane by multidrug ABC transporters where certain families of these can also act as sensors (Stock et al., 2000; Rietkötter et al., 2008). Across staphylococcal groups, differential repertoires of ABC transporters associated with antimicrobial survival are

consistent with the importance of community competition in species evolution.

Sequence variation of the NADP-dependent succinate semialdehyde dehydrogenase (SSADH) between group A staphylococci versus groups B and C was identified by the association of cluster sap_00201 (MDA 2.9, **Table 2** and **Supplementary File S2**) with group A species; this variation might be allied to differences in glutamate metabolism across the genus. Glutamate is involved in multiple metabolic processes and bacterial glutamate dehydrogenase catabolizes glutamate, which contributes to acid tolerance. NADP-SSADH catalyzes catabolism of γ -aminobutyrate, a product of glutamate dehydrogenase activity (Feehily and Karatzas, 2013); this pathway is oxidative stress sensitive owing to the catalytic cysteine residue of SSADH.

TABLE 2 | Proteins driving formation of species group A.

Group A staphylococci Random Forest Output		
PROKKA annotation	BLAST homology	MDA
Presence		
Putative cell wall associated hydrolase (epi_00542)	Hypothetical protein	2.2
Hypothetical protein (epi_02098)	Cell wall surface anchor protein	2.7
Hypothetical protein (epi_02108)	Hypothetical protein	1.9
FtsX like permease family protein (epi_02134)	ABC transporter permease	3.2
Macrolide export ATP binding/permease protein MacB (epi_02135)	Bacteriocin ABC transporter ATP-binding protein	3.0
Sensor histidine kinase GraS (epi_02136)	TCS histidine kinase	2.6
Glycopeptide resistance associated protein R (epi_02137)	TCS transcriptional regulator	2.2
Absence		
Succinate semialdehyde dehydrogenase NADP+ (sap_00201)	Succinate-semialdehyde dehydrogenase	2.9
Putative membrane protein putative toxin regulator (sap_00203)	PTS sugar transporter subunit IIC	2.7
Putative multidrug resistance ABC transporter ATP binding/permease protein Yhel (sap_00398)	Multidrug ABC transporter ATP-binding protein	3.3
Putative multidrug resistance ABC transporter ATP binding/permease protein YheH (sap_00399)	Multidrug ABC transporter ATP-binding protein	1.4
L-lactate utilization operon repressor (sap_00760)	Transcriptional regulator	2.7
Glutamate aspartate carrier protein (sap_01003)	Sodium:dicarboxylate symporter	2.7

PROKKA annotation was found by mapping clusters from the variable importance analysis to the *S. epidermidis* genome in the case of 'present' clusters and the *S. saprophyticus* genome for 'absent' clusters. The PROKKA locus tag is indicated in brackets. BLAST homology was determined from searches against the NCBI BLAST database. %MDA is representative for the cluster across the randomForest analyses, where higher values indicate increased support. Sequences corresponding to PROKKA locus tag are listed in **Supplementary File S2**.

TABLE 3 | Comparative sequence identity of the BraRS and GraRS TCS across species groups A–C.

TCS protein	Mean identity within group A	Mean identity to groups B and C GraR or GraS	Mean identity to group A GraR or GraS
BraR	77.1	39.6	44.3
BraS	62.9	26.4	29.8

TCS protein	Mean identity within group A	Mean identity within group B	Mean identity within group C	Mean identity across groups
GraR	87.8	84	97.9	66.7
GraS	69.4	66	88.2	48.2

Mean identity values of BraR and BraS within species group A and values for BraR and BraS with GraR and GraS of species group A or B/C. Mean identity values of GraR and GraS within and between species groups A, B, and C. Sequence identity was calculated from multiple sequence alignments of all protein sequences of species indicated in **Table 1**.

With respect to clusters driving formation of group B and C species, the size of species input groups B and C (**Figure 1**) limit use of the random forest algorithm. Consequently, a similar species-defined analysis of groups B and C is not included and a broader species comparison of staphylococci could be considered in future.

Diversity of Cationic AMP Survival Loci Across the Staphylococci

The described comparative genomic analysis revealed that while BraSR TCS is associated with group A species of staphylococci, the GraSR TCS is distributed across all species groups. Supporting predictions from the Random Forest analysis, low sequence identity of BraR/BraS with GraR/GraS was confirmed. BraR mean sequence identity with GraR of group A (44%) and group B/C species (40%) was greater than that of BraS compared with GraS

of group A and groups B/C (mean ~30% and ~26%, respectively) (**Table 3**).

High mean sequence identity (84–98%) of GraR regulator protein occurs within each of the three species groups (**Table 3**) with divergence of GraR between species groups identified by lower mean sequence identity (67%). GraS sensor histidine kinase was less conserved within species groups A (mean 69%) and B (mean 66%), compared with GraS of species group C that shared greatest mean sequence identity (88%), albeit that group C defined here is a small, related species set. Both BraS and GraS sensor proteins have lower sequence conservation across staphylococci than BraR and GraR (**Table 3**). The reduced divergence of these response regulators might reflect their relative isolation from selection by the external environment and differential stimuli.

Responses to cationic AMPs in the staphylococci are complex (Peschel and Collins, 2001; Herbert et al., 2007) and ligand

specificity could account for species divergence of GraSR and BraSR TCS. This evolutionary outcome could be explained with strong selection pressure driven by ubiquity and diversity of cAMPs in staphylococcal niches. One intrigue in our analysis is the absence of GraSR and presence of only BraSR TCS in the group A species, *S. pettenkoferi*, with the sole related sensor protein having a mean sequence identity of 27% with group A GraS but 58% with group A BraS. *S. pettenkoferi* BraR has a mean sequence identity of 47% with GraR and 73% with BraR from group A. These values support the *S. pettenkoferi* TCS is a BraSR ortholog. GraSR was also absent and BraSR present in the four additional publicly available *S. pettenkoferi* genome sequences (strains 1286_SHAE, 589_SHAE, UMB0834 and CCUG 51270). The absence of GraSR in *S. pettenkoferi* raises questions about the evolution of BraSR in group A staphylococci. Gene duplication of GraSR in a group A species, with subsequent sequence divergence over time to BraSR and spread throughout group A species by horizontal gene transfer, is tempting to suggest. *S. pettenkoferi* having BraSR but not GraSR presents a challenge to this paralog hypothesis. We propose two possibilities; *S. pettenkoferi* may have suffered deletion of *graSR* following acquisition of *braSR*, or *S. pettenkoferi* never acquired *braSR*, but rather its TCS evolved from ancestral genes. Such a scenario would enable group A organisms to acquire *braSR* from *S. pettenkoferi* as an additional and sufficiently divergent TCS locus.

Staphylococcus species genomes sequenced recently were investigated for their encoded GraS and BraS protein homologues, which supported the limited distribution of BraS in staphylococci as identified in the Random Forest analysis (Table 4 and Supplementary File S3). Furthermore, it revealed additional species encoding BraSR but not GraSR (*S. agnetis*, *S. auricularis*, *S. chromogenes*, *S. hyicus*, *S. massiliensis*). Regardless of the origins of both TCSs, the divergence between and within GraSR and BraSR likely reflect specificities for their ligands and selection driven by the niches to which the staphylococci are specialized.

GraSR and BraSR-Associated ABC Transporters

Both GraSR and BraSR, as members of the BceS-like IMHK family of TCS, are activated by AMP ligand bound to an associated ABC transporter (Mascher, 2014). Given the important function of these TCS, the conservation of their associated transporter protein sequences was compared across the staphylococci.

VraFG is the GraSR-associated ABC transporter (Meehl et al., 2007) and in the genomes encoding VraFG (absent from group B species and *S. pettenkoferi*) there is a high degree of shared protein sequence conservation. VraF has a mean sequence identity of 68% across the staphylococci examined (Table 1), with greatest conservation within species groups (group A, 79% identity; group B, 85.3% identity; group C, 96.8% identity). Shared sequence identity among the VraG proteins was 47.5%, with 88%, 65.2 and 61.9% identity within groups A, B, and C, respectively. The BraDE ABC transporter associated with BraSR

TABLE 4 | Presence and absence of GraS and BraS across 49 *Staphylococcus* species.

GraS-encoding only	GraS and BraS-encoding	BraS-encoding only
<i>S. arlettae</i>	<i>S. argenteus</i>	<i>S. agnetis</i>
<i>S. carnosus</i>	<i>S. aureus</i> *	<i>S. auricularis</i>
<i>S. cohnii</i>	<i>S. capitis</i> *	<i>S. chromogenes</i>
<i>S. condimentii</i>	<i>S. caprae</i> *	<i>S. hyicus</i>
<i>S. delphini</i>	<i>S. devriesei</i>	<i>S. massiliensis</i>
<i>S. edaphicus</i>	<i>S. epidermidis</i> *	<i>S. pettenkoferi</i> *
<i>S. equorum</i>	<i>S. haemolyticus</i> *	
<i>S. felis</i>	<i>S. hominis</i> *	
<i>S. fleurettii</i>	<i>S. lugdunensis</i> *	
<i>S. gallinarum</i>	<i>S. pasteurii</i>	
<i>S. intermedius</i>	<i>S. petrasii</i>	
<i>S. kloosii</i>	<i>S. saccharolyticus</i>	
<i>S. lentus</i>	<i>S. schweitzeri</i>	
<i>S. lutrae</i>	<i>S. simiae</i> *	
<i>S. microti</i>	<i>S. warneri</i> *	
<i>S. muscae</i>		
<i>S. nepalensis</i>		
<i>S. piscifermentans</i>		
<i>S. pseudintermedius</i>		
<i>S. rostri</i>		
<i>S. saprophyticus</i>		
<i>S. schleiferi</i>		
<i>S. sciuri</i>		
<i>S. simulans</i>		
<i>S. stepanovicii</i>		
<i>S. succinus</i>		
<i>S. vitulinus</i>		
<i>S. xylosum</i>		

Presence and absence were determined using BLASTp with *S. aureus* N315 SA2417 (BraS) and SA0615 (GraS) sequences confirming and extending Random Forest output. Asterisk (*) indicates species forming part of group A in Figure 1. Linked data is present in Supplementary File S3.

was identified in group A species and, similar to VraFG, revealed greater identity (68.4%) across BraD sequences compared with BraE (38.9%) protein sequences. Divergence within BraSR and GraSR-associated transporters has likely arisen from concurrent evolution of the ABC transporter specificities for AMPs.

Experimental Evolution of Nisin Resistance in *S. aureus*, *S. hominis* and *S. saprophyticus*

Previous studies demonstrated that selection by experimental evolution identified mutations conferring antimicrobial resistance in overarching regulators, notably SNPs in *braS* revealed roles for BraSR in nisin sensing and survival (Hiron et al., 2011). Following our identified species association of BraSR to group A staphylococci, we adopted an experimental evolution strategy to interrogate the contributions of GraSR and BraSR TCS under selection for nisin resistance.

Strains of group A species, *S. aureus* and *S. hominis* plus group B *S. saprophyticus* were each serially passaged

in triplicate cultures with increasing concentrations of nisin using a microtiter plate method, with an equivalent sodium citrate buffer control passaged in parallel. Stepwise increases in nisin MIC were observed for all strains tested with no obvious pattern in the rate of resistance acquisition between the species. After selection, both *S. aureus* 171 and *S. aureus* SH1000 strains exhibited ~100-fold increases in nisin MIC, a greater fold increase in resistance than that observed by Blake et al (2011; Hiron et al., 2011), which may be due to experimental design differences. Selection of both *S. hominis* strains increased nisin MIC ~25-fold, and *S. saprophyticus* strains CCM_883 and CCM_349 showed 80-fold and 5-fold increases, respectively. Multiple clones of *S. aureus* 171, *S. hominis* J31 and *S. saprophyticus* CCM883 were genome sequenced to identify sequence variants that potentially contributed to increased nisin MIC. T0 genomes were assembled and annotated, then reads from three pools (each comprising 5 independent clones) and one individual clone of each experimentally evolved species were aligned to their respective assembled genomes to identify sequence variants (SNPs, insertions/deletions) specific to nisin selection (Tables 5–7).

Nisin-Selected SNPs in Staphylococci

Experimental evolution of *S. saprophyticus* identified a SNP in *graS* (GraS: A₁₆₀S; Table 5) that was present in two clone pools, and SNP *graS* G₂₀₉C in a third pool. A single clone sequenced from the latter pool identified only one SNP in *graS* (GraS: G₂₀₉C) and an upstream variant associated with *ptsG* (Table 5). These data provide support for GraSR contributing to nisin resistance in *S. saprophyticus* given the absence of the BraSR TCS in this group B *Staphylococcus* species. Aside from TCS, other regulators may contribute to the nisin response in *S. saprophyticus* as evidenced by an identical SNP identified in two separate nisin resistance selections (pools 2 and 3) corresponding to a T₆₂I change in an uncharacterized MarR transcriptional repressor.

In both *S. aureus* and *S. hominis* there are multiple pathways to high-level nisin resistance. Each species revealed SNPs in TCS systems, but these differed across the parallel selection experiments (Tables 5–7). In *S. aureus*, a non-synonymous SNP in *braS* (BraS: T₁₇₅I) was present in 100% of reads from one sequenced pool, differing from previous work that identified a discrete *braS* SNP (BraS: A₂₀₈E) (Hiron et al., 2011). Evidence for a second TCS contributing to nisin resistance arose from a *walk* non-synonymous SNP (Walk: H₃₆₄R) within the diverse and flexible signal sensing PAS domain of Walk in *S. aureus* (Hefti et al., 2004). WalkR is essential and functions to maintain cell wall metabolism (Delaune et al., 2012) and SNPs in this TCS contribute to vancomycin and daptomycin resistance due to cell wall-thickening (Howden et al., 2011). Should this cell wall phenotype be associated with the H₃₆₄R Walk variant it could similarly limit nisin interaction with its lipid II target to abrogate pore formation. A large overlap was reported between the WalkR and GraSR regulatory networks in *S. aureus* (Falord et al., 2011).

In *S. hominis*, a *graS* SNP (GraS: S₁₂₀L) was present in 2 clones of sequence pool 2 and no SNPs or other sequence variants were identified in *braSR* (Tables 5–7). *S. hominis* has both *braSR* and *graSR* loci and therefore it is intriguing nisin resistance selection resulted in SNPs in a different TCS to *S. aureus* despite encoding both, potentially reflecting differences in their contribution across group A staphylococci. A further transcriptional regulator might contribute to nisin resistance in both *S. aureus* and *S. hominis*, where the uncharacterized *yhcF* revealed SNPs producing G₇₃R and N₄₇*, respectively; the presence of SNPs in *yhcF* of both species supports a role for this regulator. The YhcF transcriptional regulator proteins of *S. aureus* and *S. hominis* have 75% similarity and their cognate genes are adjacent to an ABC transporter locus with potential specificity for GlcNAc, which might catalyze recycling of cell wall substrates from nisin damage. The role of this operon is currently being investigated.

In summary, we have identified differential encoding and diversity of antimicrobial resistance regulators and their associated transporters across the staphylococci. Our previous studies of the nasal microbiome correlated cumulative antimicrobial production with community structure, limitation of invasion and *S. aureus* exclusion (Coates et al., 2014; Libberton et al., 2014, 2015). Further dissection of antimicrobial sensing and discrimination via the TCS systems BraSR and GraSR combined with analysis of their associated transport specificities will provide information that can be layered with niche-relevant antimicrobial activities from competing species. Such analyses are now emerging and will provide a more holistic determination of *Staphylococcus* ecology.

MATERIALS AND METHODS

Staphylococcus Orthologous Gene Content

Representative genomes of 21 different *Staphylococcus* species available at the time of analysis (Table 1) were either sequenced (see later section) or retrieved from the NCBI FTP repository¹. Complete genomes were used where possible. Draft genomes available as NCBI scaffolds were reordered against an appropriate reference using a bespoke perl script. Genomes were annotated using PROKKA (version 1.5.2) (Seemann, 2014) to ensure consistent gene calling and annotation. OrthoMCL (version 1.4) was used to cluster orthologous proteins (Li et al., 2003), with input parameters, *e*-value cut-off: 1e-5, percentage identity cut-off: 30, percentage match cut off: 20. Briefly, initial BLAST steps of orthoMCL used the latter two low stringency cut-off values; these values were used to retain more proteins for clustering from these BLAST stages. Inparalog, ortholog and co-ortholog pairwise relationships were generated through reciprocal best and better hits in subsequent stages that used the *p*-value cut-off of 1e-5. Finally, the MCL (Markov clustering) aspect of the tool was applied to these pairwise relationships to allow clustering into orthologous

¹<http://ftp.ncbi.nlm.nih.gov/>

TABLE 5 | Non-synonymous, homozygous SNPs from independent clone pools of staphylococci after nisin selection.

Gene ID (Prokka)	Protein ID	Pool	Position	Base change	Amino acid change	Allele frequency
S. aureus 171						
<i>walk</i>	Walk Sensor kinase	1	17119	A -> G	H ₃₆₄ R	1
<i>gltB_1</i>	Glutamate synthase	1	437361	A -> T	Q ₇₉₇ L	1
<i>rpoB</i>	DNA-directed RNA polymerase subunit beta	1	520176	C -> T	H ₅₀₆ Y	1
<i>mraY</i>	Phospho-N-acetylmuramoyl-pentapeptide transferase	1	1103071	G -> A	V ₂₆₆ I	1
<i>yhcF</i>	Transcriptional regulator	1	1998279	G -> A	G ₇₃ R	1
Hypothetical	Membrane protein	2	615003	C -> T	Q ₅₇ *	0.35
<i>rpoC</i>	DNA-directed RNA polymerase subunit beta	3	523690	G -> T	A ₄₄₈ S	0.99
<i>femB</i>	FemB	3	1323450	G -> A	R ₂₁₅ H	0.63
Phage terminase	Terminase	3	1491745	G -> C	G ₂₄₀ A	1
<i>greA</i>	GreA	3	1625376	A -> T	L ₇₆ *	0.37
<i>bras</i>	BraS sensor histidine kinase	3	2627088	C -> T	T ₁₇₅ I	1
S. hominis J31						
<i>rpoB</i>	DNA-directed RNA polymerase subunit beta	1	41317	G -> T	D ₁₀₄₆ Y	0.79
<i>graS</i>	GraS sensor histidine kinase	2	147503	C -> T	S ₁₂₀ L	0.42
<i>ftsH</i>	FtsH Zinc metalloprotease	2	2172470	C -> A	D ₁₇₁ E	1
<i>gmk</i>	Guanylate Kinase	3	552630	G -> A	R ₁₃₅ H	1
<i>yhcF</i>	Transcriptional regulator	3	1212777	C -> T	Q ₄₇ *	1
S. saprophyticus 883						
<i>graS</i>	GraS sensor histidine kinase	1	2068987	G -> T	G ₂₀₉ C	1
<i>codY</i>	CodY regulator	2,3	1537681	C -> A	L ₇₉ F	1,1
<i>pitA</i>	Phosphate transporter	2,3	2066724	C -> T	A ₁₉₅ V	0.99,1
<i>graS</i>	GraS sensor histidine kinase	2,3	2069134	C -> A	R ₁₆₀ S	1,1
marR family	MarR regulator	2,3	2136907	C -> T	T ₆₂ I	1,1

Names and functions of genes containing SNPs in the sequenced clone pools are shown with their locations and nucleotide change. Cognate amino acid change or stop (*) is indicated. Pools comprised 5 clones from each of three independent experiments and allele frequencies were determined from numbers of corresponding reads in these pools. Nisin MICs of clones in each pool were confirmed to ensure they were similar.

groups (Li et al., 2003; Fischer et al., 2011). A bespoke python script was used to create a table describing the presence or absence of each OrthoMCL cluster within every genome. These data were converted to a matrix for analysis in the statistical package R and a heatmap was generated from the matrix. To control for gross strain-specific effects on the heat map (and thus OrthoMCL clusters), this step was repeated by substituting with alternative strains (**Supplementary Table S1**) and all permutations were analyzed in subsequent steps of the analysis.

Drivers of OrthoMCL Group Formation

The R library, Random Forest (version 4.6-7) (Liaw and Wiener, 2003) was used to investigate the genetic inputs directing classification of the species into their OrthoMCL groups. A presence/absence table of each of the orthologous groups obtained from the United States300 permutation of the OrthoMCL analysis was generated using a bespoke python script and used as the input data for the Random Forest algorithm.

The data was split into a test and training data set with both sets including equal proportions of group A species. The optimum value for mtry was found to be 66 using the tuneRF function (ntree = 1001, stepFactor = 1.5, improve = 0.001). These mtry and ntree parameters resulted in a model with an out of bag (OOB) error rate of 9.09% and area under ROC curve (AUC) of 0.96.

Data output was summarized using the variable importance plot function and the numeric mean decrease in accuracy (MDA) resulting from the permutation of each variable was obtained through the importance function; these data were used as the measure of the importance of each variable. The maximum MDA in this analysis was 3.3. Clusters were mapped back to the genome and the annotation of protein sequence for a species representative of each cluster was retrieved. Protein sequences of clusters identified as important were retrieved and their annotations curated and verified against published annotations. In addition, outputs were generated by substituting strains of species in the analysis to compare conservation of identified clusters between the variable importance plots. Sequences of protein clusters from the single species representative in **Table 2** and identified by Random Forest output are listed in **Supplementary Files S2, S3**. Protein sequences were retrieved from their respective genomes and alignments were performed using ClustalW2 (version 2.1).

Minimum Inhibitory Concentration Assay

Nisin (Sigma-Aldrich Company Ltd, United Kingdom) was prepared as a 20 mg mL⁻¹ solution in 10 mM sodium citrate (Sigma-Aldrich Company Ltd, United Kingdom) at pH 3 and stored at 4°C. MIC assay used microtiter plates with doubling dilutions of nisin in BHI (Thermo Scientific) inoculated 1 in 2

TABLE 6 | Non-synonymous, homozygous SNPs from single clones of *S. aureus*, *S. hominis*, and *S. saprophyticus* after nisin selection.

S. aureus strain 171 (single clone from pool 1)				
Gene ID (Prokka)	Protein ID	Position	Base change	Amino acid change
<i>walk</i>	WalK Sensor histidine kinase	17119	A -> G	H ₃₆₄ R
<i>gltB_1</i>	Glutamate synthase	437361	A -> T	Q ₇₉₇ L
<i>rpoB</i>	DNA-directed RNA polymerase subunit beta	520176	C -> T	H ₅₀₆ Y
<i>mraY</i>	Phospho-N-acetylmuramoyl-pentapeptide transferase	1103071	G -> A	V ₂₆₆ I
<i>msrR</i>	Regulatory protein MsrR	1309845	G -> T	E ₁₈₁ *
<i>yurK</i>	Transcriptional regulator	1998279	G -> A	G ₇₃ R
S. hominis strain J31 (single clone from pool 2)				
<i>ftsH</i>	Zinc metalloprotease FtsH	2172470	C -> A	D ₁₇₁ E
S. saprophyticus strain 883 (single clone from pool 1)				
<i>graS</i>	GraS sensor histidine kinase	2068987	G -> T	G ₂₀₉ C

Names and functions of genes containing SNPs in single selected clones are shown with their locations and nucleotide change. Cognate amino acid change or stop (*) is indicated. Nisin MICs of clones in each pool were confirmed to ensure they were similar.

TABLE 7 | INDELS from nisin selection pools and single clones of *S. aureus*, *S. hominis*, and *S. saprophyticus*.

Source	Gene ID (Prokka)	Protein ID	Location	Base change	Effect
S. aureus 171					
Single, Pool 3	<i>lpl2_2</i>	Lipoprotein	403168	195_196 insGG	I66fs §
Single, Pool 1,3	<i>sdrE_1</i>	MSCRAMM family adhesin	554844	2672_2673 insC	K891fs §
Single			1990375	-1_-1 insCC	uv
Single, Pool 2,3			2052262	*1530_*1530 delTG	fs
Single, Pool 3	<i>deoC2</i>	Deoxyribose-phosphate aldolase 2	2126552	450_451 insAG	K151fs §
Single, Pool 1	Hypothetical	Hypothetical protein	2471437	116_117 insT	E40fs
Single, Pool 3	<i>fnbA_2</i>	Fibronectin binding protein	2490720	1763_1764 delCG	S588fs §
Pool 1	Hypothetical	Hypothetical protein	1556011	201 delT	S67fs §
Pool 2			1337112	-1_-1 insATG	
Pool 2,3	Hypothetical	Transposase	1337623	209_211 delAAG	E70del
Pool 2	Hypothetical	Transposase	1819467	1047_1048 insC	*350fs §
Pool 2	<i>leuA_2</i>		2052046	*1530 delC	dv
Pool 2	Hypothetical	Hypothetical	2471438	115_116 insATA	P39del, insHT
Pool 3	<i>lpl2_1</i>	Hypothetical	402338	212_213 insCT	Q71fs §
Pool 3	<i>sdrD_1</i>	MSCRAMM family adhesin	550856	3237_3238 insC	M1080fs §
Pool 3	Hypothetical		555082	-1_-1 insG	
Pool 3	Hypothetical	LPXTG surface protein	2480756	216 delA	T72fs §
S. hominis J31					
Single, Pool 2	<i>ftsH</i>	Zinc metalloprotease	2172279	325 delA	S109fs §
Pool 1	<i>relA</i>	GTP Pyrophosphokinase	951655	764 delA	Q255fs §
Pool 2	<i>ssaA2_2</i>	CHAP domain containing protein	1437614	575_578 delGTTA	G192fs §
S. saprophyticus 883					
Single, Pool 1,2,3	<i>ptsG</i>	PTS alpha-glucoside transporter subunit IIBC	604054	-1_-1 insAA	uv

Names and functions of genes containing INDELS in the sequenced clone pools are shown with their locations and nucleotide change. Cognate amino acid change or other sequence change is indicated: frameshift (fs); upstream variant (uv); downstream variant (dv); deletion (del); insertion (ins). INDELS marked § are predicted to have a major consequence by SnpEFF. Pools comprised 5 clones from each of three independent experiments.

with 100 μ L bacterial suspension adjusted to OD₆₀₀ 0.2 \pm 0.005. The lowest concentration with an optical density \leq to that of the initial optical density was taken as the minimum inhibitory concentration (MIC).

Selection for Nisin Resistance

Experimental evolution was performed by serial passage in broth containing doubling dilutions of nisin in triplicate wells of a

microtiter plate. For selection of *S. aureus* and *S. saprophyticus*, the maximal assay concentration of nisin was 5 mg mL⁻¹ and for *S. hominis* 50 μ g mL⁻¹. Control selection experiments with equivalent sodium citrate concentrations were performed in parallel. Experiments were initiated with inoculation of bacteria to OD₆₀₀ = 0.2 for the first passage and plates were incubated static at 37°C. Bacteria growing at the highest concentration of nisin after 24–48 h were passaged forward to the next plate;

subsequent passages were inoculated with a 1:1000 dilution of culture. Serial passage was continued until growth occurred at the maximal nisin concentration (for strains *S. saprophyticus* = 10 mg ml⁻¹, *S. aureus* = 10 mg ml⁻¹, and *S. hominis* = 250 µg ml⁻¹) or for a period of 12 days. All passaged cultures were collected and stored at -80°C in 20% (v/v) glycerol (Fisher Scientific) after each passage and the T₀ time point served as comparator strain.

Colonies were randomly selected for sequencing after plating from independent biological replicate cultures that had reached an equivalent maximum level of nisin resistance. Clones from each repeat were selected and cultured in 10 mL of BHI at 37°C with shaking at 200 rpm overnight. Increased MICs were confirmed by using the MIC assay described above at the highest nisin concentrations. Selection was performed for a corresponding citrate control time point for each of the three species.

DNA Extraction, Library Preparation and Sequencing

Cells were harvested from overnight culture and lysed in buffer containing 12.5 µg ml⁻¹ lysostaphin (Sigma-Aldrich) and 10 U mutanolysin (Sigma-Aldrich). DNA was purified using a DNeasy Blood and Tissue Kit (Qiagen). DNA (30 ng) from each of five selected clones was pooled to make Illumina Truseq DNA libraries with an insert size of 350 bp. In addition to three separate clone pools, a single clone was selected for sequencing from the clones used to constitute the pools. Single clones were selected on the basis of the highest DNA quality. The single clones and the T₀ isolates were also sequenced using Illumina Truseq nano DNA libraries with 350 bp inserts.

Identification of SNPs and INDELS

T₀ comparator strains were assembled using VelvetOptimiser (version 2.2.5; Victoria Bioinformatics Consortium) with Kmer sizes from 19 to 99 and Velvet version 1.2.06 (Zerbino and Birney, 2008). Annotation was carried out using PROKKA version 1.5.2 (Seemann, 2014). The PacBio assembly of *S. hominis* strain J31 (Accession FBVO01000000) (Coates-Brown and Horsburgh, 2017) was used as the comparator assembly for this strain. Good quality filtered reads from experimentally evolved pools and single clones were aligned to respective comparator strains using the BWA (version 0.5.9-r16) (Li and Durbin, 2009) packages aln and sampe, and also using BWA (version 0.7.5a-r405) mem package. SAM files were converted to bcf (binary variant call) files with samtools for SNP calling using the mpileup package. The bcf output file from mpileup was then converted to vcf (variant call format) files and quality filtered. For SNPs, only this quality filtered vcf file from the pooled clones, along with mpileup output without base data, were used to further filter the SNPs to include only those present in 33.33% of reads, which equates to the SNP being present in more than one clone. To reduce falsely called SNPs, SNPs not called from both alignments (from either BWA aln and sampe or BWA mem) were removed

from the data set, as recommended by Li (Li, 2014). SNPs called in the control data and evolved isolates were filtered from the data.

AVAILABILITY OF DATA AND MATERIALS

Genomes resulting from this work can be retrieved from the ENA database at EMBL-EBI (<https://www.ebi.ac.uk/ena/data/view>) under the bioproject accession PRJEB22856, including data from experimental evolution of *S. aureus* 171; Parental *S. aureus* 171 data accession: LT963437. Individual genome assembly accessions used in **Figure 1** are listed in **Table 1** and **Supplementary File S1**. Strains not already publicly archived are available on request. This manuscript was submitted to bioRxiv ahead of review (Coates-Brown et al., 2018).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.02753/full#supplementary-material>

FILE S1 | *Staphylococcus* species and strains used as substitutes in OrthoMCL analyses.

FILE S2 | Species group A, present and absent cluster protein sequences. Sequences represent clusters listed in **Table 2**.

FILE S3 | Data from BLASTp search analysis for GraS and BraS homologues in 49 *Staphylococcus* species genomes. BLASTp was performed using default settings and either *S. aureus* N315 SA0615 (GraS) or SA2417 (BraS) protein sequences.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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