SUPPLEMENTARY MATERIAL

METHODS

Bacterial isolates and growth conditions. The recent VSG isolates were collected from two clinical sources. Two (VGS007 and VGS008) were obtained from the bloodstream of patients at the MD Anderson Hospital, Houston, TX (courtesy of Dr. Sam Shelburne). One VGS isolate was obtained courtesy of Drs. Ronald Jones and Helio Sader (JMI Labs, IA) (1). They were confirmed as *S. mitis-oralis* by either MALDI-TOF or standard biochemical assays.

DAP-R derivatives of these three DAP-S parental strains were obtained by serial *in vitro* passage in DAP as previously described (2). Briefly, the parental strains (starting inocula of ~10^8 CFU/ml) were cultured overnight in brain heart infusion broth (BHIB) with DAP (20 μg/ml) plus $CaCl_2$ 50 mg/L. After each 24-hr exposure, surviving colonies were collected and passed in fresh BHIB with DAP 20 μg/ml plus $CaCl_2$ for up to 12 consecutive days. DAP-R derivatives were stored at -80°C. DAP MICs were determined by Etest as recommended by the manufacturer. Stability of DAP-R were investigated by passing DAP-R derivatives in antibiotic-free BHIB for 5 consecutive days. MICs were also determined in these isolates post-passage.

Determination of spontaneous resistance frequency. The spontaneous frequency of evolution of DAP-R was determined for each of the DAP-S parental strains (VGS007, VGS008, and 32364. In brief, each parental strain was grown overnight in BHIB at 37°C and resuspending in fresh BHIB to an OD600 of 1, which corresponds to a density of ~10^8 cfu/ml. The cfu/ml of the original bacterial suspension was determined by serial dilution. In addition, 100 μl volumes of the suspension were plated evenly only BHI agar plates (supplemented with $CaCl_2$ 50 mg/L) containing increasing concentration of DAP (0, 2, 4, 8, 16, and 32 μg/ml). Plates were incubated at 37°C for 24h and examined for colony growth.

Bodipy (BDP)-DAP binding uptake and microscopy. BDP-DAP (Merck) is a fluorescent derivative of DAP which retains microbiologic activity. We used BDP-DAP at two different concentrations (4 and 64 μg/ml) to quantify the relative amounts of BDP-DAP bound to bacterial cells as previously described (2). Briefly, VGS cells were stained with BDP-DAP at indicated concentrations for 15 minutes at 37°C. Excess unbound BDP-DAP was removed, and cells were washed twice in Luria-Bertani (LB) broth. Fluorescence was observed employing fluorometry, using a standard FITC (fluorescein isothiocyanate) filter (excitation 490 nm and emission 528 nm). Protein extraction from each sample preparation was performed and estimated using the bicinchoninic acid (**BCA**) protein assay kit (Thermo Scientific) as instructed by the manufacturer. The relative fluorescence units/mg protein were then determined at each BDP-DAP concentration. Statistics were performed using Student's unpaired *t* test comparing DAP-R derivatives to DAP-S parental strains. A minimum of 2 experimental runs on separate days was carried out.

As a companion study, direct visualization of the relative accumulation and distributions of BDP-DAP binding to individual bacterial cells within a given chain (comparing DAP-S vs DAP-R strains) was performed by fluorescence microscopy, following BDP-DAP exposures as described above. For this microscopy, after BDP-DAP exposures, cells were counter-stained with propidium iodide (**PI**; 30 μM) for 15 min at 37°C prior to microscopy (to semi-quantify BDP-DAP-induced cell membrane permeabilization and cell death). Bacterial cells were viewed and images acquired using a Keyence BZ-X700 fluorescence microcope with PlanApo N 100X objective. All microscopy was performed by one of us (TT), blinded as to the initial identification of the isolate. A minimum of 2 high-powered fields was examined to provide an overall estimation of BDP-DAP and PI cell-to-cell distributions and fluorescence. Images of the most representative fields were then obtained (**see Figure 1D-F**).

Cell membrane phospholipid content, fluidity and surface charge. Lipid extractions from VGS strains were carried out as previously described (2). Three major membrane phospholipids (**PG, PA**, and **CL**) were separated by two-dimensional thin-layer chromatography (**2D-TLC**), digested, and quantified at OD_{660} as reported previously (2). Identification and confirmation of phospholipids were done by exposure to iodine vapors, spraying with $CuSO_4$ (100 mg/ml) containing 8% (vol/vol) phosphoric acid, and heating at 180°C (2). The identity of these phospholipids has been previously validated by both internal lipid standards on 2D-TLC, as well as by mass spectrometry (data not shown [2]).

Membrane fluidity assays were done using a protocol that incorporates the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (**DPH**) into target cell membranes. The relative insertion and three-dimensional orientation of this probe is then measured and quantified as fluorescent polarization indices by spectrometry (excitation and emission wavelengths of 360 and 426 nm), as described previously (2, 3). The extent of membrane fluidity is defined as the inverse of the polarization index (i.e., the lower the polarization index, the more fluid the membrane) (2). The mean indices (+/- SD) were obtained from a minimum of 3-5 independent experiments. Statistics were performed using unpaired Student's *t* test, and a *P* value of < 0.05 was considered statistically significant.

The cytochrome *c* binding assay was done as a surrogate indicator of the relative net positive surface charge of the VGS cells. Briefly, overnight grown cells were washed, incubated with 0.5 mg/ml of cytochrome *c* for 10 minutes, and the amount of cytochrome *c* that remained in the supernatant were determined spectrophotometrically as described previously (4). The more unbound cytochrome *c* in the supernatant indicated a relatively more positive surface charge.

Whole genome analysis.

Genomic DNA was extracted using DNAeasy (QIAGEN), and libraries were prepped using Nextera-XT library preparation kit from Illumina (San Diego, CA). Each genome was sequenced using a MiSeq machine in experiments designed to obtain over 100x coverage. Parental genomes were *de novo* assembled using CLC Genomics Workbench version 8.5, after trimming the reads for quality (Q20) and removing possible contamination from Illumina adapter sequences. Assembled parental genomes were annotated using RAST (5). Mapping of the DAP-R derivative reads against the DAP-S parental assemblies was performed with using BWA (6), followed by variant calling with three different programs: SamTools/BCFTools (7), GATK (8) and CLC Workbench Genomics low frequency variant detector. A consensus from the three callers was obtained using VCFTools (9) and only the genetic variants found by all three callers were investigated manually against the alignment of mapped reads, selecting those with at least a quality score above 40, and without heterogeneous signals. Annotation of the variants was done using SnpEFF (10) based on the annotations of the parental genomes. Then the genomes of the DAP-R derivatives were assembled as the parental to verify the presence of the genetic variants. To do so, the sequences of those genes associated with DAP-R were extracted for each case and aligned with Muscle (11). The alignments were manually inspected to confirm the detected genetic variants.

Mutant construction and genetic manipulation.

To generate a *pgsA* deletion mutant in *S. oralis* strain 351, a gene replacement cassette was constructed by cloning the chromosomal regions flanking *pgsA* upstream and downstream of the *cat* gene in pC326 (12). The resulting plasmid, pKO-*pgsA*, was introduced into *S. oralis* 351 by natural transformation as previously described (13). For generation of the *pgsA* gene replacement strains, we constructed a *cat-pgsA* transcriptional fusion cassette containing the

desired point mutation within the *pgsA* coding sequence. To this end, a 514-bp fragment encompassing the sequence immediately upstream of *pgsA* was PCR amplified using the primer pair *rodZ*-F and *rodZ*-R (Table S1), digested with XhoI and HindIII and cloned upstream of the *cat* gene in pC326. A 519-bp fragment encompassing the 5' end of *pgsA* together with 10-bp of the upstream intergenic region was PCR amplified with primer pair *pgsA*-F and *pgsA*-R (Table S1), digested with PstI And NotI and cloned downstream of the *cat* cassette generating plasmid p*cat-pgsA* . Site-directed mutagenesis was used to introduce either the G52S [GGT-AGC] or G65E[GGA-GAA] point mutation within the *pgsA* sequence of p*cat-pgsA* as previously described (14), generating plasmids p*cat-pgsA*^{G52S} and p*cat-pgsA*^{G65E}*.* Each plasmid was introduced separately to *S. oralis* 351 as described above, followed by plating the transformation mixture onto blood agar containing either 5 μg ml^{-1} chloramphenicol alone or chloramphenicol with 2 or 8 μg ml^{-1} of daptomycin. Genomic DNA was isolated from chloramphenicol-resistant clones, and the *pgsA* point mutation and adjacent cat insertion was confirmed by PCR analysis and DNA sequencing.

REFERENCES

1. Huband MD, Castanheira M, Farrell DJ, Flamm RK, Jones RN, Sader HS, Mendes RE. 2016. In vitro activity of dalbavancin against multidrug-resistant *Staphylococcus aureus* and streptococci from patients with documented infections in Europe and surrounding regions (2011-2013). Int J Antimicrob Agents 47:495–499.

2. Mishra NN, Tran TT, Seepersaud R, Garcia-de-la-Maria C, Faull K, Yoon A, Proctor R, Miro JM, Rybak MJ, Bayer AS, Arias CA, Sullam PM. 2017. Perturbations of phosphatidate cytidylyltransferase (CdsA) mediate daptomycin resistance in *Streptococcus mitis/oralis* by a novel mechanism. Antimicrob Agents Chemother 61.

3. Bayer AS, Prasad R, Chandra J, Koul A, Smriti M, Varma A, Skurray RA, Firth N, Brown MH, Koo SP, Yeaman MR. 2000. In vitro resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity. Infect Immun 68:3548–3553.

4. Mishra NN, Bayer AS, Weidenmaier C, Grau T, Wanner S, Stefani S, Cafiso V, Bertuccio T, Yeaman MR, Nast CC, Yang S-J. 2014. Phenotypic and genotypic characterization of daptomycin-resistant methicillin-resistant *Staphylococcus aureus* strains: relative roles of mprF and dlt operons. PLoS One 9:e107426.

5. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards R a., Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam a. R, Xia F, Stevens R. 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 42:D206–D214.

6. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760.

7. Li H. 2011. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27:2987–2993.

8. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20:1297–303.

9. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, McVean G, Durbin R. 2011. The variant call format and VCFtools. Bioinformatics 27:2156–2158.

10. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Ruden DM, Lu X. 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Landes Biosci 1–13.

11. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acid Res 32:1792–1797.

12. Mitchell J, Siboo IR, Takamatsu D, Chambers HF, Sullam PM. 2007. Mechanism of cell surface expression of the *Streptococcus mitis* platelet binding proteins PblA and PblB. Mol Microbiol 64:844–857.

13. Seo HS, Xiong YQ, Mitchell J, Seepersaud R, Bayer AS, Sullam PM. 2010. Bacteriophage lysin mediates the binding of *Streptococcus mitis* to human platelets through interaction with fibrinogen. PLoS Pathog 6:e1001047.

14. Seepersaud R, Bensing BA, Yen YT, Sullam PM. 2012. The accessory Sec protein Asp2

modulates GlcNAc deposition onto the serine-rich repeat glycoprotein GspB. J Bacteriol 194:5564–5575.

Table S1. Additional changes identified by whole-genome sequencing

Strain set[a]	Derivative[b]		Predicted gene	Predicted function
S. mitis				
VGS007		X	*oppA*	Oliopeptide ABC transporter protein
		X	*mutL*	DNA mismatch repair protein
		X	DNA-directed RNA polymerase	RNA synthesis
		X	*cbpD*	choline binding protein D
		X	*dltD*	D-alanyl-lipoteichoic acid biosynthesis protein
		X	tRNA nucleotidyltransferase	protein synthesis
VGS008		X	methylthioadenosine deaminase	cellular metabolism
		X	Type 1 restriction-modification system	bacterial protection
		X	*trkH*	potassium uptake protein
		X	*scpC*	serine endopeptidase
		X	*cbpA*	choline binding protein A
		X	cell surface protein precursor	cell surface protein precursor
		X	translation initiation factor 2	bacterial translation
S. oralis				
	D1	**D5**		
32364	X	X	β-hexosaminidase	cellular metabolism
	X	X	T1SS secreted agglutinin	bacterial protection
	X	X	PTS system	sugar metabolism
	X	X	RNA binding methyltransferase	RNA synthesis
	X	X	*fmtB*	cell wall-associated protein
	X	X	*recN*	DNA repair protein
	X	X	*yrrC*	RecD-like DNA helicase
	X		ECF transporter	nutrient uptake
	X	X	hypothetical protein	unknown function
	X		*hflX*	GTP-binding protein
	X	X	ABC transporter permease protein	cellular metabolism
		X	*msbA*	lipid A export protein
		X	*codY*	GTP-sensing transcriptional pleiotropic repressor
		X	ribonuclease	RNA metabolism
		X	*ciaH*	putative histidine kinase

[a] compared to daptomycin-susceptible VGS007, VGS008, and 32364P, respectively
[b] X indicates presence of mutation in daptomycin-resistant derivative

Supplementary Table 2. Oligonucleotides used for *pgsA* mutagenesis of *S. mitis* 351

Oligonucleotide	Sequence[a]
rodZ-F	CCTCGAGATCTCCCCTTGTTTTATTTC
rodZ-R	GATAAGCTTATTTTTTCCTTAATCTGTAGTAAATG
pgsA-F	GCCTGCAGGAAAAATCAATGAAAAAAGAAC
pgsA-R	TTGCGGCCGCCTTGAAATAATCATAGCCAGAG

[a] Sequence of primers are shown in the 5'-3' order, with incorporated restriction sites underlined

Figure S1. Alignment of PgsA of Gram-positive and Gram-negative pathogens. Alignment of PgsA from *S. aureus*, *B. subtilis*, *S. mitis*, *S. oralis*, *E. coli*, and *A. fulgidus*. The conserved motif $D_1xxD_2G_1xxAR…G_2xxxD_3xxxD_4$ is underlined. G52S(*S. oralis* 32364 numbering) mutation of *S. oralis* 32364-D1 is highlighted.

```
S. aureus          ------------------------------------------------------------------------------
B. subtilis        ------------------------------------------------------------------------------
S. mitis           ------------------------------------------------------------------------------
S. oralis          ------------------------------------------------------------------------------
S. oralis-G52S     ------------------------------------------------------------------------------
E. coli            ------------------------------------------------------------------------------
A. fulgidus        MHHHHHHHHHHSSGVDLGTENLYFQSNAMRLAYVKNHEIYGEKLLGLTLRERIEKTLQRAGFDVRFFDELSLEEAEDYLI

S. aureus          ------------------------------------------------------------------------------
B. subtilis        ------------------------------------------------------------------------------
S. mitis           ------------------------------------------------------------------------------
S. oralis          ------------------------------------------------------------------------------
S. oralis-G52S     ------------------------------------------------------------------------------
E. coli            ------------------------------------------------------------------------------
A. fulgidus        ILEPVLILERDLLLEGRKILVSDGFTVGYFFGGDFRTVFDGNLQSSIEKYLSLNNLESYEIWAIKLSNDNLKTAEKLLLS

S. aureus          ----------------------------MNIPNQITVFRVVLIPVFILFALVDFGFGNVSFLGGYEIRIELLISGFIFIL
B. subtilis        ----------------------------MFNLPNKITLARIALIPIFMIIMLAPFDWGRLE-VGDESIPVAHLAGAILFII
S. mitis           -------------------------MKKEQIPNLLTIGRILFIPIFIFILTIGNSIE------------SHIVAAIIFAV
S. oralis          -------------------------MKKEQIPNVLTIGRILFIPLFILILTLGHSQG------------SHLLATIIFAV
S. oralis-G52S     -------------------------MKKEQIPNVLTIGRILFIPLFILILTLGHSQG------------SHLLATIIFAV
E. coli            -------------------------MQFNIPTLLTLFRVILIPFFVLVFYLPVTW-------------SPFAAALIFCV
A. fulgidus        SLIKAKRTGLKPAYYDGWIAREINRKVSLRISRLLADTSVTPNQITVFSFFLSLVGSALFLLNSY---LTTLLAGVIIQL

S. aureus          ASLSDFVDGYLARKWNLVTNMGKFLDPLADKLLVASALIVLVQL--GLTNSVVAIIIIAREFAVTGLRLLQIEQG--FVS
B. subtilis        ASTTDWVDGYYARKLNLVTNFGKFLDPLADKLLVSAALIILVQF--DLAPAWMVIVIISREFAVTGLRLVLAGTG--EVV
S. mitis           ASITDYLDGYLARKWNVVSNFGKFADPMADKLLVMSAFIMLIEL--GMAPAWIVAVIICRELAVTGLRLLLVETGG-TVL
S. oralis          ASVTDYLDGYLARKWNVVSNFGKFADPMADKLLVMSAFIMLIEL--GMAPAWVVAIIICRELAVTGLRLLLVETGG-TVL
S. oralis-G52S     ASVTDYLDSYLARKWNVVSNFGKFADPMADKLLVMSAFIMLIEL--GMAPAWVVAIIICRELAVTGLRLLLVETGG-TVL
E. coli            AAVTDWFDGFLARRWNQSTRFGAFLDPVADKVLVAIAMVLVTEHYHSWWVTLPAATMIAREIIISALREWMAELGKRSSV
A. fulgidus        HSIIDGCDGEIARLKFMESKYGAWLDGVLDRY---SDFIIVFSITYVLSASNPVYWIIG--FLAAFASLMIAYTGDKFVA

S. aureus          AAGQLGKIKTAVTMVAITWLLLGDPLATLIGLSLGQILLYIGVIFTILSGIEYFYKGRDVFKQK
B. subtilis        AANMLGKIKTWAQIIAVSALLLHNLPFELVSFPFADLALWVAVFFTVVSGWEYFSKNWEALKTSN
S. mitis           AAAMPGKIKTFSQMFAIIFLLLHWTL-------LGQVLLYVALFFTIYSGYDYFKSSAYVFKGTFGSK
S. oralis          AAAMPGKIKTFSQMFAIIFLLLHWNL-------IGQLLLYIALFFTIYSGYDYFKGSAHVFKGTFGSK
S. oralis-G52S     AAAMPGKIKTFSQMFAIIFLLLHWNL-------IGQLLLYIALFFTIYSGYDYFKGSAHVFKGTFGSK
E. coli            AVSWIGKVKTTAQMVALAWLLWRPNIWVEY---AGIALFFVAAVLTLWSMLQYLSAARADLLDQ
A. fulgidus        AYMRTYSPEGFAIPITRDFRLLIIFACSVVN--LPSLALVIIALLGNFEALRRIVALRSYTN
```

Figure S2. Growth characteristic of *S. oralis* 32364 and its DAP-R derivatives.