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Mutations in *cdsA* and *pgsA* Correlate with Daptomycin Resistance in *Streptococcus mitis* and *S. oralis*

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ABSTRACT We investigated the ability of several recent clinical viridans group streptococci (VGS) bloodstream isolates (*Streptococcus mitis/S. oralis* subgroup) from daptomycin (DAP)-naive patients to develop DAP resistance *in vitro*. All strains rapidly developed high-level and stable DAP resistance. Substitutions in two enzymes involved in the cardiolipin biosynthesis pathway were identified, i.e., CdsA (phosphatidate cytidylyltransferase) and PgsA (CDP-diacylglycerol-glycerol-3-phosphate-3-phosphatidyltransferase). These mutations were associated with complete disappearance of phosphatidylglycerol and cardiolipin from cell membranes. DAP interactions with the cell membrane differed in isolates with PgsA versus CdsA substitutions.

KEYWORDS CdsA, PgsA, *Streptococcus mitis*, daptomycin resistance

A major problem associated with infections caused by viridans group streptococci (VGS) is their relative frequency of *in vitro* antimicrobial resistance to β-lactams and tolerance to vancomycin (1–4). Daptomycin (DAP) has been proposed as a potential alternative for severe infections caused by β-lactam-resistant *Streptococcus mitis/S. oralis* strains. We previously detailed the ability of a well-known strain of *S. oralis* (351) to rapidly develop stable, high-level DAP resistance (DAP-R) on exposure to the drug both *in vitro* and in an *in vivo* model of experimental infective endocarditis (5–7). (We use the term "daptomycin resistance" [DAP-R] rather than "daptomycin nonsusceptibility" in this paper for ease of presentation.)

The mechanism of resistance was a substitution in CdsA, a phosphatidate cytidylyltransferase that is a key enzyme for the first committed biosynthetic step within the cardiolipin (CL) biosynthesis pathway. The link between CdsA and DAP-R was shortly thereafter reported by a different group, thereby confirming our findings (8).

In the current study, we evaluated whether changes in CdsA would also be readily induced during *in vitro* DAP exposures of DAP-susceptible (DAP-S) clinical isolates of *S. mitis/S. oralis* recovered from the bloodstream of patients who had never received DAP (DAP naive). We included three recent clinical strains (*S. mitis* strains VGS007 and VGS008 and *S. oralis* strain 32364) obtained from bloodstream infections in DAP-naive patients (Table 1). All isolates were subjected to *in vitro* passage using brain heart

TABLE 1 Predicted amino acid changes in PgsA and CdsA and phospholipid analyses of *S. mitis/oralis* strains

Species and strain	DAP MIC (μg/ml)	Predicted amino acid changes and enzyme	% total phospholipid content (mean ± SD) of:		
			Phosphatidylglycerol	Cardiolipin	Phosphatidic acid
S. mitis					
VGS007	0.38	Parental	38 ± 5	47 ± 5	15 ± 7
VGS007-D8	>256	D249E in CdsA	0[a]	0[a]	100[a]
VGS008	0.38	Parental	29 ± 2	69 ± 4	2 ± 1
VGS008-D12	>256	R230H in CdsA	0[a]	0[a]	100[a]
S. oralis					
32364	1	Parental	19 ± 5	70 ± 3	11 ± 4
32364-D1	>256	G221V in CdsA, G52S in PgsA	0[a]	0[a]	100[a]
32364-D5	>256	G52S in PgsA	0[a]	0[a]	100[a]

[a]P value was <0.001 DAP-R for *S. mitis/S. oralis* strains versus DAP-S *S. mitis/S. oralis* parental strains.

infusion (BHI) broth with DAP 20 μg/ml and CaCl₂ 50 mg/liter to select for DAP-R derivatives. Spontaneous frequency of DAP-R evolution was also evaluated by using BHI agar (supplemented with CaCl₂) with increasing concentrations of DAP (0 to 32 μg/ml). Selected DAP-R derivatives and their DAP-S parental strains were then compared in regard to MICs, cell surface charge, membrane fluidity, cell membrane phospholipid compositional profiling, and DAP binding assays, as described previously (5). Whole-genome sequencing (WGS) was performed in DAP-S and DAP-R pairs to evaluate the genetic basis for DAP-R (see details in the supplemental material).

Of note, we were unable to recover DAP-R colonies of streptococci on any of the DAP-containing BHI agar plates as part of the spontaneous selection experiment (frequency <1 × 10⁻⁸ for all 3 strains). For the *in vitro* passage experiment, all *S. mitis/S. oralis* strains developed DAP-R (MIC, >256 μg/ml) rapidly on DAP exposure (Table 1). DAP-R derivatives arose after 1 to 8 days of exposure to DAP. The DAP-R phenotype was stable after 5 days of passage in antibiotic-free medium. We selected DAP-R derivatives at the end of the *in vitro* passage experiments for *S. mitis* strains VGS007 and VGS008. Two DAP-R derivatives of *S. oralis* strain 32364, D1 and D5, were included in this study to capture any genetic and phenotypic changes associated with early emergence of resistance. Growth curves indicated that the DAP-R derivatives had a marked decrease in fitness (see Fig. S2 in the supplemental material). We did not find any significant differences in cell surface charge or membrane fluidity between parental and DAP-R derivatives (data not shown). Comparative analysis of the relative content of the major cell membrane phospholipids indicated complete disappearance of phosphatidylglyc-erol (PG) and CL in all DAP-R derivatives compared with the parental controls (Table 1). The loss of the PG and CL content in DAP-R strains was accompanied by a significant increase in the relative content of phosphatidic acid (PA) (Table 1), as previously described (5).

WGS of DAP-S parental versus respective DAP-R derivatives disclosed several relevant mutations (Table 1; see also Table S1 in the supplemental material). Importantly, we identified substitutions in two major phospholipid enzymes, namely, CdsA and PgsA (Table 1). These enzymes are necessary for the synthesis of the major cell membrane phospholipids PG and CL, using PA as the initial substrate (9). We identified three substitutions in CdsA among the DAP-R derivatives of *S. mitis/S. oralis*, namely, G221V, R230H, and D249E. Based on the crystal structure of *Thermotoga maritima* (10), D249E is located within the HGGxxDRxD motif (amino acids [aa] 244 to 252), which is important for the binding of CTP and phosphatidate for the synthesis of CDP-diacylglycerol (a precursor of PG and CL), along with the SPxKxxEG motif (aa 169 to 176). Similarly, the predicted R230H substitution is located in a highly conserved residue located between these two important binding motifs. The G221V substitution is located adjacent to D222, which is predicted to form part of a cation-binding Asp-Asp dyad, previously shown to be essential for enzymatic activity (10). Taken together, these data led us to predict that these substitutions resulted in a major decrease in (or abolishment of) CdsA catalytic activity.

In contrast to the multiple mutations identified in CdsA, we found that the two DAP-R derivatives of *S. oralis* strain 32364 harbored the same G52S substitution in PgsA (Table 1). This enzyme is responsible for the synthesis of PG, using phosphatidylglycerol 3-phosphate as the substrate (9). Because PG is the precursor of CL, alterations in PgsA are also expected to modulate CL synthesis. Interestingly, a crystal structure of a representative CDP-alcohol phosphotransferase (CDP-AP) from *Archaeoglobus fulgidus* identified a conserved CDP-AP signature motif ($D_1xxD_2G_1xxAR...G_2xxxD_3xxxD_4$) within the active site of such enzymes (11). We performed an alignment of CDP-AP from *A. fulgidus* and PgsA sequences from *Staphylococcus aureus* (12), *Bacillus subtilis* (13), and *Escherichia coli* isolates (14) (see Fig. S1 in the supplemental material). The alignment revealed that the G52S substitution is located in this conserved hydrophilic subdomain, likely exposed to the cytoplasm, and present in several phospholipid synthesis enzymes, such as diacylglycerol cholinephosphotransferase from *E. coli* and ethanolaminephosphotransferase in *Saccharomyces cerevisiae*, among others (14). Structural characterization of a representative member of CDP-AP from *A. fulgidus* showed that Gly218, which is represented by G_1 of the motif, provides the necessary flexibility to the enzyme active site (11). Because the absence of PG in membrane preparations of *S. oralis* 32364-D5 was evident (Table 1), we postulated that the G52S substitution (G_1) abolishes the enzymatic synthesis of PG. Indeed, Peleg et al. (12) and Hachmann et al. (13) found that an adjacent substitution (A55V, using *S. oralis* 32364 numbering) was associated with DAP-R in *S. aureus* and *B. subtilis* isolates (Fig. S1); of note, decreased PG content has been strongly associated with the DAP-R phenotype in these latter organisms (13). Given the potential importance of the G52S mutation, we aimed to create a Δ*pgsA* or *pgsA*[G52S] variant of *S. oralis* strain 32364 by allelic replacement. After multiple attempts, this strain was found to be nontransformable, so mutagenesis was not feasible. We next applied the same strategies to *S. oralis* strain 351, which was used previously for the study of DAP-R (5). Again, introduction of *pgsA*[G52S] and deletion of *pgsA* were unsuccessful in this strain. These results suggest that *pgsA* plays an essential role in the cellular function of streptococci, as has been described for *Streptococcus sanguinis* and several other species (15). In our effort to establish a link between *pgsA* and DAP-R in *S. oralis*, we searched our collection of streptococci for mutated alleles in *pgsA*. Among isolates of streptococci, a DAP-R derivative (MIC, >256 μg/ml) of *S. oralis* SF100 was found to harbor a G65E mutation in *pgsA* (unpublished data). Thus, we attempted to introduce *pgsA*[G65E] into *S. mitis* 351, using the methods described above. This approach was eventually successful, but generation of the *pgsA*[G65E] mutation required DAP selection (8 μg/ml). Note that Gly65 (represented by G_2 in the conserved signature motif of CDP-AP) and Gly52 are conserved residues that form the surface of the ligand-binding pocket of CDP-APs. Mutations in these residues were found to render the enzyme inactive in *S. cerevisiae* isolates (16). The difficulty we encountered in mutating *pgsA* is consistent with previous studies in *S. sanguinis*, *Streptococcus mutans*, *E. coli*, and *B. subtilis* isolates (13, 15, 17, 18). Indeed, work in *E. coli* isolates has shown that a *pgsA*-null mutant was only viable when a functional copy of the *pgsA* gene was carried on a plasmid (19). Likewise, an allelic replacement of *pgsA*[A55V] was not possible in a wild-type strain of *B. subtilis* until a wild-type *pgsA* was introduced (reversion of *pgsA*[A55V] to wild type) to the DAP-R derivative, which harbors other changes associated with the resistance phenotype (13). Nevertheless, our effort to introduce a mutated *pgsA* into an *S. oralis* isolate resulted in a DAP-R phenotype. Although we cannot rule out changes in other genes that contribute to the phenotype, our results and others published previously confirm the link between *pgsA* and DAP-R (12, 13, 20). Our data also suggest the occurrence of multiple pathways of phospholipid adaptation leading to marked changes in cell membrane content of streptococci on antibiotic challenge.

We previously showed that mutations in CdsA were associated with increased DAP binding but only to select cells within the overall population. This unique phenotype had not been described in relation to cationic antimicrobial peptide resistance (5). Thus, using Bodipy FL-labeled DAP (BDP-DAP), we investigated the ability of this antibiotic to
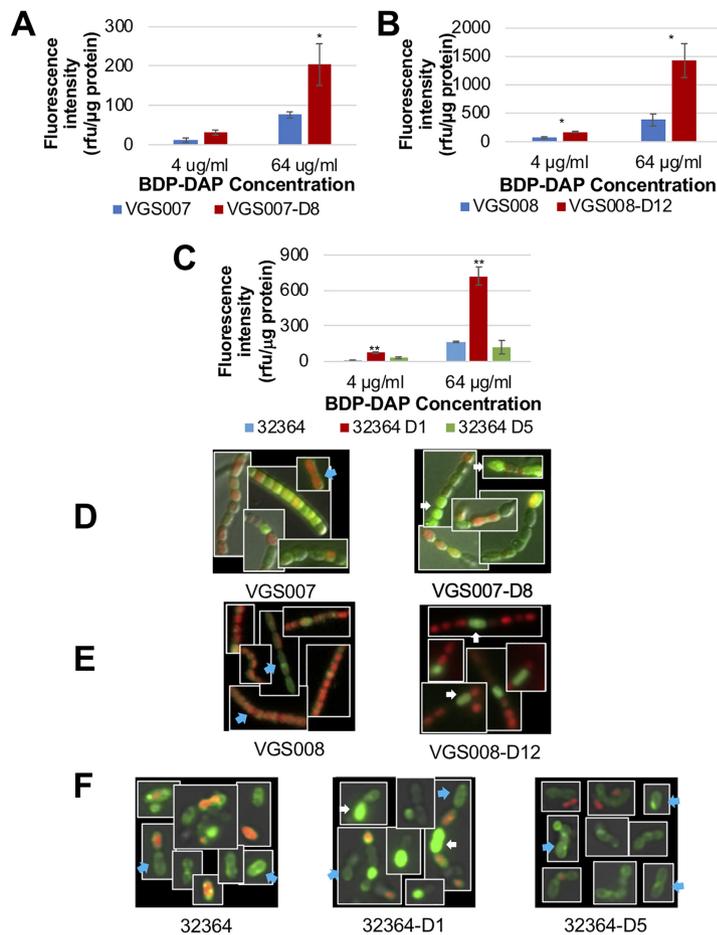
FIG 1 BDP-DAP binding of *S. mitis* and *S. oralis*. Fluorescence intensity was normalized to protein ratio, using BDP-DAP binding to *S. mitis* VGS007 and VGS007-D8 (A), *S. mitis* VGS008 and VGS008-D12 (B), and *S. oralis* 32364, 32364-D1, and 32364-D5 (C). The increased binding of BDP-DAP is seen in DAP-R derivatives with mutations in *cdsA* compared to its DAP-S parental isolate. BDP-DAP staining (64 μg/ml) of *Streptococcus* cells is shown in green, propidium iodide (red), and overlay of DAP-S parental and DAP-R derivatives of VGS007 (D), VGS008 (E), and 32364 (F). Representative of uniform/septal binding is indicated by blue arrows, and hyperaccumulative binding is indicated by white arrows. *, $P < 0.05$; **, $P < 0.01$.

bind to cell membranes of parental clinical isolates and their DAP-R derivatives, as measured by fluorescence intensities. Figure 1A to C shows that all DAP-R derivatives of *S. mitis* strains (which harbor mutations in *cdsA*) exhibited a statistically significant increase in binding of BDP-DAP, especially at higher DAP concentrations (64 μg/ml). In contrast, the DAP-R derivatives of *S. oralis* strain 32364 (D1 and D5, Table 1), exhibited a distinct phenotype of BDP-DAP binding. DAP-R isolate obtained from day 1 of *in vitro* serial passage (D1) had a pattern of increased BDP-DAP similar to that seen in our other DAP-R *S. mitis* strains. However, the binding pattern of the DAP-R derivative obtained after 5 days of *in vitro* passage (D5), which lacks mutations in CdsA but harbors a G52S substitution in PgsA, was quite different, with a trend toward lower DAP binding, albeit not statistically significant (Fig. 1C). Our results suggest that, as seen with CdsA, mutations in PgsA result in loss of PG and CL synthesis. Unlike CdsA mutations, however, the PgsA mutation additionally appears to minimally impact the binding of DAP within the target cell membrane.

In the parallel fluorescence microscopy studies of DAP-R derivatives of strains VGS007 and VGS008 (harboring CdsA substitutions alone), DAP localization was similar to that seen in our previously published studies (5), namely, selected hyperaccumulation of BDP-DAP (64 μg/ml) in individual cells throughout the chain length compared

to the uniform or septal binding of BDP-DAP in DAP-S parentals (Fig. 1D and E). We also used propidium iodide (PI) to assess cell viability after exposure to DAP. The PI images revealed that in contrast to its rather uniform uptake in DAP-S cells exposed to the antibiotic, DAP-R strains exhibited more focal uptake of PI, with a higher proportion of cells failing to take the dye, suggesting that these cells remained viable after DAP exposure (Fig. 1D and E). In contrast, for the *S. oralis* 32364-D5 derivative, which has only a substitution in PgsA, uniform binding of BDP-DAP with occasional cells stained with PI was identified (Fig. 1F). This pattern, along with data from the BDP-DAP binding assay (Fig. 1C), suggested a reduction in DAP binding to the cell membrane, in a manner similar to that of DAP-R *B. subtilis* associated with PgsA mutations (13). The DAP-R 32364-D1 derivative, which harbors substitutions in both CdsA and PgsA, exhibited a mixed pattern of hyperaccumulation and uniformed binding of BDP-DAP (Fig. 1F). Taken together, our observations suggest that distinct mechanisms of DAP-R associated with alteration in CdsA (hyperaccumulation) versus PgsA in different *S. mitis/S. oralis* strains. The exact mechanism of DAP-R (diversion, repulsion, or hyperaccumulation) associated with *pgsA* is unclear and is the object of future investigations.

In summary, our findings highlight the malleability of the VGS cell membrane in response to cationic peptide-induced stress and the multiple complex pathways involved in the emergence of DAP-R in these organisms.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .01531-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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