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Transcriptional Profiling Reveals that Daptomycin Induces the *Staphylococcus aureus* Cell Wall Stress Stimulon and Genes Responsive to Membrane Depolarization▽†

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Daptomycin is a lipopeptide antibiotic that has recently been approved for treatment of gram-positive bacterial infections. The mode of action of daptomycin is not yet entirely clear. To further understand the mechanism transcriptomic analysis of changes in gene expression in daptomycin-treated *Staphylococcus aureus* was carried out. The expression profile indicated that cell wall stress stimulon member genes (B. J. Wilkinson, A. Muthaiyan, and R. K. Jayaswal, Curr. Med. Chem. Anti-Infect. Agents 4:259–276, 2005) were significantly induced by daptomycin and by the cell wall-active antibiotics vancomycin and oxacillin. Comparison of the daptomycin response of a two-component cell wall stress stimulon regulator VraSR mutant, *S. aureus* KVR, to its parent N315 showed diminished expression of the cell wall stress stimulon in the mutant. Daptomycin has been proposed to cause membrane depolarization, and the transcriptional responses to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and nisin were determined. Transcriptional profiles of the responses to these antimicrobial agents showed significantly different patterns compared to those of the cell wall-active antibiotics, including little or no induction of the cell wall stress stimulon. However, there were a significant number of genes induced by both CCCP and daptomycin that were not induced by oxacillin or vancomycin, so the daptomycin transcriptome probably reflected a membrane depolarizing activity of this antimicrobial also. The results indicate that inhibition of peptidoglycan biosynthesis, either directly or indirectly, and membrane depolarization are parts of the mode of action of daptomycin.

Daptomycin is an acidic cyclic lipopeptide antibiotic derived from the fermentation of *Streptomyces roseosporus* (61). The agent has bactericidal activity against many clinically relevant gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *S. aureus*, coagulase-negative staphylococci, penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant enterococci, and is finding clinical application (2, 61).

Despite significant effort over the past 20 years, there is some lingering uncertainty as to the mode of action of daptomycin. The earliest suggestion was that daptomycin inhibited peptidoglycan biosynthesis in gram-positive bacteria, although the specific reaction that was inhibited was not identified (3). In later work it was proposed that daptomycin inhibited the biosynthesis of another cell surface molecule, lipoteichoic acid (6, 12), although subsequent work failed to support this idea (32). However, a number of other early studies pointed to a membrane-based mode of action for daptomycin (1, 18, 34). Daptomycin has been shown to cause calcium-dependent membrane depolarization of (1, 57) and potassium release from (57) *S. aureus*. A model for the bactericidal action of daptomycin involving oligomerization of daptomycin and disruption of the functional integrity of the cytoplasmic membrane has been proposed (57).

In recent years, novel methods for gaining insights into the mode of action of an antimicrobial agent have been developed. Proteomic and transcriptomic approaches measure changes in protein production and gene transcription, respectively (5, 27, 58), in response to challenge with antimicrobial agents. Transcriptional profiling studies showing the induction of a cell wall stress stimulon that includes genes encoding proteins involved in peptidoglycan biosynthesis in *S. aureus* and other bacteria in response to challenge with cell wall-active antibiotics have been described (28, 31, 63, 66). A considerable number of the cell wall stress stimulon member genes were part of a regulon under the control of the two-component regulator VraSR (28, 31, 66).

In this paper we report the results of transcriptional profiling studies of the action of daptomycin on *S. aureus*. The cell wall antibiotics oxacillin and vancomycin, the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and the pore former and inhibitor of the peptidoglycan biosynthesis lipid II intermediate nisin were included for comparison. Daptomycin clearly induced the cell wall stress stimulon, in contrast to CCCP and nisin, suggesting that inhibition of peptidoglycan biosynthesis is part of the mode of action of daptomycin. However, a considerable number of genes were induced by both daptomycin and CCCP, consistent with a membrane-depolarizing activity of daptomycin also.

(Parts of this work were presented previously at the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy [44].)

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MATERIALS AND METHODS

Strains and antibiotics. *S. aureus* strain ATCC 29213 was provided by Cubist Pharmaceuticals (Lexington, MA), and the *S. aureus vraSR* mutant KVR, derived from *S. aureus* N315 (31), was provided by K. Hiramatsu. Strain SH1000 was also used in some experiments. Daptomycin (Cubist Pharmaceuticals), CCCP and oxacillin (Sigma Chemicals, St. Louis, MO), vancomycin (Lilly Laboratories), and nisin (Danisco Beaminster Ltd., Beaminster, United Kingdom) were used for transcriptional profiling.

MIC determinations. MICs of daptomycin, vancomycin, nisin, and CCCP were determined for *S. aureus* ATCC 29213 and SH1000 according to the Clinical and Laboratories Standards Institute (CLSI) (14). MIC determinations were carried out using Ca^{2+}-adjusted (50 mg liter^{-1}) Mueller-Hinton broth (MHBc) (57). CCCP stock was prepared in dimethyl sulfoxide, and further working concentrations were prepared using MHBc. MIC determinations were carried out on three different occasions in duplicate.

GIC studies. Transcriptional profiling studies are typically carried out by adding the agent under study to mid-exponential-phase cultures. Given that significantly larger number of organisms are used in transcriptional profiling studies than in an MIC determination, we consider that the growth-inhibitory concentration (GIC) has more relevance than the MIC for transcriptional profiling studies. Accordingly, we first determined the GIC as a prelude to transcriptional profiling. Overnight-grown cultures were used to inoculate (1% vol/vol) 20 ml MHBc medium in a 50-ml Erlenmeyer flask and were grown at 37°C with shaking at 200 rpm. Antimicrobial agents were added to the culture in log phase (optical density at 600 nm, ~0.4). Growth was measured at 600 nm at regular intervals in a Beckman DU65 spectrophotometer. Three concentrations of daptomycin (1, 5, and 10 μg ml^{-1}), CCCP (2, 4, and 10 μg ml^{-1}), nisin (7.5, 15, 20, and 25 μg ml^{-1}), and vancomycin (2, 4, and 10 μg ml^{-1}) were used to study their GICs for strain ATCC 29213. Daptomycin concentrations of 0.8, 2, and 4 μg ml^{-1} were used for the *vraSR* mutant KVR and 1, 4, and 10 μg ml^{-1} for parent strain N315.

Transcriptional profiling. Overnight-grown *S. aureus* ATCC 29213 was inoculated in MHBc medium (20 ml) in a 50-ml Erlenmeyer flask and incubated at 37°C, with shaking at 200 rpm. Growth was measured at regular intervals at 600 nm until the optical density was ~0.4. Based on the GIC study, the following concentrations of antimicrobial agents were added separately for 15 min of challenge: daptomycin, 4 μg ml^{-1}; vancomycin, 10 μg ml^{-1}; CCCP, 2 μg ml^{-1}; and nisin, 20 μg ml^{-1}. These concentrations were chosen because they gave a similar degree of inhibition of growth. For comparison purposes, strain SH1000 was challenged with 1.2 μg oxacillin ml^{-1} for 15 min. Strain SH1000, which is an 8325-line strain with the SigB defect corrected, is our standard strain for transcriptional profiling studies (47, 63). Control cultures were not challenged with antimicrobial agents and were also incubated for 15 min. For transcriptional profiling of the *vraSR* mutant strain KVR, 2 μg daptomycin μl^{-1} was used, and 10 μg ml^{-1} was used for the KVR parent strain N315.

RNA extraction and purification. Total bacterial RNA was extracted from 3 ml of culture which was mixed with 6 ml of bacterial RNA Protect solution (Qiagen, Valencia, CA), and the mixture was centrifuged at 3000 rpm for 20 min in a swinging-bucket rotor centrifuge to collect the cells. Pellets were resuspended in 1 ml of Trizol (Invitrogen, Grand Island, NY) and the cells were broken by using the FastPrep system (Qbiogene, Irvine, CA) at a speed of 6.0 for 40 s. From the broken-cell lysate, RNA was extracted as per the manufacturer's instructions. Extracted RNA was purified with an RNeasy minikit (Qiagen). DNase-treated and purified mRNA was used for microarray analysis.

Microarray hybridization and processing. cDNA was generated by using random hexamers (Invitrogen) as primers for reverse transcription. The primers were annealed (70°C for 10 min, followed by snap-freezing in ice for 1 min) to total RNA (2 μg) and were extended with SuperScript II reverse transcriptase (Invitrogen) with 0.1 M dithiothreitol–12.5 mM deoxynucleoside triphosphate–5-(3-aminoallyl)-dUTP mix (Ambion, Austin, TX) at 42°C overnight. Residual RNA was removed by alkaline treatment followed by neutralization, and cDNA was purified with a QIAquick PCR purification kit (Qiagen). Purified aminoallyl-modified cDNA was then labeled with Cy3 or Cy5 monofunctional *N*-hydroxy-succinimide ester cyanogen dyes (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Labeled cDNA was purified using a Qiagen PCR purification kit, and the purified labeled cDNA was hybridized with *S. aureus* genome microarrays version 2.0, provided by the Pathogen Functional Genomics Resource Center of the National Institutes of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). The full genome array consists of 2,592 open reading frames (ORFs) from *S. aureus* reference strain COL plus an additional 117 ORFs from strains Mu50, MW2, and N315 that are not present in the COL strain's genome complement. Each ORF is printed in triplicate on the array.

Microarray data analysis. Hybridization signals were scanned using an Axon4000B scanner (Molecular Devices, Sunnyvale, CA) with Acuity 4.0 software, and scans were saved as TIFF images. Scans were analyzed using TIGR-Spotfinder (www.tigr.org/software/) software, and the local background was subsequently subtracted. The data set was normalized by applying the LOWESS algorithm using TIGR-MIDAS (www.tigr.org/software/) software. The normalized $\log_2$ ratio of test to reference signal for each spot was recorded. Genes with fewer than three data points were considered unreliable, and their data points were discarded. The averaged $\log_2$ ratio for each remaining gene on the six replicate slides was ultimately calculated. Significant changes in gene expression were identified with SAM (significance analysis of microarrays; http://www-stat.stanford.edu/~tibs/SAM/index.html) (62) software using one class mode. SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, i.e., the false discovery rate. A cutoff of 1.5-fold for over- and underexpressed ORFs was used (26). To examine how genes with transcript level changes are distributed with regard to their function, we further classified these genes using our in-house software Gene Sorter according to the categories described in the comprehensive microbial resource of TIGR (http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi). Several controls were employed to minimize the technical and biological variations and to ensure that the data obtained were of good quality. First, each ORF was present in triplicate on the array. Second, each RNA preparation was used to make probes for at least two separate arrays for which the incorporated dye was reversed. Finally, three independent cultures were used to prepare RNA samples.

Microarray data accession number. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE9494.

RESULTS AND DISCUSSION

Antibiotic concentrations for transcriptional profiling. The following MICs were determined for strain ATCC 29213: daptomycin, 1 μg ml^{-1}; CCCP, 2 μg ml^{-1}; nisin, 7.5 μg ml^{-1}; and vancomycin, 2 μg ml^{-1}. The result for daptomycin with this strain was within the published CLSI quality control range (14). The daptomycin MIC for the *vraSR* mutant KVR was 0.78 μg ml^{-1}, and that for its parent, N315, was 1 μg ml^{-1}. Based on the MICs, GICs were determined for the antimicrobials to choose the concentration of agent and duration of treatment for transcriptional profiling studies (Fig. 1 and 2).

As shown in Fig. 1a, 5 and 10 μg daptomycin ml^{-1} caused complete inhibition of growth of strain ATCC 29213. However, the VraSR$^-$ mutant strain KVR was significantly more susceptible to daptomycin than its parent strain N315 (Fig. 2). GIC determinations for vancomycin, CCCP, and nisin are shown in Fig. 1b to d, respectively. The following concentrations of agents were chosen for 15 min of challenge in transcriptional profiling experiments with strain ATCC 29213: daptomycin, 4 μg ml^{-1}; vancomycin, 10 μg ml^{-1}; CCCP, 2 μg ml^{-1}; and nisin, 20 μg ml^{-1}. For comparison purposes, strain SH1000 was challenged for 15 min with 1.2 μg oxacillin ml^{-1} (47, 63).

The daptomycin transcriptome reveals induction of the cell wall stress stimulon. Four hundred seventy-four ORFs were overexpressed (see Table S1 in the supplemental material), and 395 ORFs (see Table S3 in the supplemental material) were underexpressed in strain ATCC 29213 challenged with 4 μg daptomycin ml^{-1} for 15 min. Genes showing more than twofold overexpression upon daptomycin challenge and the expression of these genes in cells challenged wiht other agents, and in the VraSR mutant KVR versus its parent strain N315,

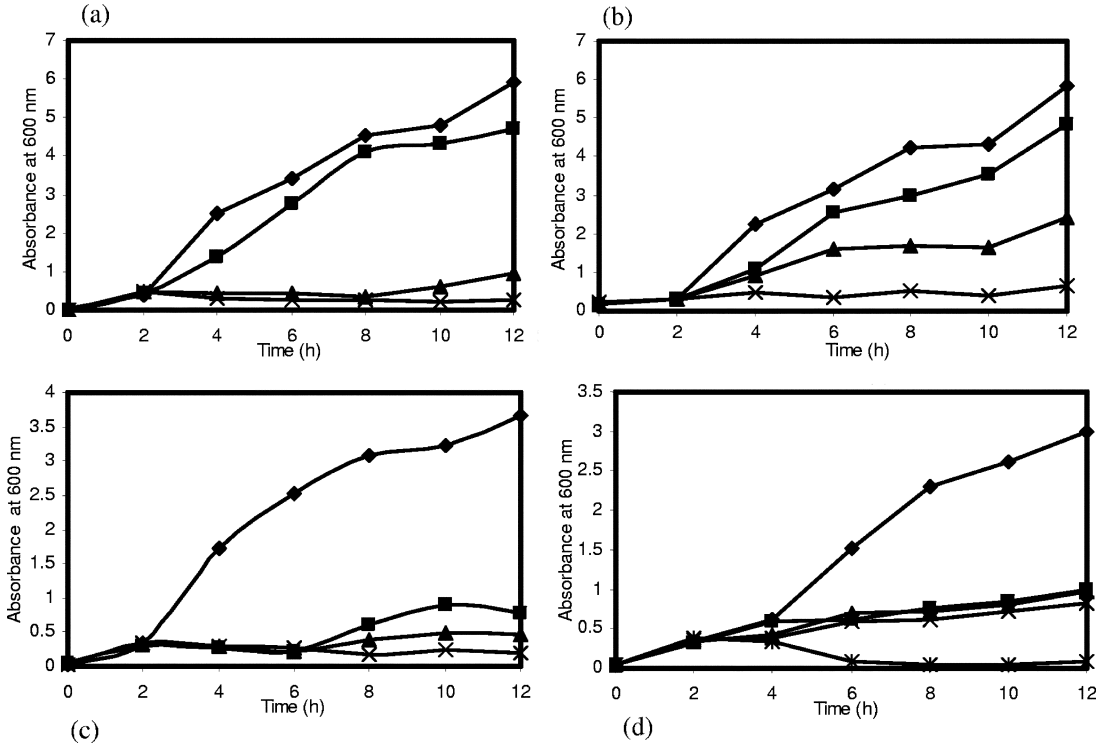

FIG. 1. GICs of the antimicrobial agents for *S. aureus* strain ATCC 29213. (a) Daptomycin. ◆, control; ■, 1 μg ml^{-1}; ▲, 5 μg ml^{-1}; ×, 10 μg ml^{-1}. (b) Vancomycin. ◆, control; ■, 2 μg ml^{-1}; ▲, 4 μg ml^{-1}; ×, 10 μg ml^{-1}. (c) CCCP. ◆, control; ■, 2 μg ml^{-1}; ▲, 4 μg ml^{-1}; ×, 10 μg ml^{-1}. (d) Nisin. ◆, control; ■, 7.5 μg ml^{-1}; ▲, 15 μg ml^{-1}; ×, 20 μg ml^{-1}; ∗, 25 μg ml^{-1}.

are shown in Table 1 and Table S2 in the supplemental material. Genes in a variety of categories were altered in their expression, including a large number encoding hypothetical proteins which are shown in Tables S1 and S3 in the supplemental material. Previous transcriptional profiling studies of the response of *S. aureus* revealed the induction of a cell wall stress stimulon by cell wall-active agents (31, 63, 66). Cell wall stress stimulon member genes are currently defined as genes altered in their expression by oxacillin, D-cycloserine, and bacitracin (63) and those shown to be controlled by VraSR upon vancomycin challenge of *S. aureus* (31). Previously designated and likely (on the basis of their induction by daptomycin, oxacillin and vancomycin) cell wall stress stimulon member genes induced by daptomycin are highlighted in Table 1 and in Tables S2 and S3 in the supplemental material.

In our initial study of the cell wall stress stimulon (63), two prominent categories of cell wall stress stimulon member genes were Cell Envelope-Related and Protein Fate. About 32 cell envelope-related genes were overexpressed upon daptomycin challenge, and recognized cell wall stress stimulon member genes include *pbpB*, *murAB*, *murI*, and *tcaA*. *pbpB* encodes penicillin-binding protein 2, which is an important *S. aureus*

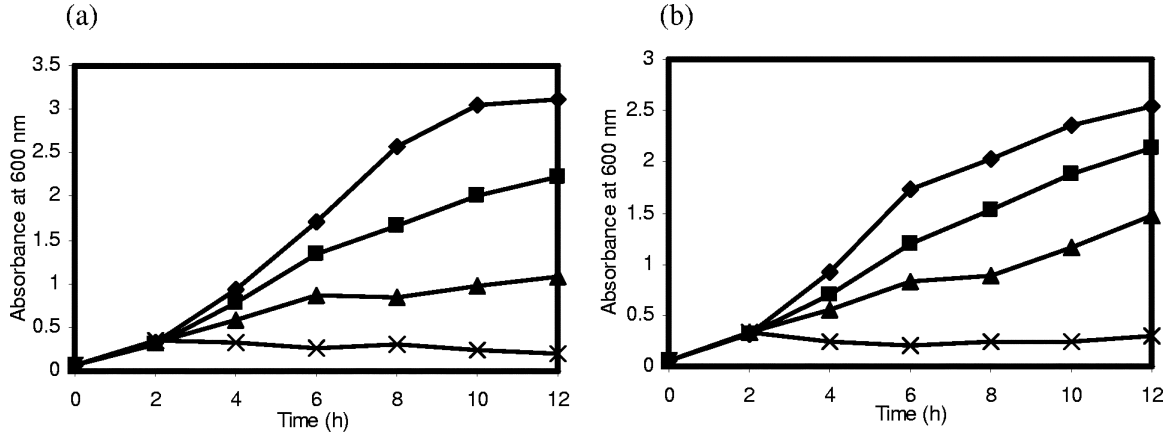

FIG. 2. Daptomycin GICs for *S. aureus* strains N315 (a) and KVR (b). (a) ◆, control; ■, 1 μg ml^{-1}; ▲, 4 μg ml^{-1}; ×, 10 μg ml^{-1}. (b) ◆, control; ■, 0.8 μg ml^{-1}; ▲, 2 μg ml^{-1}; ×, 4 μg ml^{-1}.

TABLE 1. Genes upregulated by daptomycin and their response status with other antibiotics

Category and locus[b]	Gene	Protein	Subcategory	Change (fold) in expression[a]					
				Dap	Van	Oxa	CCCP	Nis	KVR[c]
Amino acid biosynthesis									
SA1362	*hom*	Homoserine dehydrogenase	Aspartate family	5.73	4.20	3.80	15.91	0.00	−2.51
SA1430	*dapA*	Dihydrodipicolinate synthase	Aspartate family	3.76	0.00	0.00	29.98	0.00	−2.12
SA1429	*asd*	Aspartate-semialdehyde dehydrogenase	Aspartate family	3.24	2.80	1.95	24.56	0.00	−1.80
SA1363	*thrC*	Threonine synthase	Aspartate family	3.06	3.20	2.80	21.48	0.00	−2.65
SA1431	*dapB*	Dihydrodipicolinate reductase	Aspartate family	2.66	1.90	2.10	27.63	0.00	0.00
SA1364	*thrB*	Homoserine kinase	Aspartate family	2.48	0.00	2.80	19.62	0.00	−2.08
SA0600	*ilvE*	Branched-chain amino acid aminotransferase	Pyruvate family	2.18	3.41	0.00	5.52	0.00	−1.59
SA0503	NA	*trans*-sulfuration enzyme family protein	Serine family	2.80	2.62	1.69	−2.23	0.00	0.00
SA0557	*cysK*	Cysteine synthase	Serine family	2.28	0.00	0.00	0.00	0.00	−2.09
SA0502	NA[d]	Cysteine synthase/cystathionine beta-synthase family protein	Serine family	2.00	0.00	0.00	0.00	0.00	0.00
Cell envelope									
SA2451	NA	Amino acid ABC transporter, amino acid-binding protein	Other	2.65	2.25	0.00	6.75	0.00	0.00
SA1522	NA	Elastin binding protein, putative	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	2.39	0.00	0.00	5.24	0.00	−2.42
SA1066	NA	Fmt	Biosynthesis of murein sacculus and peptidoglycan	4.93	2.36	5.44	−3.13	0.00	0.00
SA2116	*murAB*	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase 2	Biosynthesis of murein sacculus and peptidoglycan	4.14	2.58	4.29	0.00	2.18	−2.54
SA0248	*lrgB*	LrgB	Biosynthesis of murein sacculus and peptidoglycan	3.56	0.00	0.00	6.62	0.00	0.00
SA2352	*tcaA*	TcaA	Biosynthesis of murein sacculus and peptidoglycan	3.03	3.06	7.16	−2.08	0.00	−1.89
SA0247	*lrgA*	Holin-like protein LrgA	Biosynthesis of murein sacculus and peptidoglycan	2.70	0.00	0.00	14.85	0.00	0.00
SA1490	*pbp2*	Penicillin-binding protein 2	Biosynthesis of murein sacculus and peptidoglycan	2.21	1.74	2.97	0.00	0.00	−2.59
SA0263	*lytM*	Peptidoglycan hydrolase	Biosynthesis of murein sacculus and peptidoglycan	2.20	0.00	0.00	0.00	0.00	0.00
SA2451	NA	Amino acid ABC transporter, amino acid-binding protein	Other	2.65	2.25	0.00	6.75	0.00	0.00
SA2554	NA	Membrane protein, putative	Other	2.03	0.00	−1.62	0.00	5.40	−2.65
Cellular processes									
SA2570	NA	Galactoside *O*-acetyltransferase	Toxin production and resistance	2.89	0.00	0.00	2.28	0.00	0.00
SA2413	NA	Drug resistance transporter, EmrB/QacA subfamily	Toxin production and resistance	2.69	0.00	0.00	1.85	0.00	−1.60
SA0754	*norA*	Multidrug resistance protein (NorA)	Toxin production and resistance	2.09	0.00	−1.58	0.00	0.00	−1.62
SA2252	NA	AcrB/AcrD/AcrF family protein	Toxin production and resistance	2.04	0.00	0.00	0.00	0.00	0.00
SA1003	NA	Negative regulator of competence MecA, putative	DNA transformation	2.96	0.00	2.19	5.90	0.00	−1.34
SA1891	NA	RNA III-activating protein TRAP	Pathogenesis	2.05	1.35	0.00	5.19	0.00	−1.98
SA1759	NA	Universal stress protein family	Adaptations to atypical conditions	2.74	2.30	1.90	11.61	2.05	−3.16
SA0958	NA	General stress protein 13	Adaptations to atypical conditions	2.14	1.90	1.80	1.87	0.00	0.00
SA1010	*relA1*	GTP pyrophosphokinase	Adaptations to atypical conditions	2.07	0.00	2.15	5.43	0.00	0.00
SA2075	*ftsW*	Cell division protein, FtsW/RodA/SpoVE family	Cell division	2.03	1.61	2.30	−2.30	−1.74	0.00
SA0876	NA	Arsenate reductase, putative	Detoxification	2.04	0.00	0.00	0.00	0.00	0.00
SA1003	NA	Negative regulator of competence MecA, putative	DNA transformation	2.96	0.00	2.19	5.90	0.00	−1.34

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TABLE 1—*Continued*

Category and locus[b]	Gene	Protein	Subcategory	Change (fold) in expression[a]					
				Dap	Van	Oxa	CCCP	Nis	KVR[c]
SA1645	NA	*comE* operon protein 2	DNA transformation	2.05	0.00	0.00	6.80	0.00	0.00
SA1440	NA	XpaC, putative	Other	3.36	2.04	1.56	4.82	0.00	−1.76
SA2126	*luxS*	Autoinducer-2 production protein LuxS	Other	2.14	1.51	0.00	0.00	0.00	0.00
SA0672	*sarA*	Staphylococcal accessory regulator A	Pathogenesis	3.81	1.60	2.83	−1.95	1.65	−1.86
SA1891	NA	RNA III-activating protein TRAP	Pathogenesis	2.05	1.35	0.00	5.19	0.00	−1.98
SA2419	*hlgA*	Gamma-hemolysin, component A	Toxin production and resistance	10.18	2.90	2.20	6.63	0.00	0.00
SA2712	*drp35*	drP35 protein	Toxin production and resistance	8.87	6.60	0.00	0.00	0.00	−8.04
SA2422	*hlgB*	Gamma hemolysin, component B	Toxin production and resistance	5.04	2.20	1.80	13.77	0.00	0.00
SA2421	*hlgC*	Gamma hemolysin, component C	Toxin production and resistance	3.52	1.80	1.70	8.97	0.00	0.00
SA1441	NA	Tellurite resistance protein, putative	Toxin production and resistance	3.42	1.35	0.00	5.45	0.00	−1.72
SA2570	NA	Galactoside *O*-acetyltransferase	Toxin production and resistance	2.89	0.00	0.00	2.28	0.00	0.00
SA0746	*norR*	Transcriptional regulator, MarR family	Toxin production and resistance	2.70	1.91	−1.53	−6.32	−1.58	0.00
SA2413	NA	Drug resistance transporter, EmrB/QacA subfamily	Toxin production and resistance	2.69	0.00	0.00	1.85	0.00	−1.60
SA0754	*norA*	Multidrug resistance protein (NorA)	Toxin production and resistance	2.09	0.00	−1.58	0.00	0.00	−1.62
SA2252	NA	AcrB/AcrD/AcrF family protein	Toxin production and resistance	2.04	0.00	0.00	0.00	0.00	0.00
Protein fate									
SA0570	*clpC*	ATP-dependent Clp protease, ATP-binding subunit ClpC, authentic frameshift	Degradation of proteins, peptides, and glycopeptides	3.98	1.62	1.90	4.48	0.00	−2.47
SA0979	*clpB*	ATP-dependent Clp protease, ATP-binding subunit ClpB	Degradation of proteins, peptides, and glycopeptides	3.87	1.80	2.30	5.82	0.00	−2.69
SA2563	NA	ATP-dependent Clp protease, putative	Degradation of proteins, peptides, and glycopeptides	3.68	2.10	1.90	7.15	6.28	−2.66
SA1777	NA	Serine protease HtrA, putative	Degradation of proteins, peptides, and glycopeptides	3.06	3.43	5.39	0.00	0.00	−2.60
SA0833	*clpP*	ATP-dependent Clp protease, proteolytic subunit ClpP	Degradation of proteins, peptides, and glycopeptides	2.97	2.01	1.50	1.74	0.00	−1.59
SA0595	NA	Peptidase, M20/M25/M40 family	Degradation of proteins, peptides, and glycopeptides	2.37	0.00	0.00	1.88	0.00	0.00
SA2463	*pepA2*	Glutamyl-aminopeptidase	Degradation of proteins, peptides, and glycopeptides	2.09	0.00	0.00	3.79	0.00	−2.06
SA1005	*pepF*	Oligoendopeptidase F	Degradation of proteins, peptides, and glycopeptides	2.06	1.99	1.80	0.00	−3.23	0.00
SA1897	NA	Protein export protein PrsA, putative	Protein and peptide secretion and trafficking	9.59	4.46	3.88	0.00	0.00	−6.29
SA0969	*spsB*	Signal peptidase IB	Protein and peptide secretion and trafficking	2.56	1.82	3.94	−2.76	0.00	−1.48
SA0418	NA	MttA/Hcf106 family protein	Protein and peptide secretion and trafficking	2.14	0.00	0.00	0.00	0.00	0.00
SA2016	*groEL*	Chaperonin, 60 kDa	Protein folding and stabilization	3.81	0.00	0.00	3.22	0.00	−2.02
SA1637	*dnaK*	DnaK	Protein folding and stabilization	3.68	1.76	1.54	3.75	0.00	−2.39
SA1638	*grpE*	Heat shock protein GrpE	Protein folding and stabilization	3.28	1.90	1.79	2.31	0.00	−1.90
SA2017	*groES*	Chaperonin, 10 kDa	Protein folding and stabilization	2.84	2.20	2.30	2.12	0.00	0.00
SA0556	NA	Chaperonin, 33 kDa	Protein folding and stabilization	2.15	1.89	2.30	0.00	0.00	1.78
SA1271	*hslU*	Heat shock protein HslVU, ATPase subunit HslU	Protein folding and stabilization	2.12	0.00	1.76	2.55	0.00	−1.61
SA2385	NA	Heat shock protein, Hsp20 family	Protein folding and stabilization	2.04	0.00	1.70	1.83	0.00	0.00
SA1459	NA	Peptide methionine sulfoxide reductase, degenerate	Protein modification and repair	3.08	2.36	4.57	0.00	0.00	−2.64
SA1034	NA	Lipoate-protein ligase A family protein	Protein modification and repair	2.48	0.00	0.00	2.68	0.00	0.00

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TABLE 1—*Continued*

Category and locus[b]	Gene	Protein	Subcategory	Change (fold) in expression[a]					
				Dap	Van	Oxa	CCCP	Nis	KVR[c]
Regulatory functions									
SA1639	*hrcA*	Heat-inducible transcription repressor HrcA	DNA interactions	3.58	1.53	0.00	2.51	0.00	−2.32
SA2147	NA	Transcriptional antiterminator, BglG family/DNA-binding protein	DNA interactions	2.39	−2.98	0.00	0.00	−2.34	−1.52
SA2517	NA	Transcriptional regulator, MerR family	DNA interactions	2.18	2.75	4.89	0.00	0.00	−2.38
SA2349	NA	Transcriptional regulator, TetR family	DNA interactions	2.14	0.00	0.00	0.00	0.00	0.00
SA0837	*gapR*	Gap transcriptional regulator	DNA interactions	2.09	0.00	0.00	5.00	3.51	−1.57
SA2302	NA	Transcriptional regulator, putative	Other	3.57	3.83	4.70	2.18	0.00	−3.39
SA1398	NA	Transcriptional regulator, putative	Other	3.17	2.24	4.26	−2.19	0.00	−1.75
SA1003	NA	Negative regulator of competence MecA, putative	Other	2.96	0.00	2.19	5.90	0.00	−1.34
SA1891	NA	RNA III-activating protein TRAP	Protein interactions	2.05	1.35	0.00	5.19	0.00	−1.98
SA2147	NA	Transcriptional antiterminator, BglG family/DNA-binding protein	DNA interactions	2.39	−2.98	0.00	0.00	−2.34	−1.52
SA1003	NA	Negative regulator of competence MecA, putative	Other	2.96	0.00	2.19	5.90	0.00	−1.34
SA1891	NA	RNA III-activating protein TRAP	Protein interactions	2.05	1.35	0.00	5.19	0.00	−1.98
Signal transduction									
SA1457	NA	PTS system, IIA component	PTS	2.92	2.88	5.44	3.37	0.00	−3.39
SA2148	NA	PTS system, mannitol-specific IIA component	PTS	2.11	2.80	2.30	0.00	−2.30	−2.17
SA0759	NA	PTS system, fructose-specific IIABC component, authentic frameshift	PTS	2.11	0.00	0.00	0.00	22.52	0.00
SA1457	NA	PTS system, IIA component	PTS	2.92	2.88	5.44	3.37	0.00	−3.39
SA1942	*vraR*	DNA-binding response regulator VraR	Two-component systems	10.39	3.09	6.61	0.00	0.00	−11.67
SA1943	*vraS*	Sensor histidine kinase VraS	Two-component systems	9.57	6.15	7.00	0.00	0.00	−17.33

[a] Expression increases and decreases for up- and down-regulated genes, respectively. 0.00 indicates data not available or no change. Dap, daptomycin; Van, vancomycin; Oxa, oxacillin; Nis, nisin.
[b] Boldface indicates genes reported as cell wall stress stimulon members by Utaida et al. (63) and/or Kuroda et al. (31).
[c] Daptomycin-challenged strain KVR versus daptomycin-challenged strain N315.
[d] NA, not available.

penicillin-binding protein (51) that has also been shown to be up-regulated in its expression by vancomycin using microarrays (31) and by Northern blotting (7). *murAB* encodes UDP-*N*-acetylglucosamine 1-carboxylvinyl transferase 2, which catalyzes the first committed step of peptidoglycan biosynthesis (38). *murI* encodes glutamate racemase, and Pucci et al. (52) have shown that this enzyme is involved in D-glutamate biosynthesis, a component of *S. aureus* peptidoglycan. *tcaA* is implicated in teicoplanin resistance in *S. aureus* and may be involved in cell wall biosynthesis (9). Other genes induced by daptomycin involved in cell wall biosynthesis were *glmM*, encoding phosphoglucosamine mutase; putative teichoic acid biosynthesis genes *tagA* (UDP-*N*-acetyl-D-mannosamine transferase), *tagX*, *tagG*, and SA0238; *femX*; and SA2074, encoding D-alanine-D-alanine ligase.

Related to the cell envelope, classified under Cellular Processes, are *ftsW*, *divIB*, *ftsZ*, and SA0555 and SA0551, encoding cell division proteins. Also classified in this category are *bacA* (encoding bacitracin resistance protein) and *drp35*, previously shown to be inducible by cell wall-active antibiotics (43, 66).

Another prominent category into which cell wall stress stimulon member genes fall is Protein Fate, which includes chaperones and proteases (31, 63). Known cell wall stress stimulon member genes induced by daptomycin included *clpC* (chaperone/protease), *clpB* (chaperone/protease), *spsA* and *spsB* (type 1 signal peptidases A and B), *prsA* (peptidyl-prolyl *cis*/*trans* isomerase), *htrA* (heat shock protease), and *msrA* (methionine sulfoxide reductase). In addition, genes encoding the major heat shock proteins GroEL, GroES, DnaK, and DnaJ had increased expression. Treatment of cells with cell wall-active antibiotics is believed to result in the accumulation of damaged, misfolded, and aggregated proteins (58, 63, 66).

A variety of genes involved in DNA metabolism were up-regulated by daptomycin challenge (see Table S2 in the supplemental material). *recU* is a cell wall stress stimulon member gene, and upregulation of genes involved in DNA metabolism is now recognized as a feature of β-lactam action on *S. aureus* (37) and on *Escherichia coli* (42).

The TRAP (target of RNA III-activating protein) gene was induced by daptomycin. This protein was shown to be induced in a proteomic study of cell wall-active antibiotic action in *S. aureus* (58). TRAP plays an important role in signal transduction and expression of virulence factors in *S. aureus* (4) and may be involved in cell lysis of *S. aureus* (68).

The genes encoding the LrgA and LrgB proteins were up-regulated. Rice and Bayles (53) have proposed that these proteins act as antiholins that regulate the activity of the holin CidA and CidB proteins, which enhance peptidoglycan hydrolase activity and β-lactam sensitivity. Induction of *lrgA* and *lrgB* along with downregulation of expression of the major autolysin *atl* can be viewed as a response of the cell to preserve peptidoglycan when faced with the challenge of a cell wall-active agent (66).

Genes in a wide variety of functional categories were down-regulated by daptomycin challenge (see Table S3 in the supplemental material), including those in Purines, Pyrimidines, Nucleosides and Nucleotides; Energy Metabolism; Transport; Binding Proteins; and Protein Synthesis. Genes SA0270, SA2291, and SA2581, encoding proteins involved in biosynthesis of the characteristic carotenoid pigment of *S. aureus* staphyloxanthin, were underexpressed 3.5- to 7.1-fold. We suspect that downregulation of carotenoid biosynthesis genes is an attempt by the cell to shut down this branch of the isoprenoid biosynthetic pathway (48) and divert isoprene units to the biosynthesis of undecaprenol to increase the biosynthesis of peptidoglycan (35). In this context, *bacA*, which encodes undecaprenol kinase, was up-regulated by daptomycin (see Table S1 in the supplemental material). Decreased production of carotenoids (staphyloxanthin) would be expected to impact membrane fluidity, increasing it according to Chamberlain et al. (13), which may affect the interaction of daptomycin with the membrane. As noted previously (63), the gene encoding the cell's major autolysin (*atl*) was down-regulated by daptomycin, as was *lytS*. Also down-regulated was SA2298, encoding *N*-acetylmuramoyl-L-alanine amidase. Presumably the cell reacts to protect peptidoglycan from destruction by autolytic peptidoglycan hydrolases (36, 63, 66).

The gene expression pattern of *vraSR* mutant KVR in response to daptomycin confirms the induction of the cell wall stress stimulon by daptomycin. Two highly significant genes that were increased in expression 10.4- and 9.6-fold were *vraR* and *vraS*, which are classified under Signal Transduction. VraSR is a two-component signal transduction system that is induced by cell wall-active agents and controls the expression of a regulon of which many cell wall stress stimulon genes are members (31, 63, 66). Homologs with a similar function quite specific to cell wall-active agents are present in a wide variety of gram-positive bacteria (28). This constitutes strong evidence that daptomycin induces the cell wall stress stimulon, and it has been proposed that inhibition of peptidoglycan synthesis is somehow sensed by VraS and signal transduction to VraR involves the membrane (28, 47, 66). VraR is believed to be a transcriptional regulator. The VraSR regulon induced by cell wall-active antibiotics is part of the cell wall stress stimulon (8, 20, 31, 66). Concentrations of daptomycin giving similar partial inhibition of growth were added to strains KVR and N315 for 15 min. In this experiment, daptomycin-challenged KVR was used as the experimental sample and daptomycin-challenged strain N315 was used as the control. Thus, if a gene is up-regulated in strain N315 but is not up-regulated or is up-regulated to a lesser extent in strain KVR, it will appear as an underexpressed gene.

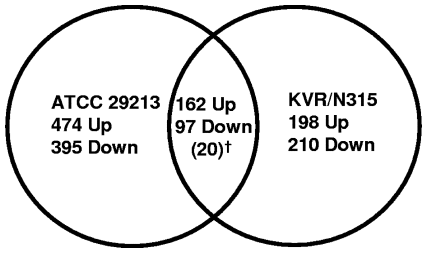

FIG. 3. Comparison of daptomycin-induced genes in *S. aureus* ATCC 29213 and *S. aureus* N315 *vraSR* mutant KVR. †, genes reported by Kuroda et al. (31).

The results showed that in the *vraSR* mutant KVR, 198 and 210 ORFs were up- and down-regulated, respectively, and all the important cell wall stress stimulon members that were controlled by *vraSR* were down-regulated (Table 1; see also Tables S2 and S3 in the supplemental material). In addition, we found that 249 of the 408 genes induced or repressed by daptomycin in KVR were also altered in their expression in daptomycin-treated ATCC 29213. These included 20 genes from various functional groups, including *cysK*, *thrC*, *thrB*, *asd*, *dapA*, *ilvE*, *opuD2*, *recU*, *prsA*, *htrA*, *vraS*, *vraR*, *murAB*, *pbp2*, *tcaA*, *drp35*, the teichoic acid biosynthesis protein gene (SA0238), the MerR family transcriptional regulator gene (SA2517), and a glycerate kinase gene (SA2435), which were shown by Kuroda et al. (31) to be under VraSR control. These genes were up-regulated in daptomycin-treated ATCC 29213 and down-regulated in KVR. In addition, we found that another 162 and 97 genes from different functional groups were up- and down-regulated, respectively, in daptomycin-treated ATCC 29213, which has an intact *vraSR* background, whereas expression of these genes was vice versa in KVR due to the absence of functional VraSR (Fig. 3). This indicates that the two-component system VraSR plays an important role in the induction of the cell wall stress stimulon by daptomycin. On the other hand, 14 genes were up- or down-regulated regardless of the *vraSR* status in both strains. These genes were directly induced by daptomycin without the participation of the *vraSR* system and thus appear not to be controlled by this system. Previous studies of various cell wall-active antibiotics revealed that in addition to the cell wall stress stimulon, each agent altered expression of additional genes that were uniquely responsive to that agent (31, 63).

Comparison of the daptomycin transcriptome with the oxacillin and vancomycin transcriptomes. In comparable experiments with the cell wall-active antibiotics vancomycin (strain ATCC 29213, 4 μg ml^{-1}, 15 min) and oxacillin (strain SH1000, 1.2 μg ml^{-1}, 15 min), 95 and 143 ORFs were overexpressed

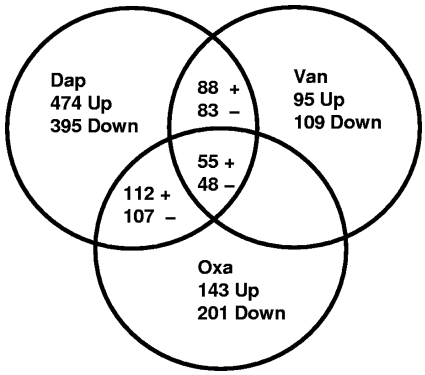

FIG. 4. Venn diagram analysis of genes induced in response to daptomycin (Dap) and cell wall-active antibiotics vancomycin (Van) and oxacillin (Oxa) (+, up-regulated genes; −, down-regulated genes).

and 109 and 201 ORFs were underexpressed in response to vancomycin (see Table S4 in the supplemental material) and oxacillin (see Table S5 in the supplemental material), respectively.

Comparison of daptomycin and vancomycin expression profiles revealed that 88 and 83 ORFs were up- and down-regulated, respectively, by both antibiotics. Similar comparison of daptomycin and oxacillin revealed 112 and 107 ORFs up- and down-regulated by both agents. When ORFs common to all three antibiotics were compared, 55 and 48 ORFs were up- and down-regulated (Fig. 4).

The expression responses of genes shown to be up-regulated by daptomycin to oxacillin and vancomycin are presented in Table 1 and in Table S2 in the supplemental material. The oxacillin and vancomycin transcriptomes observed are compatible with previous reports (31, 63). The expression of *vraS* and *vraR* was increased strongly by all three agents. In addition, *murAB*, *pbpB*, *tcaA*, and various *tag* genes were up-regulated by all three agents. Other cell wall stress stimulon member genes and VraSR regulon genes increased in expression by all three agents included *recU*, *spsB*, SA1457 (encoding a phosphotransferase [PTS] system IIA component), SA1459 (encoding methionine sulfoxide reductase) (58), *drp35*, and *bacA*. *atl* was down-regulated 3.1-, 2.1-, and 3.1-fold by daptomycin, vancomycin, and oxacillin, respectively (see Table S3 in the supplemental material). From these results it is clear that daptomycin causes an induction of the cell wall stress stimulon, similarly to the other cell wall-active antibiotics.

Transcriptomes of the membrane-active agents CCCP and nisin. Given the reported effects of daptomycin on membrane depolarization, we determined the changes in gene expression due to the membrane-active agents CCCP and nisin (57). CCCP had dramatic effects resulting in the overexpression of 450 ORFs and underexpression of 500 ORFs (see Table S6 in the supplemental material). Nisin treatment resulted in the over- and underexpression of 100 and 180 ORFs, respectively (see Table S7 in the supplemental material).

CCCP caused massive increases in expression of a considerable number of genes involved in amino acid biosynthesis, with particularly dramatic effects on some aspartate family genes—more than 20-fold induction in some cases. ORFs in the following categories, as well as a large number of ORFs encoding hypothetical proteins, were up-regulated: Fatty Acid and Phospholipid Metabolism, Biosynthesis of Cofactors, Prosthetic Groups, and Carriers, Central Intermediary Metabolism, Transport and Binding Proteins, DNA Metabolism, Protein Synthesis, Protein Fate, Regulatory Functions, Signal Transduction, Cell Envelope, Cellular Process, and Enzymes of Unknown Function.

Various genes encoding glycolysis pathway proteins were up-regulated, perhaps as a response to depletion of the proton gradient. Fuchs et al. (19) have reported that glycolysis genes are up-regulated on switching *S. aureus* from aerobic to anaerobic growth conditions. *kdpA* and *kdpB*, which encode the A and B subunits, respectively, of the potassium-transporting ATPase, were heavily induced. The two-component sensor response genes *saeS* and *saeR* were heavily induced by CCCP, as was the sensor gene *kdpD*. Genes encoding various components of gamma hemolysin were induced more than sixfold. Interestingly, daptomycin treatment resulted in significant induction of *hlgA*, *hlgB*, and *hlgC*, which encode components of gamma hemolysin. Kuroda et al. (30) have described the SaeRS regulon and shown that it includes gamma hemolysins. This two-component regulation system also shows some up-regulation by β-lactams and vancomycin (30). However, *vraS* and *vraR*, controllers of the cell wall stress regulon, were not induced.

Large numbers of genes in various categories were down-regulated by CCCP, and all in all CCCP had a massive impact on the transcriptome of the organism, which is perhaps understandable in that it depletes the proton gradient, which is fundamental to cellular bioenergetics.

CCCP is an uncoupler that collapses the pH and electrical gradients by shuttling protons in their protonated form across the cell membrane, releasing protons inside the cell (22). Patton et al. (46) have reported that CCCP significantly enhanced the transcription of *lrgAB*, encoding putative antiholin proteins. It was proposed that reduction in $\Delta\Psi$ was the main component of the proton motive force that increased *lrgAB* transcription. *lrgA* and *lrgB* were also significantly induced by daptomycin, whereas they were not induced by oxacillin or vancomycin (or nisin). This example of overlap between the daptomycin and CCCP transcriptomes suggests that in addition to inducing the cell wall stress stimulon, daptomycin also causes membrane depolarization. Other examples of overlap in the daptomycin and CCCP transcriptomes include induction, by both agents, of: various aspartate family genes, the TRAP gene, gamma hemolysin genes, *dinP*, various energy metabolism genes, and several transport and binding protein genes, including *kdpA*, *kdpB*, and *kdpC*.

Nisin had much less dramatic effects on gene expression than CCCP. A number of genes encoding nitrate and nitrite reductase (*narG*, *narB*, *narH*, *narJ*, and *narI*) and the transcriptional regulator gene *nirR* were up-regulated 4.5- to 12-fold. Other anaerobic metabolism genes heavily up-regulated were *pflB*, encoding formate acetyltransferase, and *pflA*, encoding pyruvate formate-lyase-activating enzyme. A number of glycolysis/gluconeogenesis genes were up-regulated. The sugar PTS system fructose-specific IIABC component was up-regulated 22-fold. *murAA* and *murAB* were also up-regulated. A considerable number of genes involved in energy metabolism and transport and binding proteins were down-regulated.

Nisin is a lantibiotic antimicrobial peptide that contains intramolecular rings formed by the thioether amino acids lanthionine and 3-methyllanthionine (55). It has been proposed that nisin inserts into the membrane and forms a short-lived pore, resulting in depolarization of the membrane and the rapid efflux of cytoplasmic ions, amino acids, and nucleotides (17). It is believed that nisin uses the lipid II molecule of peptidoglycan biosynthesis as a docking molecule for subsequent pore formation (10, 11). In addition to pore formation, nisin is believed to inhibit peptidoglycan biosynthesis through its interaction with lipid II (24, 65). Perhaps surprisingly, in our transcriptional profiling studies we did not find that nisin induced the cell wall stress stimulon. This might indicate that the changes in transcription due to nisin challenge may be more reflective of pore formation and membrane depolarization than inhibition of peptidoglycan biosynthesis, especially early in the interaction of nisin with susceptible cells. Hasper et al. (24) have proposed that nisin kills bacteria primarily by forming pores, whereas other lantibiotics too short to span the bacterial bilayer inhibit peptidoglycan biosynthesis by removing lipid II from its functional location.

Waite and Hutkins (64) have reported that treatment of *Listeria monocytogenes* with nisin led to the efflux of intracellular metabolites, in particular phosphoenolpyruvate, and adenine nucleotides. This may have significant bearing on the induction of glycolysis, nitrate reduction, and fermentation genes by nisin in the present study.

There was not a great deal of overlap between the genes altered in their expression by CCCP or nisin and the cell wall-active agents oxacillin and vancomycin (Table 1; see also Table S2 in the supplemental material). The critical controller of the cell wall stress stimulon *vraSR* was not induced by CCCP or nisin.

The mode of action of daptomycin appears to include inhibition of cell wall synthesis. Genome-wide transcriptional responses to challenge with antimicrobial agents are now accepted as a source of information on the mode of action of an agent (27). In the present study, daptomycin showed a clear induction of the cell wall stress stimulon, a characteristic of antimicrobial agents that cause inhibition of peptidoglycan biosynthesis (31, 63, 66). A significant proportion of the cell wall stress stimulon genes are members of the VraSR regulon, a two-component system that appears to be dedicated to responding to inhibition of peptidoglycan biosynthesis in gram-positive bacteria (28, 66). The non-pore-forming cationic antimicrobial peptide Lcn972 is known to inhibit peptidoglycan biosynthesis and has been shown to upregulate the expression of *cesSR*, a *vraSR* homolog in *Lactococcus lactis* (39). Dermicidin, like daptomycin, is an anionic peptide. However, the dermicidin transcriptome bears little or no resemblance to that of daptomycin (33), indicating that the daptomycin transcriptome is not simply due to its anionic nature. These results indicate that part of the mode of action of daptomycin includes a cell wall mode of action. In this respect, it recalls earlier studies that indicated that daptomycin inhibited peptidoglycan biosynthesis (3).

An effect of daptomycin on cell wall synthesis is compatible with previous electron microscopy studies of the effect of daptomycin treatment on the morphology of *S. aureus* and other gram-positive bacteria (23, 57). Formation of aberrant division septa was a characteristic effect of daptomycin treatment of *S. aureus*, *Enterococcus faecalis*, and *Bacillus subtilis*, in addition to species-specific effects probably reflective of the unique modes of cell wall growth in these three genera. Furthermore, Stubbings et al. (60) in a proteomic investigation of the mode of action of daptomycin on *S. aureus* showed that VraR and other member proteins of the cell wall stress stimulon were up-regulated and concluded that interference with peptidoglycan biosynthesis may be part of the mode of action of daptomycin. In recent transcriptional profiling studies of *B. subtilis*, it has been shown that daptomycin induces *liaRS* and its regulon and genes responsive to membrane depolarization (21). LiaSR is a homolog of VraSR.

Currently the general consensus for the mode of action of daptomycin is that it binds to the cytoplasmic membrane in a Ca^{2+}-dependent manner and oligomerizes in the membrane, leading to an efflux of potassium from the cell (57, 59). Jung et al. (29) and Straus and Hancock (59) have proposed a modified model of daptomycin action. It is proposed that in the presence of Ca^{2+} daptomycin oligomerizes to form a 14- to 16-mer and arranges itself into a micelle. It is proposed that in close proximity to the membrane, the multimer dissociates and daptomycin inserts into the membrane, and possibly oligomerization occurs. Induction of positive membrane curvature with potential disruption of membrane function is proposed to be particularly significant in this model.

Our studies have shown that daptomycin induces the cell wall stress stimulon, which is known to occur upon the inhibition of peptidoglycan biosynthesis. It is not clear whether this occurs through the direct interaction of daptomycin with an enzyme or substrate in the peptidoglycan biosynthetic pathway or whether it is a secondary effect due to its interaction with the membrane causing structural alterations that impair the normal function of the membrane.

Overlap in the daptomycin and CCCP transcriptomes is compatible with a membrane-depolarizing action of daptomycin. Some genes were induced by both daptomycin and CCCP but not by oxacillin or vancomycin. These included genes involved in aspartate biosynthesis, *lrgA*, *lrgB*, gamma hemolysin genes, energy metabolism genes, protein fate genes, and genes encoding various transport and binding proteins. Clearly, the daptomycin transcriptome may also reflect a membrane-depolarizing action of this antimicrobial agent. It should be noted that CCCP did not induce the cell wall stress stimulon including *vraSR*, the two-component regulator responding to inhibition of peptidoglycan biosynthesis. Hence, our studies are compatible with the idea that the daptomycin mode of action includes both inhibition of peptidoglycan biosynthesis and membrane depolarization. A similar dual mode of action has also been suggested for the lipoglycopeptide telavancin (25). Comparison of daptomycin- and telavancin-induced transcriptional changes may be of great interest. Mascio et al. (40) have reported that daptomycin is bactericidal against stationary-phase and other nondividing bacteria. Such bacteria are not expected to actively biosynthesize peptidoglycan, and the membrane-depolarizing activity of daptomycin is more likely to be responsible for its activity against them.

Pietiäinen et al. (50) have reported that in a transcriptional profiling study in *B. subtilis*, various antimicrobial peptides

(cationic peptides in this case) elicited complex stress responses compatible with multiple modes of action.

Relation of induction of the cell wall stress stimulon by daptomycin to decreased daptomycin susceptibility of vancomycin-intermediate *S. aureus* (VISA). A number of recent reports have presented evidence that a decrease in the susceptibility of *S. aureus* to vancomycin is accompanied by a decrease in susceptibility to daptomycin (16, 45, 54, 56, 67).

The mechanism of vancomycin resistance in VISA is far from clear (49). A thickened cell wall is common to many VISA strains (15), and clearly a thickened cell wall may impede the access of daptomycin to the cytoplasmic membrane. However, McAleese et al. (41) have reported that a VISA strain (MIC, 8 $\mu g\ ml^{-1}$) from a patient had increased expression of a significant number of cell wall stress stimulon member genes in drug-free medium compared to a vancomycin-susceptible parent strain. Thus, increased expression of the cell wall stress stimulon correlates with decreased vancomycin susceptibility, possibly due to a more robust peptidoglycan synthesis machinery. Given that the mode of action of daptomycin appears to include an effect on cell wall synthesis, the decreased daptomycin susceptibilities of VISA strains could be due to increased peptidoglycan biosynthesis in these strains. In connection with this, Mwangi et al. (45) have reported that the *vraR* operon is mutated in diverse VISA strains. Clearly, these mutations, which are correlated with decreased vancomycin susceptibility, may also play a role in decreased daptomycin susceptibility in such strains, given the likely part inhibition of peptidoglycan biosynthesis plays in the mode of action of daptomycin.

Conclusion. The potential dual mode of action of daptomycin as revealed by transcriptional profiling studies is compatible with previous reports invoking both cell wall inhibition and membrane depolarization as modes of action of daptomycin.

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