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Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon

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The molecular events following inhibition of bacterial peptidoglycan synthesis have not been studied extensively. Previous proteomic studies have revealed that certain proteins are produced in increased amounts upon challenge of *Staphylococcus aureus* with cell-wall-active antibiotics. In an effort to further those studies, the genes upregulated in their expression in response to cell-wall-active antibiotics have been identified by genome-wide transcriptional profiling using custom-made Affymetrix *S. aureus* GeneChips™. A large number of genes, including ones encoding proteins involved in cell-wall metabolism (including *pbpB*, *murZ*, *fmt* and *vraS*) and stress responses (including *msrA*, *htrA*, *psrA* and *hslO*), were upregulated by oxacillin, D-cycloserine or bacitracin. This response may represent the transcriptional signature of a cell-wall stimulon induced in response to cell-wall-active agents. The findings imply that treatment with cell-wall-active antibiotics results in damage to proteins including oxidative damage. Additional genes in a variety of functional categories were upregulated uniquely by each of the three cell-wall-active antibiotics studied. These changes in gene expression can be viewed as an attempt by the organism to defend itself against the antibacterial activities of the agents.

INTRODUCTION

Staphylococcus aureus is a leading cause of hospital- and community-acquired infection. In the pre-antibiotic era, the mortality associated with serious systemic staphylococcal infections was 80 % (Smith & Vickers, 1960). Methicillin-resistant *S. aureus* (MRSA) strains that have a tendency to acquire multiple antibiotic resistance determinants are now very common in hospitals. Two recent developments have further raised the anxiety level about antibiotic-resistant *S. aureus*. First, MRSA-associated infections, which were unusual in community-onset *S. aureus* infections, are occurring more frequently (Baba *et al.*, 2002). Second, since 1997, strains showing reduced susceptibility to vancomycin, the sole antibiotic to which *S. aureus* remained uniformly susceptible, have been encountered in various countries around the world (Hiramatsu *et al.*, 2002). However, *S. aureus* strains with high-level vancomycin resistance have been described with a vancomycin minimum inhibitory concentration (MIC) of greater than 128 μg ml^{-1} (Anon, 2002). Given the above information, there is a need for increased knowledge about the mechanism(s) of action and resistance to cell-wall-active antibiotics, and for the development of new agents active against *S. aureus*.

It is axiomatic that cell-wall-active antibiotics inhibit bacterial growth by inhibiting peptidoglycan biosynthesis (Gale *et al.*, 1982; Walsh, 2003). However, following inhibition of peptidoglycan biosynthesis, various secondary cellular responses are possible: cells may lyse, cells may die and lyse, cells may die without lysis or growth may be inhibited but the cells survive (tolerance) (Mychajlonka *et al.*, 1980). The molecular events occurring after treatment of bacteria with a cell-wall-active antibiotic have not been studied extensively. Mychajlonka *et al.* (1980) showed that the gross synthesis of RNA and protein is inhibited subsequent to inhibition of peptidoglycan biosynthesis by a cell-wall-active antibiotic. However, subsequent work by Jablonski & Mychajlonka (1988) showed increased production of certain proteins in response to oxacillin treatment of a tolerant strain of *S. aureus*. Recently, Singh *et al.* (2001a) used a proteomic approach to study proteins produced by *S. aureus* in response to cell-wall-active antibiotics. Two-dimensional gel electrophoresis of oxacillin-treated

Abbreviation: PBP, penicillin-binding protein.

Details of the genes upregulated by treatment of *S. aureus* cultures with oxacillin (Table I), bacitracin (Table II) and D-cycloserine (Table III) are available in *Microbiology* Online.

S. aureus revealed at least nine proteins that were produced in elevated amounts compared to control cultures. Five of the proteins produced in response to oxacillin were identified by N-terminal sequencing. This set of proteins appeared to be produced in response to other cell-wall-active antibiotics, but not antibiotics affecting other cellular targets (Singh *et al.*, 2001a), suggesting there may be a 'proteomic signature' (VanBogelen *et al.*, 1999) characteristic of the cellular response to cell-wall-active antibiotics.

The development of DNA microarray technology provides an opportunity to capture a genome-wide picture of changes within the bacterial transcriptome in response to environmental perturbations (Conway & Schoolnik, 2003). Recently, Dunman *et al.* (2001) have described transcriptional-profiling studies utilizing a first-generation Affymetrix *S. aureus* GeneChip™ representing 86 % of the *S. aureus* strain COL genome sequence. In this report, we describe our transcriptional-profiling studies of the methicillin-susceptible strain RN450 (8325-4) genes that are upregulated in response to the cell-wall-active antibiotics oxacillin, bacitracin and D-cycloserine, which inhibit different steps in peptidoglycan biosynthesis. The studies indicate the existence of a cell-wall-stress stimulon.

METHODS

S. aureus* strain and culture conditions.** *S. aureus* RN450 (8325-4) was grown overnight in 5 ml of tryptic soy broth (TSB) medium at 37 °C with aeration. Overnight cultures were used to inoculate (1 : 100 dilution) 100 ml fresh TSB medium in a 250 ml flask. Cultures were incubated with aeration at 200 r.p.m. at 37 °C to early-exponential phase (OD_{600} 0·3). Cells were then exposed to the indicated cell-wall-active antibiotic for 60 min at concentrations giving significant inhibition of growth (Singh *et al.*, 2001a). Control cultures were grown for 1 h in the absence of antibiotic. Cells were pelleted by centrifugation at 10 000 ***g for 20 min at 4 °C, washed with fresh TSB and used for isolation of RNA.

RNA extraction. Cell pellets were resuspended in 2·5 ml lysis buffer (20 mM Tris/HCl, pH 7·5, 145 mM NaCl) containing 0·5 mg lysostaphin ml^{-1} (Sigma) and incubated at 25 °C for 30 min for lysis to occur. Total RNA was subsequently isolated using a Qiagen RNeasy Maxi kit according to the manufacturer's recommendations for prokaryotic RNA isolation. RNA concentrations were determined by spectrophotometry (OD_{260} value of 1·0 = 40 μg RNA ml^{-1}). Five micrograms of each RNA sample were electrophoresed in a 1·2 % agarose/0·66 M formaldehyde gel to assess RNA integrity, according to Qiagen RNA electrophoresis recommendations.

RNA processing

GeneChip™ hybridization and washing. RNA samples were processed then hybridized and washed on *S. aureus* GeneChips™ as described previously (Dunman *et al.*, 2001).

GeneChip™ analysis. To increase reproducibility, each experiment was performed in duplicate (RNA extraction from control and antibiotic-treated cells). Each labelled RNA sample was then hybridized to at least one GeneChip™. Following hybridization, washing and staining, GeneChips™ were scanned at 570 nm, 3 μm resolution in an Affymetrix GeneChip™ scanner. Affymetrix algorithms (MICROSUITE 4.0) calculated signal intensities (mean difference) and 'Present' or 'Absent' determinations for each gene, as described previously (Dunman *et al.*, 2001). To normalize for global systematic variations caused by inconsistencies in labelling and/or loading, each mean difference value was divided by the median mean difference for a given GeneChip™. Normalized intensity values were then averaged for each gene at each assay condition. GENESPRING version 3.2.11 software (Silicon Genetics) was used to plot normalized intensity values across samples. Genes demonstrating at least a twofold increase in transcripts titres in response to each antibiotic treatment as compared to control samples were first identified. Genes that were subsequently determined to be 'Present' by Affymetrix algorithms in antibiotic-treated samples and that were also found to be significantly different (*t*-test; *P* cut-off value of 0·05) from control samples were considered to be upregulated in response to challenge by a particular cell-wall-active antibiotic. Based on these lists of genes that were putatively upregulated by an individual antibiotic, Venn diagrams were then used to identify genes that demonstrated increased transcript titres in response to all, an individual or combinations of antibiotics tested. A similar process was used to identify genes that were downregulated in response to cell-wall-active antibiotic challenge. However, in this case, genes had to be considered 'Present' by Affymetrix criteria in control samples as opposed to treated conditions.

Northern blot analysis. Equal amounts of RNA (10 μg) from control and antibiotic-treated cultures were separated on a 1·2 % agarose/0·66 M formaldehyde gel and transferred to a nylon membrane by standard procedures (Sambrook *et al.*, 1989). High-stringency hybridization was performed in a heat-sealable bag containing 10 ml of hybridization buffer [5 × SSC, 5 × Denhardt's, 50 % formamide, 1 % (w/v) SDS, 100 μg denatured herring sperm DNA ml^{-1}] and radiolabelled randomly primed DNA synthesized in the presence of [α-^{32}P]dCTP [specific activity > 3000 Ci $mmol^{-1}$ (> 111 TBq); ICN Pharmaceuticals] products as a probe at 42 °C (Sambrook *et al.*, 1989). Membranes were subjected to low- and moderate-stringency washes prior to autoradiography. The following PCR primers were used to generate PCR templates. 16S rRNA (478 nt of GenBank accession no. Y15856), 5′-AAATCTTGACATCCTTTGACAACTC-3′ and 5′-CTAGCTCCTAAAAGGTTACTCCACC-3′; *vraS* (1044 nt of GenBank GI: 9501766), 5′-TGAGCGTTCAATGGAAGG-3′ and 5′-GCACAACTTTCATTGGCAC-3′; *tcaA* (1383 nt of GenBank GI: 15925705), 5′-GAAATCTTGCCCGAAGG-3′ and 5′-TTGGTTGC-TTGGTAGGTG-3′. All PCR products were gel-purified prior to being radiolabelled using the Prime-a-Gene labelling system (Promega) in the presence of [α-^{32}P]dCTP and used to probe the membrane. The *msrA* gene fragment was generated from the construct pRSETa-*msrA* (Singh *et al.*, 2001b). These fragments were then radiolabelled as above before being used for probing the membrane.

RESULTS AND DISCUSSION

Inhibition of growth by antibiotics

In previous proteomic studies, mid-exponential-phase cultures were exposed to cell-wall-active antibiotics and the proteins produced were studied (Singh *et al.*, 2001a). In the present studies, oxacillin, bacitracin and D-cycloserine were added separately to mid-exponential-phase cultures. After 1 h incubation, RNA was extracted from control and antibiotic-treated cultures for transcriptional profiling. Control cultures increased from an OD_{600} value of 0·31 to 0·96 (mean of eight cultures) during 1 h incubation at 37 °C. Oxacillin (1·2 μg ml^{-1}) caused 32 % inhibition of

the increase in the optical density, bacitracin (6·7 units ml^{-1}) 77·5 % inhibition and D-cycloserine (150 μg ml^{-1}) 44 % inhibition (duplicate experiments for each antibiotic).

Genes upregulated by oxacillin, bacitracin and D-cycloserine

One hundred and five genes were upregulated by all three antibiotics and are shown in Table 1. Genes belong to various functional categories, which include amino-acid transport and metabolism, carbohydrate transport and metabolism, cell-envelope biogenesis, DNA replication, recombination and repair, post-translational modification, protein turnover and chaperones, signal-transduction mechanisms, and transcription. Forty-six genes encoding hypothetical proteins were found to be putatively induced by each antibiotic.

msrA, which encodes methionine sulfoxide reductase, and the gene encoding glucose-specific enzyme IIA of the phosphotransferase system, which are present in the same transcriptional unit (Singh *et al.*, 2001b), were strongly induced by all three cell-wall-active antibiotics. MsrA and glucose-specific enzyme IIA were previously shown to be produced in increased amounts upon oxacillin challenge of *S. aureus*, by two-dimensional gel electrophoresis (Singh *et al.*, 2001a). These data provide validation of the GeneChip™ data. *msrA* is classified in the post-translational modification, protein turnover and chaperones category. Other strongly induced genes in this category were *psrA*, encoding a peptidyl-prolyl *cis*/*trans* isomerase homologue, and the genes for the heat-shock-protein homologues HtrA and Hsp33 (*htrO*).

Several cell-wall-related genes were upregulated by each of the antibiotics. These included *pbpB*, the gene encoding penicillin-binding protein (PBP) 2, *sgtB*, encoding a hypothetical protein similar to PBP1A/1B, *murZ*, encoding UDP-*N*-acetylglucosamine-1 carboxylvinyl transferase 2, and *murI*, encoding glutamate racemase. Possibly also related to cell-wall metabolism was *gltD*, encoding the small subunit of glutamate synthase. In addition, the gene encoding Fmt, an autolysis- and methicillin-resistance-related protein, was upregulated. Furthermore, a hypothetical protein similar to the *lyt* divergon expression attenuator LytR was strongly upregulated. *vraS*, which encodes a vancomycin-resistance-associated two-component histidine kinase sensor, was strongly expressed in response to cell-wall-active antibiotics.

In addition, two genes involved in DNA replication, recombination and repair were strongly expressed. These were *recU*, encoding a recombination protein U homologue, and one encoding a conserved hypothetical protein.

Two genes involved in the regulation of transcription were strongly induced. These were the above-mentioned *lytR* homologue and *hrcA*, encoding a heat-inducible transcriptional repressor.

The three cell-wall-active antibiotics studied are each active at different steps in peptidoglycan biosynthesis (Gale *et al.*, 1982; Walsh, 2003). Oxacillin binds to PBPs and inhibits the final cross-linking step of peptidoglycan biosynthesis. D-Cycloserine inhibits alanine racemase and D-alanine : D-alanine synthetase in a true competitive manner. Bacitracin binds to the carrier molecule in peptidoglycan biosynthesis C_{55}-isoprenyl pyrophosphate and prevents its dephosphorylation. Even though the agents affect different stages of peptidoglycan biosynthesis, 105 genes were upregulated when *S. aureus* cells were challenged with each of the three antibiotics. This suggests that the cell responds to inhibition of peptidoglycan biosynthesis in general, and the genes comprise a cell-wall-stress stimulon. A stimulon refers to the entire set of genes responding together to an environmental stimulus (Smith & Neidhardt, 1983), the environmental stimulus being a cell-wall-active antibiotic in this case. Cao *et al.* (2002) have referred to a vancomycin stimulon in *Bacillus subtilis*. Two prominent functional categories to which genes belonged were cell-wall-related genes, and post-translational modification, protein turnover and chaperones.

Each antibiotic stimulated transcription of the *pbpB* gene encoding PBP2 (Table 1). Murakami *et al.* (1999) and Boyle-Vavra *et al.* (2003) have demonstrated by Northern blot analysis that oxacillin and vancomycin increase the level of *pbpB* transcripts. Vancomycin also upregulated *pbpB* in transcriptional-profiling studies (data not shown). PBP2 plays a critical role in the peptidoglycan metabolism of *S. aureus*. Cooperation of the transglycosylase domain of PBP2 is required for the PBP2a of methicillin-resistant *S. aureus* to carry out cell-wall synthesis in the presence of methicillin (Pinho *et al.*, 2001). PBP2 is believed to play a role in borderline resistance to methicillin in the absence of *mecA* (Chambers, 1997). Increased PBP production has been noted in laboratory and clinical glycopeptide-intermediate *S. aureus* (Hanaki *et al.*, 1998; Moreira *et al.*, 1997). We can propose that the cell responds to inhibition of peptidoglycan synthesis by increasing the transcription of the *pbpB* gene in order to boost PBP2 production, and presumably then the rate of peptidoglycan synthesis, to restore the damaged and missing wall. Perhaps glucose-specific-enzyme-IIA induction serves to increase the rate of glucose transport into the cell to provide necessary energy for increased peptidoglycan biosynthesis. Peptidoglycan synthesis is believed to be activated in a clinical glycopeptide-intermediate *S. aureus* (Hanaki *et al.*, 1998).

murZ is upregulated by the cell-wall-active antibiotics and is the first step committed to biosynthesis of UDP-*N*-acetylmuramyl pentapeptide. The enzyme UDP-*N*-acetylglucosamine 1-carboxylvinyl transferase 2 catalyses the condensation of phosphoenolpyruvate with UDP-*N*-acetylglucosamine (Marquardt *et al.*, 1992). This may also be a response by the cell to increase the rate of peptidoglycan biosynthesis. Two genes related to D-glutamate production that were upregulated were *murI*, encoding

Table 1. Genes upregulated in response to the cell-wall-active antibiotics bacitracin, D-cycloserine and oxacillin

Chip ORF*	Fold increase†			N315 ORF‡	Gene§	Function‖	Role category
	Bacitracin	D-Cycloserine	Oxacillin				
774	Up	Up	Up	SA0008	*hutH*	Histidine ammonia-lyase	Amino-acid transport and metabolism
5433	Up	Up	Up	SA0953	*potD*	Spermidine/putrescine-binding protein precursor homologue	Amino-acid transport and metabolism
2460	4·0	3·1	Up	SA1166	*thrB*	Homoserine kinase homologue	Amino-acid transport and metabolism
3239	Up	Up	Up	SA1165	*thrC*	Threonine synthase	Amino-acid transport and metabolism
2639	Up	Up	3·7	SA1679		Hypothetical protein – similar to D-3-phosphoglycerate dehydrogenase	Amino-acid transport and metabolism
5094	6·2	5·3	Up	SA0638	*bacA*	Bacitracin-resistance protein (putative undecaprenol kinase) homologue	Antibiotic resistance
4523	Up	Up	13·2	SA1187		Antibacterial protein	Antibiotic resistance
4524	Up	Up	Up	SA1186		Antibacterial protein	Antibiotic resistance
2166	9·2 (0·9)	5·1 (0·6)	7·8 (1·3)	SA1255		PTS system, glucose-specific enzyme II, A component	Carbohydrate transport and metabolism
3374	Up	Up	Up	SA0903			Carbohydrate transport and metabolism
4565	16·5	9·2	14·8	SA2220			Carbohydrate transport and metabolism
4788	Up	Up	Up	SA0909	*fmt*	Fmt, autolysis and methicillin-resistance-related protein	Cell-envelope biogenesis
1100	3·2	2·8	Up	SA0997	*murI*	Glutamate racemase	Cell-envelope biogenesis
2896	7·2	3·1	8·9	SA1926	*murZ*	UDP-*N*-acetylglucosamine 1-carboxylvinyl transferase 2	Cell-envelope biogenesis
3368	4·9 (0·7)	2·8 (0·4)	4·2 (0·7)	SA1283	*pbp2*	PBP2	Cell-envelope biogenesis
1676	10·9	5·2	8·1	SA1691	*sgtB*	Hypothetical protein – similar to penicillin-binding protein 1A/1B	Cell-envelope biogenesis
1271	3·9	2·5	3·5	SA1105			Cell-envelope biogenesis
3937	Up	Up	Up	SA2405	*betA*	Choline dehydrogenase	Cell motility and secretion
1199	Up	Up	Up	SA0826	*spsB*	Type-1 signal peptidase 1B	Cell motility and secretion
2991	Up	Up	Up	SA1651	*hemH*	Ferrochelatase homologue	Coenzyme metabolism
1073	2·1	2·6	2·9	SA1587	*ribA*	Riboflavin biosynthesis protein	Coenzyme metabolism
2972	4·9 (0·4)	3·0 (0·2)	4·3 (0·2)	SA1282	*recU*	Recombination protein U homologue	DNA replication, recombination and repair
1626	Up	Up	Up	SA1710		Hypothetical protein – similar to DNA polymerase III, α chain PolC type	DNA replication, recombination and repair
2061	5·9 (0·3)	2·4 (0·1)	13·8 (4·8)	SA0481		Conserved hypothetical protein	DNA replication, recombination and repair
2612	2·0	3·9	Up	SA1093		DNA topoisomerase I *topA* homologue	DNA replication, recombination and repair
5245	Up	Up	Up	SA0827		Hypothetical protein – similar to ATP-dependent nuclease subunit B	DNA replication, recombination and repair
1085	Up	Up	Up	SA1141	*glpK*	Glycerol kinase	Energy production and conversion
37	Up	Up	Up	SA2343			Hypothetical protein

Table 1. cont.

Chip ORF*	Fold increase†			N315 ORF‡	Gene§	Function‖	Role category
	Bacitracin	D-Cycloserine	Oxacillin				
378	Up	Up	Up	SA1049			Hypothetical protein
475	Up	Up	2·1	SA0427			Hypothetical protein
511	Up	Up	Up	SA2360			Hypothetical protein
644	5·6	5·0	26·6	SAS016			Hypothetical protein
762	Up	Up	Up	SA1703			Hypothetical protein
766	Up	Up	Up	SA1432			Hypothetical protein
924	Up	Up	Up	SA0591			Hypothetical protein
1178	Up	Up	Up	SAS043			Hypothetical protein
1295	Up	Up	Up	SA1712			Hypothetical protein
1339	Up	Up	Up	SA1240			Hypothetical protein
1711	Up	Up	Up	SA2246			Hypothetical protein
1891	Up	Up	2·4	SA1274			Hypothetical protein
1894	Up	Up	Up	SA1133			Hypothetical protein
2025	Up	Up	Up	SA0550			Hypothetical protein
2141	3·0	4·6	2·5	SA0648			Hypothetical protein
2165	9·3	4·7	14·1	SA1254			Hypothetical protein
2534	Up	Up	Up	SA0513			Hypothetical protein
2599	3·8	3·2	Up	SA1215			Hypothetical protein
2695	Up	Up	2·2 (0·1)	SA0629			Hypothetical protein
3621	Up	Up	Up	SA0507			Hypothetical protein
3815	4·4	2·6	5·7	SA2481			Hypothetical protein
4002	6·1	3·1	Up	SA0433			Hypothetical protein
4163	5·6	4·0	3·2	SA2139			Hypothetical protein
4240	7·8	3·4	7·1	SA1702			Hypothetical protein
4275	Up	Up	2·9 (<0·1)	SA1931			Hypothetical protein
4392	3·5	2·5	Up	SA0830			Hypothetical protein
4399	Up	Up	Up	SA1582			Hypothetical protein
4536	Up	Up	Up	SA1295			Hypothetical protein
4564	10·6	6·2	18·7	SA2221			Hypothetical protein
4634	2·6	2·1	2·2	SA1851			Hypothetical protein
5131	4·0 (1·1)	2·6 (0·4)	Up	SA2297			Hypothetical protein
5310	Up	Up	Up	SA1173			Hypothetical protein
5481	3·5	2·0	2·8	SA1490			Hypothetical protein
3141	2·6	2·4	2·3	SA1654			Hypothetical protein – similar to ABC transporter
5095	Up	Up	Up	SA0639			Hypothetical protein – similar to ABC transporter
170	Up	Up	Up	SA1988			Hypothetical protein – similar to alginate lyase
1399	7·6	4·9	5·5	SA0914			Hypothetical protein – similar to chitinase B

Table 1. cont.

Chip ORF*	Fold increase†			N315 ORF‡	Gene§	Function‖	Role category
	Bacitracin	D-Cycloserine	Oxacillin				
3503	Up	Up	Up	SA0357			Hypothetical protein – similar to exotoxin 2
990	Up	Up	Up	SA0172			Hypothetical protein – similar to integral membrane protein
2796	Up	Up	3·2	SA1970			Hypothetical protein – similar to multidrug-resistance protein
2173	Up	Up	Up	SA0846			Hypothetical protein – similar to oligopeptide transporter
3167	3·0	3·6	4·9	SA0849			Hypothetical protein – similar to oligopeptide transporter
991	Up	Up	Up	SA0173			Hypothetical protein – similar to surfactin synthetase
5128	2·2	2·9	2·9	SA0244			Hypothetical protein – similar to teichoic acid biosynthesis protein
1450	4·9 (0·5)	3·0 (0·9)	2·8 (<0·1)	SA0758			Hypothetical protein – similar to thioredoxin
5248	6·6	2·6	Up	SA2344	*copA*	Copper-transporting ATPase *copA*	Inorganic-ion transport and metabolism
3716	7·7	2·7	3·5	SA1948	*czrB*	Cation-efflux system membrane protein homologue	Inorganic-ion transport and metabolism
197	Up	Up	Up	SA2489		Hypothetical protein – similar to high-affinity nickel-transport protein	Inorganic-ion transport and metabolism
548	Up	Up	Up	SA0547	*mvaK1*	Mevalonate kinase	Lipid metabolism
4924	Up	Up	Up	SA1103	*uppS*	Undecaprenyl pyrophosphatase synthetase	Lipid metabolism
5059	Up	Up	Up	SA1434		Acetyl-CoA carboxylase (biotin carboxylase subunit), *accC* homologue	Lipid metabolism
4482	3·7	3·7	Up	SA1310	*ansA*	Probable L-asparaginase	Miscellaneous
5638	4·2 (<0·1)	3·3 (0·3)	Up	SA0122	*butA*	Acetoin(diacetyl)reductase	Miscellaneous
2217	Up	Up	Up	SA1127	*cinA*	Competence-damage inducible protein *cinA*	Miscellaneous
3360	3·3	3·2	10·3	SA0431	*gltD*	NADH-glutamate synthase small subunit	Miscellaneous
4021	Up	Up	Up	SA1861	*ilvC*	α-Keto-β-hydroxylacil reductoisomerase	Miscellaneous
4283	Up	Up	Up	SA2146	*tcaA*	TcaA protein	Miscellaneous
2192	Up	Up	Up	SA1734		Pyrazinamidase/nicotinamidase homologue	Miscellaneous
3043	Up	Up	Up	SA0379		Bacteriophage L54a, M23/M37 peptidase domain protein	Miscellaneous
4690	Up	Up	Up	SA1172		Hypothetical protein – similar to GMP reductase	Nucleotide transport and metabolism
5119	Up	Up	Up	SA1211	*opp-2F*		Oligopeptide transporter
4967	Up	Up	Up	SA0847	*oppD*	Oligopeptide transport system ATP-binding protein OppD homologue	Oligopeptide transporter

Table 1. cont.

Chip ORF*	Fold increase†			N315 ORF‡	Gene§	Function‖	Role category
	Bacitracin	D-Cycloserine	Oxacillin				
4966	2·1	3·0	4·1	SA0848	*oppF*	Oligopeptide transport system ATP-binding protein OppF homologue	Oligopeptide transporter
5133	5·8	3·7	Up	SA1146	*bsaA*	Glutathione peroxidase	Post-translational modification, protein turnover and chaperones
2168	7·6 (4·7)	4·4 (2·4)	11·9 (6·6)	SA1257	*msrA*	Peptide methionine sulfoxide reductase	Post-translational modification, protein turnover and chaperones
4537	5·2 (1·3)	2·7 (0·6)	7·7 (0·4)	SA1659	*prsA*	Peptidyl-prolyl *cis*/*trans* isomerase homologue	Post-translational modification, protein turnover and chaperones
2167	6·3 (1·6)	3·6 (1·1)	8·3 (0·9)	SA1256			Post-translational modification, protein turnover and chaperones
2420	11·0 (1·2)	4·2 (0·8)	11·0 (4·7)	SA1549		Hypothetical protein – similar to heat-shock protein HtrA	Post-translational modification, protein turnover and chaperones
5480	Up	Up	Up	SA0470		Heat-shock protein HSP33 homologue	Post-translational modification, protein turnover and chaperones
199	Up	Up	Up	SA2490		Hypothetical protein – similar to *N*-hydroxyarylamine *O*-acetyltransferase	Secondary metabolites biosynthesis, transport and catabolism
764	7·2	2·8	10·4	SA1701	*vraS*	Two-component sensor histidine kinase	Signal-transduction mechanisms
2503	Up	Up	Up	SA1947	*czrA*	Repressor protein	Transcription
940	6·2	3·5	12·0	SA1411	*hrcA*	Heat-inducible transcriptional repressor	Transcription
1504	Up	Up	2·2	SA0461	*mfd*	Transcription-repair coupling factor	Transcription
4386	4·3	3·2	2·5	SA1195	*msrR*	Peptide methionine sulfoxide reductase regulator MsrR	Transcription
2201	8·8 (2·0)	4·6 (0·7)	14·0 (0·8)	SA2103		Hypothetical protein – similar to *lyt* divergon expression attenuator LytR	Transcription
2850	2·2	2·1	2·0	SA0908			Transcription

*Designated *S. aureus* GeneChip™ ORF number.

†Fold increase in transcript titre as compared to control cells. Several genes have been tiled in a redundant manner on the GeneChip™ used in these studies; number in parentheses represents the standard deviation of fold change where applicable. 'Up' represents genes for which an accurate fold increase could not be calculated; transcripts were considered absent in control but present in treated cells, based on Affymetrix algorithm predictions.

‡Corresponding designated *S. aureus* strain N315 gene (Kuroda *et al.*, 2001).

§Previously described gene name.

‖Previously described gene product function.

glutamate racemase, and *gltD*, encoding the small subunit of glutamate synthase.

tca has been described by Brandenberger *et al.* (2000) as being a three-cistronic operon that increases teicoplanin resistance in *S. aureus*. It is speculated that *tcaRAB* may be involved in cell-wall biosynthesis.

Two genes possibly related to the suppression of autolysis, which would serve to protect the cell from damage caused by the triggering of these enzymes by cell-wall-active antibiotics, were *fmt* and *lytR*. Fmt is a protein whose inactivation leads to a greater rate of autolysis of *S. aureus* in the presence of 0·02 % Triton X-100 (Komatsuzawa *et al.*, 1997). Fmt is also believed to be involved in the expression of oxacillin resistance in the presence of Triton X-100. LytR is a potential response regulator of a two-component regulatory system that regulates a dicistronic operon encoding two proteins, LrgA and LrgB. They are hypothesized to control peptidoglycan hydrolase activity (Brunskill & Bayles, 1996; Groicher *et al.*, 2000).

vraS is a vancomycin-resistance-associated gene with homology to the histidine kinase family (Kuroda *et al.*, 2000), located immediately upstream of *vraR*, a response-regulator homologue. The transcription of *vra* was upregulated in a clinical vancomycin-resistant isolate. Overexpression of *vraR* in a susceptible strain increased vancomycin resistance.

The cell-wall-active antibiotics also caused strong induction of genes encoding the stress molecular chaperones/proteases HtrA and Hsp33. HtrA is a protease (Pallen & Wren, 1997). Hsp33 (encoded by *hslO*) is a potent molecular chaperone (Graf & Jakob, 2002) that is under heat-shock control at the transcriptional level. On the post-translational level, Hsp33 is under oxidative stress control (Jakob *et al.*, 1999). The redox sensor is a zinc-coordinating cysteine centre that forms intramolecular disulfide bonds under oxidizing, activating conditions. *msrA* also encodes a protein that protects against oxidative damage, reducing methionine sulfoxide residues to methionine (Singh *et al.*, 2001b). An implication of these results is that treatment of cells with cell-wall-active antibiotics causes the accumulation of damaged, misfolded and aggregated proteins, necessitating the production of stress-chaperone and protease proteins to deal with the accumulation of aberrant proteins. Furthermore, the results imply that treatment with cell-wall-active antibiotics causes oxidative damage to proteins, as indicated by the induction of *msrA* and *hslO* (Storz & Zheng, 2000). This appears to be a previously unappreciated aspect of the mode of action of cell-wall-active agents. *prsA* encodes a peptidyl-prolyl *cis*/*trans* isomerase. Such enzymes catalyse *cis*–*trans* isomerization around X-Pro peptide bonds and facilitate envelope protein folding (Raivio & Silhavy, 2000). These proteins participate in the response to envelope stress in *Escherichia coli*. In *B. subtilis*, PrsA is an extracellular lipoprotein and is considered to be dedicated to assisting the folding and stability of exported proteins at the cytoplasmic membrane–cell wall interface (Wahlström *et al.*, 2003).

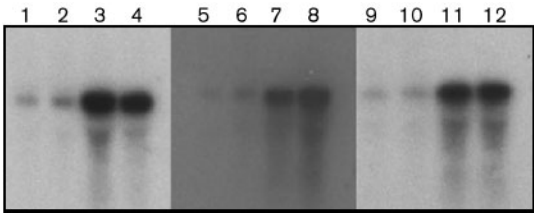

Fig. 1. Northern blot hybridization of the *msrA* gene with total RNA isolated from the *S. aureus* RN450 cells and separated on a 1·2 % denaturing agarose gel. Lanes 1, 2, 5, 6, 9 and 10, total RNA from *S. aureus* RN450 grown in TSB for 1 h after reaching an OD_{600} value of 0·3. Lanes 3, 4, 7, 8, 11 and 12, total RNA from *S. aureus* RN450 cells stressed with 1·2 μg oxacillin ml^{-1} (lanes 3 and 4), 6·7 U bacitracin ml^{-1} (lanes 7 and 8) and 150 μg D-cycloserine ml^{-1} (lanes 11 and 12), respectively.

Northern blot analysis of gene induction

Northern blot analysis was used to further validate the results obtained from transcriptional profiling. As expected, the *msrA* gene was demonstrated to show increased transcription in response to oxacillin, bacitracin and D-cycloserine as revealed by Northern blot analysis (Fig. 1). This is consistent with the transcriptional-profiling results which demonstrated that *msrA* is upregulated in response to cell-wall-active antibiotic stress. Northern analysis also showed that transcripts from *vraS* and *tcaA* were increased when bacteria were challenged with oxacillin (Fig. 2). This is in agreement with the transcriptional-profiling results that these genes are highly induced in response to oxacillin stress.

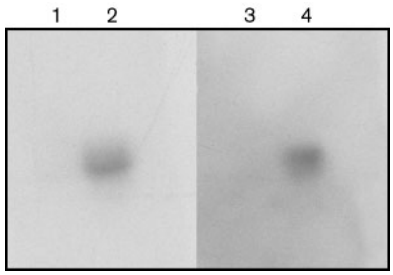

Fig. 2. Northern blot hybridization of the *vraS* and *tcaA* genes with total RNA isolated from the *S. aureus* RN450 cells and separated on a 1·2 % denaturing agarose gel. Lanes 1 and 3, total RNA from *S. aureus* RN450 grown in TSB for 1 h after reaching an OD_{600} value of 0·3 probed with *vraS* and *tcaA*, respectively. Lanes 2 and 4, total RNA from *S. aureus* RN450 cells stressed with 1·2 μg oxacillin ml^{-1} for 1 h after reaching an OD_{600} value of 0·3 probed with *vraS* and *tcaA*, respectively.

Genes upregulated by oxacillin but not bacitracin or D-cycloserine

Ninety-eight genes were upregulated by oxacillin treatment of cultures at least twofold, but were not induced by the other cell-wall-active antibiotics tested (Table I, http://mic.sgmjournals.org). Most of these genes were increased in expression by a modest amount, i.e. two- to threefold. In addition to genes in the role categories in Table 1, genes involved in coenzyme metabolism, energy production and conversion, lipid metabolism, secondary metabolites biosynthesis, transport and catabolism, and translation, ribosomal structure and biogenesis showed increased expression. Genes potentially related to cell-wall biosynthesis included *ragB* (glucosamine-6-phosphate isomerase), *mvaK2* (phosphomevalonate kinase) and *uppS* (undecaprenyl pyrophosphatase synthetase). The chaperone/protease genes *clpP* and *clpB* were also upregulated.

Genes upregulated by bacitracin but not by oxacillin or D-cycloserine

Forty-six genes were up regulated twofold or more by bacitracin but not by the other antibiotics (Table II, http://mic.sgmjournals.org). Strikingly, ORF 1764 was upregulated 50-fold, and this ORF is believed to represent an integral recombinase, core domain family protein. Three genes encoding proteins involved in lysine biosynthesis were upregulated, *dapB*, *dapD* and *lysA*. Lysine is a component of *S. aureus* peptidoglycan. The *pbp4* gene was increased in expression, as was *mvaK1*, encoding mevalonate kinase.

Genes upregulated by D-cycloserine but not by oxacillin or bacitracin

An enormous number (218) of genes were upregulated by D-cycloserine (Table III, http://mic.sgmjournals.org), including genes involved in amino-acid transport and metabolism (27), carbohydrate transport and metabolism (6), cell-envelope biogenesis (6), cell motility and secretion (5), coenzyme metabolism (4), DNA replication, recombination and repair (4), energy production and conversion (4), hypothetical protein (106), inorganic-ion transport and metabolism (8), lipid metabolism (8), miscellaneous (18), nucleotide transport and metabolism (2), post-translational modification, protein turnover and chaperones (2), secondary metabolites biosynthesis, transport and catabolism (6), transcription (7), and translation, ribosomal structure and biogenesis (1).

This is one of the first reports of transcriptional profiling of the response of a human bacterial pathogen to cell-wall-active antibiotics. We believe that our studies have uncovered a cell-wall-stress stimulon, which may have characteristic transcriptome and proteome signatures. Although the focus of this body of work was to identify genes that are collectively upregulated by cell-wall-active antibiotics, Table 2 lists 87 genes that were determined to be downregulated by each antibiotic. Recently, Ng *et al.* (2003) have reported on microarray analysis of the response of *Streptococcus pneumoniae* to challenge with sublethal concentrations of translation inhibitors. Transcript levels of ribosomal proteins and translation factors were upregulated, whereas tRNA charging and amino-acid biosynthesis enzyme transcript levels were downregulated. Global transcription patterns formed a signature that could be used to classify the mode of action of translation inhibitors. Subinhibitory concentrations of erythromycin and rifampicin were shown to alter global transcription patterns through studies of promoter–*lux* reporter constructs in a *Salmonella typhimurium* library (Goh *et al.*, 2002).

Cell-wall-active antibiotics have been shown to induce regulons controlled by specific RNA polymerase sigma factors in two non-pathogenic soil bacteria. These are the SigE regulon in *Streptomyces coelicolor* (Hong *et al.*, 2002), and the SigW and SigM regulons of *B. subtilis* (Cao *et al.*, 2002). A variety of cell-wall-active antibiotics acted as inducers of the *sigE* signal transduction system, and evidence was provided that SigE directs transcription of the *cwg* operon containing genes predicted to specify the biosynthesis of a cell-wall glycan (Hong *et al.*, 2002).

The *B. subtilis* SigW and SigM sigma factors are part of the extracytoplasmic function subfamily of sigma factors, members of which control cell-envelope-related functions (Helmann, 2002). Cao *et al.* (2002) showed that vancomycin induced 19 SigW-dependent genes as revealed by DNA microarray studies. In addition, vancomycin induced genes that are not known to be controlled by extracytoplasmic function sigma factors, including 10 cell-wall-related genes. Thirty-three members of the large SigB general stress regulon were induced later during vancomycin treatment.

Extracytoplasmic function sigma factors have not been discovered in *Staphylococcus aureus* genome sequencing (Kuroda *et al.*, 2001), with the only sigma factors described to date being housekeeping sigma factor SigA and SigB, which regulates the expression of various virulence factors and stress genes (Cheung *et al.*, 1999; Gertz *et al.*, 2000). The extent of the involvement of SigB in the expression of the cell-wall-stress stimulon we have described is unknown at this point.

The strain that we used in these studies, *S. aureus* 8325-4, is a derivative of strain 8325, which is the line of strains most frequently used in genetics-based staphylococcal studies (Novick, 1991). Giachino *et al.* (2001) reported that 8325 derivatives have an 11 bp deletion in the 5′ part of the *rsbU* gene. This generates a stop codon giving an ORF of 74 aa compared to the uninterrupted 323 aa ORF of intact RbsU. RbsU is a protein activator of SigB. Thus, 8325 derivatives are defective in SigB activation, although SigB is detected in the strains by Western blotting (Giachino *et al.*, 2001). Our proteomic studies of the response of *S. aureus* to cell-wall-active antibiotics were carried out using strain 8325-4, as were the present transcriptomic studies. Clearly, future studies should evaluate the role of

Table 2. Genes downregulated in response to the cell-wall-active antibiotics bacitracin, D-cycloserine and oxacillin

Chip ORF*	Fold decrease†			N315 ORF‡	Gene§	Function‖	Role category
	Bacitracin	D-Cycloserine	Oxacillin				
4473	4·5	3·1	Down	SA0821	*argH*	Argininosuccinate lyase	Amino-acid transport and metabolism
953	2·9 (<0·1)	2·6 (0·2)	Down	SA1674		Glutamate ABC transporter ATP-binding protein	Amino-acid transport and metabolism
2344	Down	Down	Down	SA1945		Hypothetical protein – similar to mannose-6 phosphate isomerase *pmi*	Carbohydrate transport and metabolism
4485	Down	2·3	Down	SA0249	*scdA*	Cell division and morphogenesis-related protein	Cell division and chromosome partitioning
595	Down	Down	Down	SA0519	*sdrC*	Fibrinogen-binding, bone sialoprotein-binding protein	Cell-envelope biogenesis and outer membrane
4081	6·7 (0·6)	14·2 (7·6)	Down	SA0107	*spa*	Immunoglobulin G binding protein A precursor	Cell-envelope biogenesis and outer membrane
4040	3·6	4·5	9·2	SA2505		LPXTG-motif cell wall anchor domain protein	Cell-envelope biogenesis and outer membrane
470	3·0	2·8	8·5	SA0423		Hypothetical protein – similar to autolysin	Cell motility and secretion
5345	4·0	2·3	2·4	SA0965	*ctaB*	Cytochrome *caa3* oxidase (assembly factor) homologue	Coenzyme metabolism
5082	2·1	2·4	3·6	SA1496	*hemA*	Glutamyl-tRNA reductase	Coenzyme metabolism
5053	6·8	4·7	Down	SA2186	*nasF*	Uroporphyrin-III C-methyl transferase	Coenzyme metabolism
515	44·1 (5·9)	5·0 (0·4)	9·1 (2·7)	SA0232	*lctE*	L-Lactate dehydrogenase	Energy production and conversion
3524	Down	6·0 (0·2)	Down	SA2185	*narG*	Respiratory nitrate reductase α chain	Energy production and conversion
621	Down	3·5 (0·9)	Down	SA2182	*narI*	Nitrate reductase γ chain	Energy production and conversion
15	12·6	8·5	Down	SA0218	*pflB*	Formate acetyltransferase	Energy production and conversion
5619	2·6 (0·3)	2·1 (0·1)	3·8 (0·5)	SA0912	*qoxB*	Quinol oxidase polypeptide I QoxB	Energy production and conversion
1846	3·0	2·5	3·4	SA0911	*qoxC*	Quinol oxidase polypeptide III QoxC	Energy production and conversion
513	5·6 (2·6)	4·3 (0·6)	Down	SA0231		Hypothetical protein – similar to flavohaemoprotein	Energy production and conversion
1845	2·7	2·3	3·2	SA0910		Hypothetical protein – similar to quinol oxidase polypeptide IV QoxD	Energy production and conversion
4792	2·9	2·0	3·4	SA0913		Hypothetical protein – similar to quinol oxidase polypeptide II QoxA	Energy production and conversion
3362	Down	9·2 (4·4)	15·8 (3·6)	SA2156		L-Lactate permease *lctP* homologue	Energy production and conversion
622	Down	10·8 (2·8)	Down	SA2183		Hypothetical protein – similar to nitrate reductase δ chain	Energy production and conversion
3960	5·7	4·0	Down	SA1984	*asp23*	Alkaline-shock protein 23	Function unknown
3029	Down	2·1 (0·1)	Down	SA2479			Hypothetical protein
1740	10·5 (1·9)	6·7 (1·7)	Down	SA0562	*adh1*	Alcohol dehydrogenase I	General function prediction only
583	Down	4·6 (0·4)	Down	SA0309	*geh*	Glycerol ester hydrolase	General function prediction only
3759	3·1	2·5	2·0	SA1307		Hypothetical protein – similar to GTP-binding protein	General function prediction only
2874	3·7	2·5	2·2	SA1975		Hypothetical protein	General function prediction only
1666	6·0	5·9	Down	SA2303		Hypothetical protein – similar to membrane-spanning protein	General function prediction only
3289	6·7	3·3	Down	SA0285			Hypothetical protein

Table 2. cont.

Chip ORF*	Fold decrease†			N315 ORF‡	Gene§	Function‖	Role category
	Bacitracin	D-Cycloserine	Oxacillin				
2078	3·6	2·3	2·6	SA0292			Hypothetical protein
392	Down	2·5	Down	SA0739			Hypothetical protein
2675	5·1	4·1	2·2	SA0772			Hypothetical protein
167	7·1	5·8	Down	SA1985			Hypothetical protein
3961	5·6	3·1	Down	SA1986			Hypothetical protein
620	5·7	4·0	Down	SA2181			Hypothetical protein
1914	29·2	9·6	53·8	SA2268			Hypothetical protein
1353	Down	3·8	4·3	*SA0850*			Hypothetical protein
4462	2·4	3·7	6·4	SA2284			Hypothetical protein – similar to accumulation-associated protein
343	4·8	5·4	9·1	SA2285			Hypothetical protein – similar to accumulation-associated protein
628	Down	Down	Down	SA2189			Hypothetical protein – similar to NirR
4168	7·8 (2·8)	5·3 (2·0)	Down	SA2179			Hypothetical protein – similar to response regulators of two-component regulators
876	2·3	2·3	2·3	SA2074	*modA*	Probable molybdate-binding protein	Inorganic-ion transport and metabolism
4173	Down	4·7 (1·2)	Down	SA2184	*narH*	Nitrate reductase β chain	Inorganic-ion transport and metabolism
3786	Down	10·7 (8·0)	Down	SA2176	*narK*	Nitrite extrusion protein	Inorganic-ion transport and metabolism
2095	3·6	2·4	2·3	SA0453		Hypothetical protein – similar to 4-diphospho-cytidyl-2-C-methyl-D-erythritol kinase	Lipid metabolism
2462	Down	2·2	Down	SA2336	*clpL*	ATP-dependent Clp proteinase chain *clpL*	Post-translational modification, protein turnover and chaperones
16	14·5	7·3	Down	SA0219	*pflA*	Formate acetyltransferase activating enzyme	Post-translational modification, protein turnover and chaperones
619	Down	3·9 (0·7)	Down	SA2180		Hypothetical protein – similar to two-component sensor histidine kinase	Signal-transduction mechanisms
2283	Down	4·0	2·4	SA0322		Hypothetical protein – similar to transcription regulator	Transcription
2401	2·0	2·3	2·6	SA1504	*infC*	Translation initiation factor IF-3 *infC*	Translation, ribosomal structure and biogenesis
4469	2·6	2·3	2·9	SA0816		Hypothetical protein – similar to polyribonucleotide nucleotidyltransferase	Translation, ribosomal structure and biogenesis
5239	Down	2·9	Down	SA0905	*atl*	Autolysin	
626	Down	Down	Down	SA2187	*nasE*	Assimilatory nitrite reductase	
2353	Down	Down	Down	SA1269		Blt-like protein	

Table 2. cont.

Chip ORF*	Fold decrease†			N315 ORF‡	Gene§	Function‖	Role category
	Bacitracin	D-Cycloserine	Oxacillin				
5004	2·8	2·6	2·5	SA2053		Glucose uptake protein homologue	
2343	Down	2·4	Down	*SA0608*			
4175	Down	2·7	Down	*SA2396*			

*Designated *S. aureus* GeneChip™ ORF number.
†Fold increase in transcript titre as compared to control cells. Several genes have been tiled in a redundant manner on the GeneChip™ used in these studies; number in parentheses represents the standard deviation of fold change where applicable. 'Down' represents genes for which an accurate fold decrease could not be calculated; transcripts were considered absent in control but present in treated cells, based on Affymetrix algorithm predictions.
‡Corresponding designated *S. aureus* strain N315 gene (Kuroda *et al.*, 2001). Italic *SA* numbers correspond to COL genes (ORF not within N315 genome).
§Previously described gene name.
‖Previously described gene product function.

SigB in the expression of the putative cell-wall-stress stimulon we have described. Horsburgh *et al.* (2002) have recently described the construction of an RbsU$^+$ derivative of strain 8325-4. The *msrA* gene, which is strongly induced by oxacillin in strain 8325-4 (Singh *et al.*, 2001a, b), is also strongly induced in 8325-4 RbsU$^+$ (R. Pechous, N. Ledala, B. J. Wilkinson & R. K. Jayaswal, unpublished observations).

Recently, Chan *et al.* (2003) have reported on their studies on the microarray identification of genes responsive to cell-wall-active antibiotics using a different strain of *S. aureus*, which is presumably SigB$^+$, and different cell-wall-active antibiotics to the ones used in the present study. They found several of the same genes to be upregulated that we found to be induced, including *vraS*, *vraR*, *murZ*, *sgtB*, *prsA*, *msrA*, *lytR* and *tcaA*.

We expect that full delineation and definition of the cell-wall-stress stimulon will be an iterative process. The mechanism of sensing cell-wall stress or damage and the signal-transduction pathways remain to be worked out. Chan *et al.* (2003) have provided evidence that a VraS/VraR controlled regulon is part of the cell-wall-stress stimulon.

Recognition of a cell-wall-stress stimulon should prove useful in antibacterial drug development, including allowing investigators to identify a cell-wall-inhibitory mode of action of novel active compounds. Also, it will be interesting to evaluate the transcription response to cell-wall-active antibiotics in strains resistant to these agents.

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