Extensive and Genome-Wide Changes in the Transcription Profile of *Staphylococcus aureus* Induced by Modulating the Transcription of the Cell Wall Synthesis Gene *murF*^{▽†}

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A *murF* conditional mutant was used to evaluate the effect of suboptimal transcription of this gene on the transcriptome of the methicillin-resistant *Staphylococcus aureus* strain COL. The mutant was grown in the presence of optimal and suboptimal concentrations of the inducer, and the relative levels of transcription of genes were evaluated genome wide with an Affymetrix DNA microarray that included all open reading frames (ORFs) as well as intergenic sequences derived from four sequenced *S. aureus* strains. Using a sensitivity threshold value of 1.5, suboptimal expression of *murF* altered the transcription of a surprisingly large number of genes, i.e., 668 out of the 2,740 ORFs (close to one-fourth of all ORFs), of the genome of *S. aureus* strain COL. The genes with altered transcription were distributed evenly around the *S. aureus* chromosome, and groups of genes involved with distinct metabolic functions responded in unique and operon-specific manners to modulation in *murF* transcription. For instance, all genes belonging to the *isd* operon and all but 2 of the 35 genes of prophage L54a were down-regulated, whereas all but one of the 21 members of the *vraSR* regulon and most of the 79 virulence-related genes (those for fibronectin binding proteins A and B, clumping factor A, gamma hemolysin, enterotoxin B, etc.) were up-regulated in cells with suboptimal expression of *murF*. Most importantly, the majority of these altered gene expression profiles were reversible by resupplying the optimal concentration of IPTG (isopropyl-β-D-thiogalactopyranoside) to the culture. The observations suggest the coordinate regulation of a large sector of the *S. aureus* transcriptome in response to a disturbance in cell wall synthesis.

Transcriptomic approaches are powerful tools for the identification of groups of genes with similar expression patterns and for the establishment of putative regulatory relations between them. Using microarray technology, recent reports have described extensive changes in gene expression profiles when *Staphylococcus aureus* was challenged by different cell wall inhibitors such as oxacillin, bacitracin, D-cycloserine, and vancomycin (12, 19, 20, 34). The group of genes whose transcription was altered during treatment with antibiotics that target different steps in cell wall synthesis was proposed to represent members of a cell wall stimulon responding to a specific form of stress in a coordinate manner.

The large number and diversity of genes that respond to cell wall inhibitors by altered transcription suggest that many functions of the bacterial cell are connected through regulatory loops to one or more steps in cell wall synthesis which may also serve as control points of metabolism. Such a control should also be operational in the absence of antibiotic treatment, and

we searched for a system in which the existence of such a regulatory system could be experimentally demonstrated.

The method of choice became available with the isolation of an *S. aureus murF* conditional mutant in which the rate of transcription of *murF* depended on the concentration of the IPTG (isopropyl-β-D-thiogalactopyranoside) inducer in the medium (30). The MurF ligase catalyzes the last cytoplasmic step in the synthesis of the peptidoglycan precursor: it adds the D-alanyl-D-alanine dipeptide to the UDP-linked MurNAc-tripeptide, thus forming the peptidoglycan building block, the UDP-linked MurNAc pentapeptide. The assembly of this peptidoglycan building block is essential for all the cell wall synthesis reactions that take place on the outer surface of the plasma membrane.

The biochemical consequences of suboptimal transcription of *murF* include reduction in the amounts of UDP-linked MurNAc-pentapeptide in the cell wall precursor pool and accumulation of a tripeptide precursor which incorporates into the peptidoglycan in the form of un-cross-linked monomers. Bacteria with suboptimal *murF* transcription also grow slower and have reduced beta-lactam antibiotic resistance (30). In fact, the *murF* gene had been previously added to the wide list of auxiliary genes that are essential for the optimal expression of methicillin resistance in *S. aureus* (6, 31).

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We used custom-made DNA microarray chips to profile the effect of suboptimal transcription of *murF* on gene expression in the methicillin-resistant *S. aureus* (MRSA) strain COL, including the reversibility of the effects.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Staphylococcus aureus* strain COL and its derivative, the *murF* conditional mutant COL*spacmurF* (30), were used for all the experiments described in this paper. The cells were grown at 37°C with aeration in tryptic soy broth (TSB) or tryptic soy agar (Difco Laboratories, Detroit, MI). The growth medium of the conditional mutant strain COL*spacmurF* was supplemented with IPTG (100 µM) (Sigma, St. Louis, MO), erythromycin (10 µg/ml) (Sigma), and chloramphenicol (10 µg/ml) (Sigma) unless otherwise described.

Growth conditions used in the microarray analysis. COL*spacmurF* was grown overnight in TSB supplemented with erythromycin, chloramphenicol, and 100 µM IPTG. The overnight culture was washed twice to remove the antibiotics and then back diluted to an optical density at 620 nm (OD₆₂₀) of 0.01 into fresh prewarmed medium supplemented with 100 µM IPTG. The culture was incubated until the OD₆₂₀ reached 0.7 (mid-log phase). A 10-ml portion of the culture was used for RNA preparation (condition A of the experiment).

The culture grown with the optimal IPTG concentration was back diluted to an OD₆₂₀ of 0.01 into fresh prewarmed TSB supplemented with a suboptimal concentration of IPTG (2.5 µM) and was incubated until the OD₆₂₀ reached 0.7. A 10-ml portion of this culture was used for RNA preparation (condition B of the experiment). IPTG was added to the culture grown in condition B, to the optimal concentration (100 µM). After 30 min of incubation, 10 ml of this culture was used for RNA preparation (condition C of the experiment).

Sampling and RNA preparation. At the time of sampling, a double volume of RNAProtect Bacteria reagent (QIAGEN, Hilden, Germany) was added to the culture. The mixture was incubated for 5 min at room temperature, the cells were centrifuged for 10 min at 3,500 rpm and 4°C, and the pellets were frozen in dry ice and kept at -70°C. Total RNA was extracted as described previously (31). The RNA quality and quantity was determined by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm. Each of the cultures grown in conditions A, B, and C was repeated three times.

DNase I purification of total RNA. Total RNA from all samples were treated with DNase I by using the RNase-free DNase set protocol and RNeasy minikit protocol (QIAGEN, Valencia, CA). Total RNA was quantitated using the NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE).

The protocols for staphylococcal genomic chip design, *Staphylococcus aureus* array probe selection, Affymetrix probe preparation and array processing, data quality control, and statistical analysis are described in the supplemental material.

Microarray validation by real-time reverse transcription-PCR (RT-PCR). Total RNA preparation was performed exactly as described for the microarray assay. Residual DNA was removed from the samples by performing two on-column DNase digestion steps with RNase-free DNase (QIAGEN). cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) and random hexamers. Specific primers for the genes tested and for 16S rRNA, used as endogenous control, were designed using Primer Express software (Applied Biosystems) and are listed in Table S1 in the supplemental material. Real-time PCR was performed with the ABI PRISM 7000 sequence detection system (Applied Biosystems) and SYBR green technology. The three biological replicates were analyzed in duplicate and normalized against 16S rRNA gene expression. Each assay included samples for the three conditions and standard curves, both for the target gene and for the 16S rRNA control gene. For all experiments, the amounts of target and control genes were determined from the appropriate standard curve. In order to obtain a normalized relative gene expression value, the amount of target gene for each condition was divided by the respective amount of control gene.

Determination of oxacillin MIC. COL*spacmurF* cultures were plated on tryptic soy agar with 100 µM and 2.5 µM IPTG and incubated overnight at 37°C. Oxacillin Etests (AB Biodisk, Solna, Sweden) were used to determine the MICs.

In vivo autolysis assay. Triton X-100-stimulated autolysis was determined as described previously (5). COL cultures were grown to an OD₆₂₀ of 0.7. COL*spacmurF* cultures were grown exactly as described for the microarray assay conditions A, B, and C.

Preparation of autolytic extracts. Crude autolytic extracts were prepared as described previously (28). Bacterial cultures were grown as described for the microarray assay conditions A, B, and C. The bacterial pellets were frozen to

-70°C and extracted with 4% sodium dodecyl sulfate (SDS) at room temperature or with 3 M LiCl and 0.1% Triton X-100 at 4°C, and the supernatants were used as autolytic extracts.

Crude cell wall preparation. Bacteria were grown as described for the microarray assay conditions A and B, chilled rapidly, harvested by centrifugation, and boiled in 8% SDS for 30 min. After cooling to room temperature, the samples were washed with water until all traces of SDS were removed and were lyophilized.

In vitro autolysis assay. Crude cell walls were suspended in 50 mM Tris-Cl (pH 7.5) to an initial OD₆₂₀ of 0.5 and incubated with 10 µg/ml of each LiCl autolytic extract. Lysis was measured as the decrease in OD₆₂₀ during incubation at 37°C.

Zymogen profiles. Crude cell walls were incorporated in polyacrylamide resolving gels (10% acrylamide-0.2% bisacrylamide) at a final concentration of 1 mg/ml. The SDS-extracted supernatants were separated by the technique of Laemmli (13) at a constant current of 20 mA at 4°C. The lytic effect of the enzymes was visualized as described previously (29). After a washing step in water, the gels were incubated at 37°C in zymogen buffer for 1 h.

Metabolic pattern determination. Strain COL was grown to an OD₆₂₀ of 0.7, and COL*spacmurF* cultures were grown exactly as described above for microarray assay conditions A and B. The cells were washed twice and resuspended in the same volume of buffer (0.15 M NaCl, 0.1 mM NaPO₄, pH 7.2). Biolog GP2 microplates (Biolog, Hayward, CA) were inoculated with 150 µl of the cell suspension in each well and incubated overnight.

RESULTS AND DISCUSSION

The structure and biosynthesis of the bacterial peptidoglycan are the targets of some of the most powerful antimicrobial agents. Inhibition of these targets not only causes interference with bacterial growth but also invokes in the cells rapid changes in the pattern of transcription of a large number of genes (11, 22, 35), suggesting the existence of complex regulatory loops which link a wide variety of steps in metabolism to some steps in cell wall biosynthesis.

In all these studies with antibacterial agents, the altered gene expression profiles are the direct or indirect consequences of inhibition of enzymes involved in peptidoglycan synthesis. Furthermore, in the case of oxacillin, not one but several enzymes (penicillin binding proteins) are affected. The same is true for vancomycin and D-cycloserine, each of which inhibits at least two cell wall synthesis enzymes. Also, depending on the dose used, these inhibitions can also cause irreversible effects leading to loss of viability and/or lysis. Therefore, at least some of the regulatory abnormalities observed in antibiotic-treated cells may be related to delayed indirect effects on bacterial cell structure metabolism.

The availability of a mutant with a conditional mutation in the cell wall synthetic gene *murF* offered an opportunity to explore these regulatory circuits by a novel method that does not involve the use of antimicrobial agents. In the mutant COL*spacmurF*, constructed in the background of the highly and homogeneously methicillin-resistant *Staphylococcus aureus* strain COL (30), transcription of the *murF* gene is under the control of an IPTG-inducible promoter, which allowed us to compare the transcriptomes of *S. aureus* grown with optimal and suboptimal transcription of a key determinant of cell wall precursor synthesis.

Experimental design. The experimental design consisted of growing COL*spacmurF* under optimal (100 µM) (condition A) and suboptimal (2.5 µM) (condition B) concentrations of the inducer for six generations (mass doubling times). In order to test the reversibility of transcriptional changes, a portion of the culture grown with the suboptimal IPTG concentration was

exposed for a single generation time to optimal levels of the inducer (condition C). Cells were harvested at the same optical density in the mid-exponential phase of growth, RNA was prepared, and the number and nature of genes which underwent a change in transcriptional pattern were evaluated using a custom-made Affymetrix DNA microarray.

The impact of the decreased transcription of the *murF* gene has been previously described in detail for several concentrations of inducer (30). For the purposes of this study, only two IPTG concentrations were used. Cultures grown in 100 μ M IPTG (condition A) showed properties which were similar to those of the parental strain COL, in which *murF* is under the control of its native promoter. Cultures grown at the suboptimal concentration of 2.5 μ M IPTG (condition B) showed extensive changes in various properties, which included a reduced growth rate, a reduction in the cellular amounts of the cell wall precursor pentapeptide to about 50% of the normal level, and an increase in the concentration of the abnormal cell wall precursor tripeptide, which also incorporated as an unsubstituted monomer into the peptidoglycan of the cell wall. Cultures grown with 2.5 μ M IPTG also showed decreased and heterogeneous resistance to oxacillin (Fig. 1a to c).

Changes in the transcriptional profile of cells grown in suboptimal concentrations of IPTG. The reduced rate of transcription of *murF* caused alterations in a surprisingly large number of genes. With a threshold value set at 1.5, as many as 668 genes were affected (393 were up-regulated and 275 were down-regulated), out of the total 2,740 described open reading frames (ORFs) for *S. aureus* strain COL. With a threshold value set at 2.0, the total number of genes affected was 316 (203 overexpressed and 113 repressed). With a threshold set at 5.0, the number of genes affected was reduced to 59 (44 up-regulated and 15 down-regulated). The genes involved were homogeneously distributed along the entire chromosome (see Fig. S1 in the supplemental material).

All 668 genetic determinants identified in the microarray experiment using the lower threshold value are listed in Table S2 in the supplemental material.

The surprisingly large number of genes, representing almost one-fourth of all the genetic determinants, that responded to the *murF* stress provides a striking view of the scope and complexity of the regulatory circuitry interconnecting functionally diverse aspects of the *S. aureus* cell to a single cell wall synthesis function. Most importantly, the majority of transcriptional changes were reversible even after a relatively short time of restoring suboptimal *murF* transcription to normal levels.

Activation of the cell wall stimulon. In pioneering studies, Utaida and colleagues (34) and Kuroda and colleagues (12) described extensive and unique changes in the gene expression profile of *S. aureus* strains after exposure to inhibitory concentrations of cell wall targeting antibiotics. In the study by Utaida et al. a derivative of *S. aureus* strain 8325 was exposed to oxacillin, D-cycloserine, and bacitracin at concentrations that reduced growth to 70% of the control value (34). A total of 163 genes showed altered transcription. In the study by Kuroda et al., the MRSA strain N315 was exposed to 10 times the MIC of vancomycin for 10 min, which caused altered expression of 169 genes. It was proposed that genetic determinants whose expression was altered under these conditions belonged to a coordinately regulated "cell wall stimulon" (34).

More recently, McAleese and colleagues tested the number and nature of genes with altered expression induced by brief (10-min) treatment of the MRSA strain JH1 with 1 or 8 times the MIC for vancomycin (19). The same authors also observed changes in the transcription of a large number of genes (224) when cultures of strain JH1 and its isogenic vancomycin-resistant derivative JH9 were compared by microarray analysis during growth in drug-free medium (19). Most recently, McCollum and colleagues described altered transcription of 109 genes when the methicillin-susceptible *S. aureus* strain Newman was treated with 10 times the MIC of vancomycin for 10 min (20).

A substantial proportion of the determinants that responded to the *murF* stress were similar to genes that show altered expression after treatment with wall targeted antibiotics. Table 1 shows a selection of genes that showed altered transcription both during *murF* stress and also in at least two of the published studies in which antibiotic treatment was used to induce the cell wall stimulon. The match between genes detected in the *murF* transcriptional study and any of the other studies was between 40 and 62%. However, the direction of gene expression (up- versus down-regulation) and the degree of change were not necessarily the same in the studies compared. McAleese et al. proposed a "core" cell wall stimulon composed of 15 genes that were commonly observed with altered expression in each one of the four previous studies that used antibiotic treatment for the induction (19). Table 2 shows that 11 of these 15 genes were also up-regulated in the *murF* system.

The most obvious differences between the natures of genes that responded to the *murF* stress and the ones that were up-regulated during treatment with the cell wall inhibitors were a group of genetic determinants that included cell wall synthesis genes such as *pbpB*, *murA*, *murZ*, *fntA*, *atl*, which appear to be up-regulated in most of the studies using antibiotics but remained unchanged during the suboptimal *murF* expression (Table 2). The lack of transcriptional changes in these determinants during *murF* stress fits the radically different types of perturbation of wall synthesis in the antibiotic-treated bacteria: the cells are apparently trying to compensate for cell wall synthesis enzymes that are inactivated by the antibiotics, while in the *murF* system the synthetic apparatus of cell walls remains intact.

Expansion of the cell wall stimulon. Table S3 in the supplemental material lists genes of the cell wall stimulon identified in the *murF* system in the order of their chromosomal location. Also shown in that table are the corresponding genes detected by Utaida et al. (34) and Kuroda et al. (12). This exhibit allows one to expand the list of genes that belong to the cell wall stimulon by the addition of new genes localized in the direct vicinity of previously identified cell wall stimulon determinants. The proposed new members of the cell wall stimulon belonged to operons predicted by an algorithm developed at TIGR (http://www.tigr.org/tigr-scripts/operons/pairs.cgi?taxon_id=40141) which detects conserved gene pairs in two or more bacterial genomes and estimates a probability for the genes to belong to the same operon (see Table S3 in the supplemental material). It seems that genetic determinants which are transcribed in the same mRNA molecule will tend to respond to the induced stress in a similar manner, either up- or down-regulated, and also will follow the same pattern of reversibility upon readdition of IPTG.

FIG. 1. Phenotypic characterization of strain COL and the *COLspacmurF* conditional mutant grown with the optimal IPTG concentration (100 μ M) (condition A) and a suboptimal IPTG concentration (2.5 μ M) (condition B). (a) Growth curves were monitored by OD₆₂₀, and samples were taken for microarray analysis at mid-exponential phase at the same OD₆₂₀ value of $\sim$ 0.7, as indicated by the arrows. (b) The cell wall precursor pool composition was determined by reverse-phase high-pressure liquid chromatography (30). Components of peaks 1 and 2 were determined by mass spectrometry. Structure 1, UDP-MurNac-Ala-Glu-Lys; structure 2, UDP-MurNac-Ala-Glu-Lys-Ala-Ala (31). (c) The oxacillin MIC was determined by Etest.

TABLE 1. Genes whose expression was altered in conditional mutant *COLspacmurf* when grown in the presence of a subinhibitory inducer concentration (condition B) and which also showed differential expression for at least other two of the specific conditions tested in other studies (12, 19, 20, 34)

COL ORF (SACOL)	Gene	Gene function	Change in expression level						
			COL <i>spacmurf</i> (present study) ^a		Strain NCTC 8325 (oxacillin bacitracin and D-cycloserine) (34)	Strain JH1 (vancomycin) (19)	Strains JH1 and JH9 (JH1 vs JH9) (19)	Strain Newman (vancomycin) (20)	
			Suboptimal IPTG (B/A)	Return to optimal IPTG (C/B)					
0008	<i>hutH</i>	Histidine ammonia-lyase	1.9	R	Up			3.0	
0095	<i>spa</i>	Immunoglobulin G binding protein A precursor	5.0	No	Down	-2.9	-48.9	-4.9	
0111	<i>hutA</i>	Acetoin (diacetyl) reductase	3.1	r	Up				
0191		M23/M37 peptidase domain protein	2.9	R		Up	4.5	8.2	
0204	<i>pflB</i>	Formate acetyltransferase	51.3	R	Down			-4.2	
0205	<i>pflA</i>	Pyruvate formate-lyase-activating enzyme	14.3	R	Down			-4.6	
0209	<i>coa</i>	Staphylocoagulase precursor	4.1	R	Down		-13.9		
0244	<i>scdA</i>	Cell division and morphogenesis-related protein	3.5	r			-15.4		
0478	<i>set-15</i>	Exotoxin 15	2.5	R			-13.5		
0479		Surface protein, putative	2.0	R				Down	
0504		ABC transporter, ATP binding protein	3.3	No			-2.4		
0505		ABC transporter, permease protein	3.7	No			-2.3		
0515		Glutamate synthase, small subunit	2.3	No	Up				
0625		Conserved hypothetical protein	12.2	R	Up	224.7	22.7	-5.7	
0660	<i>adhI</i>	Alcohol dehydrogenase, zinc-containing	15.3	R	Down				
0692		Conserved hypothetical protein	4.9	R	Up	Up	5.3		
0802		Conserved hypothetical protein	-2.7	No		Down	-2.1		
0967		Conserved hypothetical protein	1.8	R		5.8	2.1	4.1	
0992	<i>appC</i>	Oligopeptide ABC transporter, permease protein	4.7	No	Up				
0993	<i>appD</i>	Oligopeptide ABC transporter, ATP binding protein	4.8	No	Up		-2.1		
0994	<i>appF</i>	Oligopeptide ABC transporter, ATP binding protein	4.7	No	Up				
0995	<i>appA</i>	Oligopeptide ABC transporter, oligopeptide-binding protein	5.2	No	Up			-3.1	
1071 ^b		Chitinase-related protein	2.5	R	Up	10.6	2.2	3.2	
1384 ^b	<i>opuD</i>	Osmoprotectant transporter, BCCT family	-1.8	No					
1455 ^b	<i>cipA</i>	Carboxyl-terminal protease	1.5	No					
1456 ^b		Conserved hypothetical protein	1.6	R	Up		-2.8	3.7	
1478	<i>ald</i>	Alanine dehydrogenase	3.9	r	Up			-4.0	
1659		Conserved hypothetical protein	1.7	R	Up				
1661		Acetyl-coenzyme A carboxylase, biotin carboxylase, putative	1.8	r	Up				
1705 ^b		Hypothetical protein	4.1	R	Up	20.9	4.0	8.5	
1777 ^b	<i>htrA</i>	Putative serine protease	2.0	R	Up	6.8		6.6	
1897 ^b	<i>prsA</i>	Putative export protein	2.3	R	Up	12	3.2	6.4	
1915		Glutamate ABC transporter, ATP binding protein	2.3	r	Down		-2.0		
1932 ^b	<i>sgtB</i>	Transglycosylase domain protein	3.5	R	Up	Up	2.9	9.4	
1942 ^b	<i>vraS</i>	DNA binding response regulator VraR	1.9	R	Up	8.7	2.5	6.2	
1943 ^b		Sensor histidine kinase	1.8	R	Up	8.7	2.5	10.1	
1944 ^b	<i>vraR</i>	Conserved hypothetical protein	1.9	R	Up	8.1	2.6	9.8	
1945 ^b		Conserved hypothetical protein	2.3	R	Up	6.5	3.1	9.7	
1956 ^b		Conserved hypothetical protein	1.5	No	Up	Up	2.0	15.5	

2145	<i>glmS</i>	Glucosamine-fructose-6-phosphate aminotransferase	1.6	R		0.45			4.0
2295		Staphyloxanthin biosynthesis protein, putative	-5.8	R				4.8	
2302 ^b	<i>hydR</i>	Transcriptional regulator, putative	1.6	R	Up	4.06	Down	2.7	
2315 ^b		Conserved hypothetical protein	2.8	R		2.65	10.4	3.8	11.7
2328		Abortive infection protein family	-2.4	R		0.38		-13.3	
2363	<i>narK</i>	L-Lactate permease	23.8	r	Down			-2.7	Down
2386	<i>nirR</i>	Nitrite extrusion protein	30.6	r	Down				Down
2399		Transcriptional regulator	68.8	R	Down				
2403		ABC transporter, substrate-binding protein	-1.6	No		0.22		-3.0	
2418	<i>shi</i>	Immunoglobulin G-binding protein	2.3	No		3.36		-6.7	
2436 ^b		Conserved hypothetical protein	2.7	R	Up	5.37	9.5	2.4	6.5
2491		Conserved hypothetical protein	21.9	r	Down				-3.0
2505	<i>saxG (uap)</i>	Cell wall surface anchor family protein	3.6	No	Down			-3.2	Down
2517 ^b		Transcriptional regulator, MerR family	2.2	r		3.87	Up	2.0	3.7
2518 ^b		Conserved hypothetical protein	2.5	r	Up	3.85	8.6	2.0	3.2
2519 ^b		Conserved hypothetical protein	3.9	No		2.04			
2571 ^b		Conserved hypothetical protein	25.9	R	Up	6.55	Up	84.4	22.6
2619		Amino acid permease	3.7	No	Down	3.44			
2712 ^b	<i>drp35</i>	Drp35 protein	1.8	R		2.94			7.2
2713		Rhodanese-like domain protein	-1.8	No	Up		3.4		
Total			59		37	39	24	35	32
No. up			53		25	36	21	20	21
No. down			6		12	3	3	15	11
Total no. of genes			668		163	169	60	224	109
% Match between <i>murF</i> study and each of the other studies					40.5	47.9	40	42	62.4

^a Values for determinants which were found to be overexpressed in the suboptimal condition are listed in boldface, and the numerical value refers to the overexpressed value divided by the value for the same gene grown with optimal IPTG (condition B/condition A). If this condition causes repression of transcription, the same values are listed in normal type. Also listed for each gene is the reversibility of the response upon readdition of optimal concentrations of IPTG to bacteria which were growing with suboptimal inducer. R, high or complete reversibility; r, low reversibility; no, lack of reversibility.

^b Gene which belongs to the *VraSR* regulon.

TABLE 2. Genes belonging to the “core” cell wall stimulon and cell wall synthesis-related genes which show altered expression in this and the other studies (12, 19, 20, 34)

Group	COL ORF (SACOL)	Gene	Gene function	Change in gene expression					
				COLspacmurF (present study) ^a		Strain NCTC 8325 (oxacillin, bacitracin, and D-cycloserine) (34)	Strain JH1 (vancomycin) (19)	Strains JH1 and JH9 (JH9 vs JH1) (19)	Strain Newman (vancomycin) (20)
				Suboptimal IPTG (B/A)	Return to optimal IPTG (C/B)				
Core cell wall stimulon genes ^b	1066	<i>fml</i>	Autolysis and methicillin resistance-related protein			Up	Up		
	1777	<i>htrA</i>	Putative serine protease	2.0	R	Up	3.62	6.8	6.6
	1897	<i>prsA</i>	Putative protein export protein	2.3	R	Up	5.74	12.0	6.4
	1932	<i>sgtB</i>	Transglycosylase domain protein	3.5	R	Up	5.64	Up	9.4
	1943	<i>vraS</i>	Sensor histidine kinase	1.8	R	Up	7.41	8.7	10.1
	1944		Conserved hypothetical protein	1.9	R	Up	7.27	8.1	9.8
	1945		Conserved hypothetical protein	2.3	R	Up	7.27	6.5	9.7
	1956		Conserved hypothetical protein		No	Up	4.73	Up	15.5
	2116	<i>murZ</i>	UDP-N-acetylglucosamine 1- carboxyvinyl transferase 2	1.5		Up	2.52	5.4	4.1
	2302	<i>lytR</i>	Transcriptional regulator, putative			Up	4.06	3.4	
	2352	<i>tcaA</i>	Teicoplanin resistance-associated protein	1.6	R	Up	2.40	4.1	4.0
	2435		Conserved hypothetical protein			Up	3.33	Up	
	2436		Conserved hypothetical protein	2.7	R	Up	5.37	9.5	6.5
	2518		Conserved hypothetical protein	2.5	r	Up	3.85	8.6	3.2
	2571		Conserved hypothetical protein	25.9	R	Up	6.55	Up	22.6
Cell wall synthesis- related genes showing altered expression in 5 studies	0033	<i>mecA</i>	Penicillin binding protein 2A			Up		−2.7	
	0636	<i>mvaK1</i>	Melavonate kinase				2.08		
	0693	<i>tagA</i>	Putative teichoic acid biosynthesis protein			Up			
	0743	<i>bacA</i>	Bacitracin resistance protein			Up			
	1066	<i>fmlA</i>	Autolysis and methicillin resistance-related protein			Up	2.86	Up	
	1161	<i>murI</i>	Glutamate racemase			Up			
	1279	<i>upps</i>	Undecaprenyl pyrophosphate synthetase			Up			
	1396	<i>fmlC</i>	Autolysis and methicillin resistant- related protein					2.0	
	1490	<i>phpB</i>	Penicillin binding protein 2			Up			3.3
	2116	<i>murZ</i>	UDP-N-acetylglucosamine enolpyruvate transferase			Up	1.87	5.4	4.1
	2540	<i>strA</i>	Sortase A				2.52	−2.6	
	1194	<i>phpA</i>	Penicillin-binding protein 1	1.7	R	Up		2.9	9.4
	1932	<i>sgtB</i>	Monofunctional glycosyltransferase	3.5	R		5.64	10.6	

^a See Table 1, footnote a.

^b Genes belonging to the “core” wall stimulon identified previously (19). Of the total 15 “core” genes, 11 were found to be up-regulated in COLspacmurF in condition B.

TABLE 3. Members of the *VraRS* regulon (12) which were found to be differentially expressed when the *COLspacmurF* conditional mutant was grown with a suboptimal inducer concentration (condition B)

Change in gene expression										
COL ORF (SACOL) ^b	Gene	Gene function	Functional category	COLspacmurF (present study) ^a		Strain NCTC 8325 (oxacillin, bacitracin, and D-cycloserine) (34)	Strain N315 (vancomycin) (12)	Strain JH1 (vancomycin) (19)	Strains JH1 and JH9 (JH1 vs JH9) (19)	Strain Newman (vancomycin) (20)
				Suboptimal IPTG (B/A)	Return to optimal IPTG (C/B)					
1071		Chitinase-related protein	Unknown function	2.5	R	Up	1.94	10.6	2.2	3.2
1384	<i>opuD</i>	Osmoprotectant transporter	Transport and binding proteins	−1.8	No					
1455	<i>cip4</i>	Carboxyl-terminal protease	Protein fate	1.5	No					
1456		Conserved hypothetical protein	Hypothetical proteins	1.6	R					
1705		Hypothetical protein	Hypothetical proteins	4.1	R					
1777	<i>hmr4</i>	Putative serine protease	Protein fate	2.0	R	Up	5.74	12	3.2	6.4
1897	<i>pxs4</i>	Putative export protein	Protein fate	2.3	R	Up	5.64	Up	2.9	9.4
1932	<i>sgtB</i>	Transglycosylase domain protein	Unknown function	3.5	R					
1942	<i>vraR</i>	DNA binding response regulator	Signal transduction system	1.9	R					
1943	<i>vraS</i>	Sensor histidine kinase	Signal transduction systems	1.8	R	Up	7.41	8.7	2.5	10.1
1944		Conserved hypothetical protein	Hypothetical proteins	1.9	R	Up	7.27	8.1	2.6	9.8
1945		Conserved hypothetical protein	Hypothetical proteins	2.3	R	Up	7.27	6.5	3.1	9.7
1956		Conserved hypothetical protein	Hypothetical proteins	1.5	No	Up	4.73	Up	2.0	15.5
2302	<i>lyrR</i>	Putative transcriptional regulator	Regulatory functions	1.6	R					
2315		Conserved hypothetical protein	Hypothetical proteins	2.8	R					
2436		Conserved hypothetical protein	Hypothetical proteins	2.7	R	Up	5.37	9.5	2.4	6.5
2517		Transcriptional regulator	Regulatory functions	2.2	R					
2518		Conserved hypothetical protein	Hypothetical proteins	2.5	R	Up	3.85	8.6	2.0	3.2
2519		Conserved hypothetical protein	Hypothetical proteins	3.9	No					
2571		Conserved hypothetical protein	Hypothetical proteins	25.9	R	Up	6.55	Up	84.4	22.6
2712	<i>dpr35</i>	DPr35 protein	Unknown function	1.8	R					

^a See Table 1, footnote *a*.
^b Underlining indicates genes whose expression is commonly altered (up-regulated) in the present study and previous studies (12, 19, 20, 34).

FIG. 2. (a) Twenty sortase-anchored surface proteins were identified in the *S. aureus* COL genome. All the listed surface proteins are anchored through a sortase A-dependent mechanism, except for IsdC, which is sortase B dependent. Determinants which were found to be overexpressed in cells growing in the suboptimal condition are listed in boldface, and the numerical value shown is the overexpressed value divided by the value for the same gene grown with optimal IPTG (condition B/condition A). If this condition causes repression of transcription, the values are listed in normal type. Also listed for each gene is the reversibility of the response upon readdition of optimal concentrations of IPTG to bacteria which were growing with suboptimal inducer; complete reversibility (R) and lack of reversibility are indicated. When COL*spacmurF* was grown with the suboptimal inducer concentration (condition B), half of the genes corresponding to sortase A-anchored proteins were overexpressed and only the IsdC gene was underexpressed. (b) Organization of the *isd* (iron-regulated surface determinant) members in *S. aureus* (18). The genes *isdC*, *isdD*, *isdE*, *isdF*, *srtB*, and *isdG* (grey arrows) belong to the same operon. *isdA* and *isdB* (white arrows) belong to independent transcriptional units. (c) Members of the *isd* determinant, including the genes encoding the surface-anchored protein IsdC, ferrichrome transporter IsdE-IsdF, and sortase B, were found to be underexpressed in condition B. These genes belong to the same operon. The other *isd* genes, *isdA* and *isdB*, are transcribed independently and were not found to be affected. (d) The absolute values of gene expression are shown for the *isd* genes in the three conditions tested, i.e., in cells grown in the presence of the optimal IPTG concentration (condition A), cells grown with a suboptimal IPTG concentration (“*murF* stress”) (condition B), and cells that received an optimal concentration of IPTG following growth under the *murF* stress condition (condition C). This way of illustrating differential expression levels allows an easier comparison of the responses of individual genetic determinants to the *murF* stress, including the degree of change in transcription and the presence or lack of reversibility. The absolute values of expression are shown for the *isd* genes which showed transcription changes in condition B. For the *isd* genes belonging to the same transcriptional unit, the expression reversibility upon readdition of inducer (condition C) occurs consistently.

Changes in expression of the *vraSR* regulon. Kuroda and colleagues identified within the cell wall stimulon a group of 46 determinants in which altered transcription depended on the presence of an intact *vraSR*, a two-component sensory/regulatory system of *S. aureus* (12). As many as 21 of the members of the *vraSR* regulon also responded to the decreased transcription of *murF*, and all but two of these genes were up-regulated (Table 3). Except for *opuD*, *ctpA*, SACOL1956, and SACOL2519, the

altered transcription was reversible in the rest of the determinants.

Changes in the expression of sortase-dependent surface-anchored proteins and proteins involved with iron metabolism. The effect of *murF* stress on the transcription of surface-attached proteins suggests functional specificity. Out of the 20 previously described sortase-dependent *S. aureus* proteins (17, 18, 25), the genetic determinants of 11 were differentially ex-

TABLE 4. Genes related to iron scavenging and metabolism whose expression was found to be altered when COL*spacmurF* was grown with suboptimal IPTG (condition B)^a

COL ORF (SACOL)	Gene	Gene function	Change in gene expression					
			COL <i>spacmurF</i> (present study) ^b		Strain NCTC 8325 (oxacillin, bacitracin, and D-cycloserine) (34)	Strain N315 (vancomycin) (12)	Strains JH1 and JH9 (JH1 vs JH9) (19)	Strain JH1 (vancomycin) (19)
			Suboptimal IPTG (B/A)	Return to optimal IPTG (C/B)				
0098	<i>sirB</i>	Iron compound ABC transporter, permease protein	−2.1	R			Down	
0099	<i>sirA</i>	Iron compound ABC transporter, iron compound binding protein	−3.5	R			Down	
0796		Iron compound ABC transporter, permease protein	−1.8	No				
0797		Iron compound ABC transporter, permease protein	−1.9	No				
0798	<i>sstC</i>	Iron compound ABC transporter	−2.2	No			Down	
0799		Transferrin receptor	−1.6	No				
0997	<i>sirC</i>	Iron compound ABC transporter, permease protein	−1.9	No				
1114		Mn ²⁺ /Fe ²⁺ transporter, NRAMP family	1.6	No				
1124	<i>ctaA</i>	Cytochrome oxidase assembly protein	2.2	r				
1141	<i>isdC</i>	NPQTN cell wall surface anchor protein	−2.1	R			Down	
1142	<i>isdD</i>	Hypothetical protein	−1.7	R				
1143	<i>isdE</i>	Iron compound ABC transporter, iron compound binding protein	−1.6	R				
1144	<i>isdF</i>	Iron compound ABC transporter, permease protein	−1.6	R				
1145	<i>srtB</i>	Sortase B	−2.2	R			Down	
1146		Conserved hypothetical protein	−2.0	R				
1308		Pyruvate ferredoxin oxidoreductase, alpha subunit	−1.6	R				
1525		Ferredoxin	−1.9	No				
1611		Transcriptional regulator, Fur family	−1.8	No				
1612		ABC transporter, permease protein	−1.7	No				
1613		ABC transporter, ATP binding protein	−2.0	No				
1640		Oxygen-independent coproporphyrinogen III oxidase, putative	−1.8	No				
2165		Iron compound ABC transporter, permease protein	−2.0	R				
2166		Iron compound ABC transporter, permease protein	−2.3	R				
2167		Iron compound ABC transporter, iron compound binding protein	−2.2	R				
2170		Transporter, putative	−1.7	R				
2277		Iron compound ABC transporter, iron compound binding protein	−1.7	No				
2396		Uroporphyrinogen III methylase SirB, putative	4.8	R	Down			
2541		Acetyltransferase, GNAT family	−1.7	No				
2544	<i>sdaAA</i>	L-Serine dehydratase, iron-sulfur-dependent, alpha subunit	−2.3	No				
2545	<i>sdaAB</i>	L-Serine dehydratase, iron-sulfur-dependent, beta subunit	−3.1	No				
2546		Perfringolysin O regulator protein, putative	−2.9	No				
2638		Siroheme synthase, putative	−1.5	r				

^a The vast majority of these genes were found to be down-regulated in condition B and were not found to be affected in the other referred studies.^b See Table 1, footnote a.

pressed under the suboptimal expression of *murF*. Of these, 10 of the genetic determinants were overexpressed in condition B, while one gene, *isdC*, was repressed (Fig. 2a).

This differential response appears to correspond to the two kinds of sortases used for anchoring these proteins. Each one of the 10 overexpressed proteins is attached to the cell wall at the lipid 2 level by a sortase A-dependent process (26, 33). Overexpression of the 10 sortase A-dependent genes may be a response by the cells compensating for the shortage of normal, pentapeptide cell wall precursors which are replaced in the

condition B cells by the abnormal tripeptide precursor which does not carry the oligoglycine branches needed for the attachment of these proteins (32).

In contrast, the protein product of the solitary gene *isdC*, the expression of which was repressed, is anchored to the cell wall by a sortase B-dependent reaction in which the acceptors are in the polymerized cell wall and are equipped with the oligoglycine branches (16, 18).

A unique pattern of response to the perturbation of wall synthesis is also apparent in Fig. 2b to d, which shows that the

TABLE 5. Virulence-related genes found to be differentially expressed in the COL*spacmurF* conditional mutant when grown with suboptimal inducer concentrations (condition B)^a

Virulence-related determinants			Change in expression level					
COL ORF (SACOL)	Gene	Gene function	COL <i>spacmurF</i> (present study) ^b		Strain NCTC 8325 (oxacillin, bacitracin, and D-cycloserine) (34)	Strain N315 (vancomycin) (12)	Strains JH1 and JH9 (JH1 vs JH9) (19)	Strain JH1 (vancomycin) (19)
			Suboptimal IPTG (B/A)	Return to optimal IPTG (C/B)				
vSaα	0095	<i>spa</i> Immunoglobulin G binding protein A precursor	5.0	No	Down		Down	Down
	0119	<i>sasD</i> Cell wall surface anchor family protein	9.6	R	Down			
	0209	<i>coa</i> Staphylocoagulase precursor	4.1	R				
	0261	Putative drug transporter	−1.5	No				
	0276	<i>yukA</i> Diarrheal toxin	−1.6	R				
	0390	<i>geh</i> Lipase precursor, interruption-carboxy	−1.9	R				
	0462	Hypothetical protein	2.6	No				
	0478	<i>set3</i> Putative exotoxin 3	2.5	R				
	0479	Putative surface protein	2.0	R				
	0480	Hypothetical protein	7.7	No				
	0482	Tandem lipoprotein	−1.7	No				
	0483	Tandem lipoprotein	−1.6	No				
	0609	<i>sdrD</i> SdrD protein	4.6	No				
	0726	<i>sarX</i> Staphylococcal accessory protein X	1.6	No				
	0765	<i>saeS</i> Sensor histidine kinase	1.9	No				
	0766	<i>saeR</i> DNA binding response regulator	1.6	No				
	0856	<i>clfA</i> Clumping factor A	2.6	No				
	0857	Putative staphylocoagulase precursor	3.0	R				
	0858	<i>empbp</i> Secretory extracellular matrix plasma binding protein	1.7	No				
vSa1	0885	Pathogenicity island protein, integrase	−4.0	r				
	0886	<i>sek</i> Staphylococcal enterotoxin	−2.0	R				
	0887	<i>sei</i> Staphylococcal enterotoxin type I	−1.5	R				
	0888	Putative pathogenicity island, lipoprotein	−1.6	R				
	0889	Pathogenicity island protein	−2.0	R				
	0890	Transcriptional regulator, Cro/C1 family	−1.6	R				
	0892	Pathogenicity island protein	−1.6	R				
	0907	<i>seb</i> Staphylococcal enterotoxin B	3.8	No				
	0908	Conserved hypothetical protein	1.6	r				
	1056	<i>sspB</i> Cysteine protease precursor	2.2	No				
1057	<i>sspA</i> Serine V8 protease	2.0	No					
1060	Transcriptional regulator, MarR family	1.7	No					
vSaγ	1141	<i>isdC</i> NPQTN cell wall surface anchor protein	−2.1	R			Down	
	1164	<i>fib</i> Fibrinogen binding-related protein	5.6	No			Down	
	1165	Conserved hypothetical protein	5.5	No			Down	
	1167	Conserved hypothetical protein	1.7	No			Down	
	1168	<i>efb</i> Fibrinogen binding protein	8.8	r				
	1169	Fibrinogen binding precursor-related protein	11.2	R			Down	
	1171	Conserved hypothetical protein	1.5	No			Down	
	1173	<i>hly</i> Alpha-hemolysin precursor	7.0	No				
	1175	Conserved hypothetical protein	3.3	No				
	1184	<i>et</i> Putative exfoliative toxin	5.4	R			Down	
1185	Conserved hypothetical protein	1.7	No			Down	Down	
1357	<i>nuc</i> Thermonuclease precursor family protein	−4.2	No					
1522	<i>ebp</i> Elastin binding protein, putative	1.8	No					

Continued on following page

TABLE 5—Continued

Virulence related determinants			Change in expression level										
COL ORF (SACOL)	Gene	Gene function	COL <i>spacmurF</i> (present study) ^b		Strain NCTC 8325 (oxacillin, bacitracin, and D-cycloserine) (34)	Strain N315 (vancomycin) (12)	Strains JH1 and JH9 (JH1 vs JH9) (19)	Strain JH1 (vancomycin) (19)					
			Suboptimal IPTG (B/A)	Return to optimal IPTG (C/B)									
vSaβ	1534	<i>srrB</i>	2.1	r	Up	Up	Down	Up					
	1535	<i>srrA</i>	2.4	r									
	1777	<i>htrA</i>	2.0	R									
	1848	Conserved hypothetical protein	1.7	No					Down				
	1871	<i>epiG</i>	2.0	R									
	1872	<i>epiE</i>	2.5	R									
	1873	<i>epiF</i>	2.5	r									
	1883	Conserved hypothetical protein	1.5	No									
	2002	<i>map</i>	5.6	No									
	2003	<i>hlyB</i>	7.4	No									
	2004	Putative leukocidin subunit precursor	8.4	R									
	2006	<i>lukM</i>	11.3	r									
	2150	<i>sasB</i>	1.6	No						Down			
	2194	<i>hysA</i>	−1.5	No						Down			
	2289	<i>sarY</i>	2.1	No						Down			
	2347	Drug resistance transporter, EmrB/QacA subfamily	−1.7	No						Down			
	2348	Putative drug transporter	−1.9	No									
	2418	<i>sbi</i>	2.3	No							Up	Down	
	2421	<i>hlyC</i>	6.3	No							Up		
	2422	<i>hlyB</i>	3.9	No							Up		
	2449	Putative drug transporter	1.8	No									
	2505	<i>sasG</i>	3.6	No							Down	Down	
	2509	<i>fnbB</i>	12.5	R							Down		
	2511	<i>fnbA</i>	8.0	R							Down		
	2524	Transcriptional regulator, MarR family	−1.7	R							Down		
	2652	<i>clfB</i>	1.5	No							Down		
	2659	<i>aur</i>	−1.6	No									
	2660	<i>isaB</i>	2.7	No								Up	
	2676	<i>sasA</i>	2.4	No								Down	
	2688	<i>icaR</i>	3.3	R								Down	
	2689	<i>icaA</i>	−3.9	No									
	2690	<i>icaD</i>	−2.0	r									Up
	2691	<i>icaB</i>	−3.7	No									
	2692	<i>icaC</i>	−1.5	No									
	2694	<i>lip</i>	−1.6	No									

^a Genes are listed by chromosomal order. The virulence genes affected were searched among the data described in the five studies (12, 19, 20, 34; this study). Highlighted by boxes are the genes which were also affected in at least two of the compared studies. None of the genes listed were found to be affected in one study (20). The genes belonging to COL pathogenicity islands are grouped and identified by vertical bars.

^b See Table 1, footnote a.

determinants *isdC* through *-G* and *srtB* are transcribed from a single promoter and respond in a uniform manner (by down-regulation and reversibility) to the suboptimal transcription of *murF*. No changes were observed in the transcription of *isdA* and *isdB*, each of which is transcribed from a separate promoter and in the opposite direction (Fig. 2b) (18).

Several of the genes of the *S. aureus isd* locus (iron-surface determinant) are involved in iron acquisition (15). Interest-

ingly, the transcription of over 30 additional genes involved in iron metabolism of *S. aureus* were also found to be down-regulated under the condition of suboptimal transcription of *murF* (Table 4; genes marked with asterisks in Table S2 in the supplemental material).

Repression of the *ica* operon and the *pur* operon. Figure S2a in the supplemental material shows the rapid and reversible overexpression of *icaR*, the repressor of the *ica* operon, in

FIG. 3. (a) Absolute values of expression for the genes related to the autolysis process described for *S. aureus*. For details, see the legend to Fig. 2d. (b) Zymogen assays. Cell wall extracted from strain COL (left panel) and from COLspacmurF grown with a suboptimal inducer (condition B) (right panel) was incorporated into each gel. Autolytic crude extracts were from COL (lanes 1 and 2), COLspacmurF grown in condition A (lanes 3), COLspacmurF grown in condition B (lanes 4), and COLspacmurF grown in condition C (lanes 5). (c) In vivo autolysis assays performed for COL and COLspacmurF in conditions A, B, and C. (d) In vitro autolysis assays performed for COL and COLspacmurF in conditions A, B, and C.

response to modulation of *murF* transcription. The pattern of changes suggests that the primary responder to the reduced transcription of *murF* was the *icaR* repressor, the overexpression of which presumably was responsible for the repression of the rest of the operon, which has been associated with the capacity of *S. aureus* to form biofilms (4).

Figure S2b in the supplemental material shows that the entire purine biosynthetic pathway underwent powerful and reversible repression under the conditions when cell wall synthesis was perturbed and slowed down in the *murF* system. Previous studies showed that the reduced transcription of *murF* results in a thinning of the bacterial cell wall due to a reduced incorporation of

peptidoglycan (30). It is interesting that a recent study with *S. aureus* strains selected for reduced susceptibility to vancomycin showed exactly the opposite pattern of transcription of the purine biosynthesis operon: the entire operon was overexpressed in the bacteria with reduced vancomycin susceptibility, which showed a substantially increased thickness of the cell wall. It has been proposed that the increased transcription of the purine pathway in this system reflects the increased needs of the vancomycin-resistant bacteria for ATP, due to the excess cell wall material produced (21).

Changes in the transcription of a mobile element. *S. aureus* strain COL carries a chromosomally located prophage, L54a,

FIG. 4. Biolog plate assay performed for strain COL (a) and mutant COLspacmurF (b) grown with the optimal inducer concentration (condition A) and mutant COLspacmurF grown with suboptimal inducer concentration (condition B) (c).

which until now had been found to be specific to this strain (8). Interestingly, 33 of the 72 genetic determinants of the prophage showed decreased transcription under the conditions of cell wall stress induced by suboptimal transcription of *murF* (see Fig. S3 in the supplemental material). Each of these changes was reversible upon readdition of the optimal concentration of the IPTG inducer to the medium. The only exceptions to this uniform pattern of repression were two genetic determinants, ORFs SACOL0328 and SACOL0332, which showed overexpression in the bacteria growing with the suboptimal IPTG concentration. It appears, therefore, that the perturbation of cell wall synthesis caused reinforcement of the lysogenic state of the prophage for most of the phage genetic determinants. The two exceptional genes showing overexpression may represent repressors.

The uniform down-regulation of all but two of the 35 prophage-affected determinants shows a striking contrast with recent reports that documented activation of the SOS system and induction of lysogenic phages in bacteria treated with cell wall-active antibiotics (1, 14).

Changes in the expression of virulence-related determinants. Modulation of *murF* expression also had a powerful and selective effect on the transcription of 79 virulence-related genes. The 55 genes overexpressed in cultures grown with suboptimal transcription of *murF* included determinants of several pathogenicity islands and such important virulence determinants as fibronectin binding proteins A and B, clumping

factor A, enterotoxin B, etc. On the other hand, expression of several genes, such as *sek*, *sei*, etc., on the pathogenicity island *uSa1* showed reversible repression in bacteria growing with suboptimal transcription of *murF* (Table 5). The surprisingly large number of virulence related determinants that were up-regulated during *murF* stress suggests perhaps a more enhanced “defensive” posture by the bacteria during a compromised state of synthetic capacities.

Repression of autolytic activity. Figure 3a shows the responses of several known regulators of autolytic activity to the reduced rate of cell wall synthesis in the *S. aureus* conditional mutant cultivated at suboptimal concentrations of IPTG. It may be seen that the *lrgA* and *-B* genes, encoding a putative antiholin (10), and the genes *cidC* and *cidB*, proposed to encode a regulator of hydrolase activity (24), are overexpressed, while the genetic determinant of *lytM* is repressed, under conditions of *murF* stress. These regulatory changes would be expected to result in lower concentrations of autolytic enzymes and lower autolytic activity in the bacteria.

Follow-up experiments were done in an attempt to confirm these expectations by direct determination of the relative amounts of autolysins, using the zymogen assay and comparison of the relative activities of autolysins in lithium chloride extracts. In addition, the proneness of cells to Triton X-100-induced autolysis was also determined.

Bacteria grown in the presence of optimal expression of *murF* (condition A) and bacteria grown with reduced *murF* transcription (condition B) were used in these experiments. Figure 3b shows that crude autolytic extracts prepared from cells grown in condition B (lane 4) showed substantially less-intensive autolytic bands in the zymogen extract irrespective of whether the cell wall substrate incorporated into the gel was derived from the parental strain COL or from the bacteria grown in condition B. As a comparison we used autolytic extracts from the parental strain COL (lanes 1 and 2), bacteria grown in condition A (lane 3), and bacteria which received the optimal concentration of IPTG (condition C) (lane 5). Suggestive evidence for a somewhat increased intensity of the autolytic bands may be apparent in lane 5.

Figure 3c shows the substantially decreased lysis of cells grown under condition B in the Triton X-100 autolysis assay, and Fig. 3d compares the relative activities of lithium chloride extracts prepared from bacteria grown in condition A, B, or C. Also included are extracts prepared from the parental strain COL. Decreased activity of the lithium chloride extractable autolysins is apparent in bacteria grown under condition B, and evidence for increased autolytic activity is apparent in the extracts derived from the condition C cells.

Validation of the microarray assays. A selected group of 10 genetic determinants showing extensive up- or down-regulation during *murF* stress were chosen to test the validity of the microarray data using real-time RT-PCR. Table S4 in the supplemental material shows the results, which confirm both the degree and direction as well as reversibility of the transcriptional changes observed in the microarray system.

Operon-specific changes. Examination of the nature of genes with modified transcription in the *murF* system allows several interesting conclusions. The transcriptional changes appear to be operon specific. In fact, by applying this principle it was possible to enlarge the number of genes that belong to

a “cell wall stimulon” by over 80 new members (see Table S3 in the supplemental material). Operon-specific responses were also striking in the case of genes belonging to the *ica* group of determinants and the purine synthesis operon (see Fig. S2 in the supplemental material) and genes involved with iron metabolism (Table 4), all of which were down-regulated during *murF* stress.

Changes in intergenic regions. The Affymetrix microarrays used in these experiments included all intergenic sequences derived from ORF clusters of the four genomes. In 55 of these cases we noted that the expression of intergenic sequences underwent extensive and reversible alterations in response to the modulation in *murF* expression. In none of these cases was there an observable change in the expression in either one of the two ORFs directly neighboring the particular intergenic sequence (see Table S5 in the supplemental material). The nature of these intergenic sequences is not known. Whether or not some of these represent RNAs with possible regulatory roles is currently under investigation (9, 23).

Altered fermentation patterns in bacteria grown with suboptimally expressed *murF*. The transcriptional changes left their mark on the physiology and biochemical capacity of the bacteria also. The most striking documentation of the extent of metabolic shifts precipitated by the slowdown in the transcription of *murF* was the demonstration that bacteria grown under conditions of optimal or suboptimal *murF* transcription had different fermentation capacities as shown by the Biolog assay (Fig. 4). Bacteria grown in the presence of the optimal IPTG concentration showed the capacity to ferment eight distinct carbon sources (*N*-acetyl-D-glucosamine, α -D-glucose, D-mannose, D-trehalose, D-lactic acid methyl ester, L-lactic acid, sucrose, and glycerol). The same bacteria grown under conditions of suboptimal *murF* expression could utilize the same eight carbon sources but also acquired the additional capacity to ferment nine other carbon sources. Besides the compounds normally used by strain COL (and by the conditional mutant grown with optimal expression of *murF*), bacteria grown with suboptimal expression of *murF* were also able to metabolize dextrin, maltose, α -hydroxybutyric acid, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, and pyruvic acid methyl ester. These expanded fermentation patterns are consistent with the findings of the microarray data, which indicate that in bacteria grown at suboptimal inducer concentrations, there is an up-regulation of genes which code for anaerobic stress-related enzymes (pyruvate formate lyase and formate acetyltransferase), enzymes of nitrogen metabolism (urease and nitrite and nitrate reductases), and enzymes in the intermediary metabolism of pyrimidines and purines. The striking activation of the anaerobic stress pathway, including overexpression of SACOL0204 and SACOL0205 (genes *pflB* and *pflA*), encoding homologues of formate acetyltransferase and pyruvate/formate lyase which catalyze the nonoxidative transformation of pyruvate to acetyl coenzyme A and formate, corresponds to the typical response of a bacterium to oxygen-limiting conditions (27). These two genes were also overexpressed when *S. aureus* 8325 was challenged with hydrogen peroxide, i.e., during oxidative stress (2). Besides the *pflBA* genes, the *murF* stress also led to the activation of genes related to fermentative metabolism such as *ldh*, *fruK*, and lactate permease and formate/nitrite transporter protein genes.

These observations indicate that *S. aureus* is responding to the deficit of *murF* transcription in a way similar to what was described for oxidative stress. Yet the cultures used in the experiments with the *murF* conditional mutant were vigorously aerated throughout the course of the studies. Also, the key components of the SOS system, such as *lexA*, *uvrA*, *uvrB*, and *recA* (3, 7), remained unchanged during *murF* stress. Therefore, the activation of the anaerobic pathway by limiting the source of oxygen is excluded as an explanation. The mechanism of this major shift of energy metabolism is not clear at the present time.

It has been postulated (35) that the cell wall stimulon senses a low-molecular-weight peptidoglycan metabolite. This metabolite could be sensed at the cytoplasmic, membrane, or cell wall level. In fact, in the *murF* mutant the abnormal tripeptide structure should be present in large amounts in these three cellular compartments. Therefore, it is conceivable that the metabolite involved is either the tripeptide or the concentration of the normal pentapeptide cell wall precursor.

It is widely agreed that in bacteria the primary control of metabolism occurs at the level of transcription. The wide range of determinants responding to the *murF* stress is a striking demonstration of the multitude and complexity of transcription level links that connect a large number of diverse cellular functions to a single step in cell wall synthesis in a reversible manner. The alterations in the *S. aureus* transcriptome described in this study may be a model for the type of control that allows bacteria to rapidly adapt to changing conditions in the nutritional or physical chemical environment.

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REFERENCES

1. Beaber, J. W., B. Hochhut, and M. K. Waldor. 2004. SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**:72–74.
2. Chang, W., D. A. Small, F. Toghrol, and W. E. Bentley. 2006. Global transcriptome analysis of *Staphylococcus aureus* response to hydrogen peroxide. *J. Bacteriol.* **188**:1648–1659.
3. Courcelle, J., A. Khodursky, B. Peter, P. O. Brown, and P. C. Hanawalt. 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. *Genetics* **158**:41–64.
4. Cramton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Gotz. 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect. Immun.* **67**:5427–5433.
5. De Jonge, B. L. M., H. de Lencastre, and A. Tomasz. 1991. Suppression of autolysis and cell wall turnover in heterogeneous Tn551 mutants of a methicillin-resistant *Staphylococcus aureus* strain. *J. Bacteriol.* **173**:1105–1110.
6. de Lencastre, H., S. W. Wu, M. G. Pinho, A. M. Ludovice, S. Filipe, S. Gardete, R. Sobral, S. Gill, M. Chung, and A. Tomasz. 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. *Microb. Drug Resist.* **5**:163–175.
7. Fernandez de Henestrosa, A. R., T. Ogi, S. Aoyagi, D. Chafin, J. J. Hayes, H.

- Ohmori, and R. Woodgate. 2000. Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. *Mol. Microbiol.* **35**:1560–1572.
8. Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson, and C. M. Fraser. 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J. Bacteriol.* **187**:2426–2438.
 9. Gottesman, S. 2005. Micros for microbes: non-coding regulatory RNAs in bacteria. *Trends Genet.* **21**:399–404.
 10. Groicher, K. H., B. A. Firek, D. F. Fujimoto, and K. W. Bayles. 2000. The *Staphylococcus aureus* *lrgAB* operon modulates murein hydrolase activity and penicillin tolerance. *J. Bacteriol.* **182**:1794–1801.
 11. Jablonski, P. E., and M. Mychajlonka. 1988. Oxacillin-induced inhibition of protein and RNA synthesis in a tolerant *Staphylococcus aureus* isolate. *J. Bacteriol.* **170**:1831–1836.
 12. Kuroda, M., H. Kuroda, T. Oshima, F. Takeuchi, H. Mori, and K. Hiramatsu. 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol. Microbiol.* **49**: 807–821.
 13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
 14. Maiques, E., C. Ubeda, S. Campoy, I. Salvador, I. Lasa, R. P. Novick, J. Barbe, and J. R. Penades. 2006. β -Lactam antibiotics induce the SOS response and horizontal transfer of virulence factors in *Staphylococcus aureus*. *J. Bacteriol.* **188**:2726–2729.
 15. Maresso, A. W., and O. Schneewind. 2006. Iron acquisition and transport in *Staphylococcus aureus*. *Biometals* **19**:193–203.
 16. Marraffini, L. A., and O. Schneewind. 2005. Anchor structure of staphylococcal surface proteins. V. Anchor structure of the sortase B substrate IsdC. *J. Biol. Chem.* **280**:16263–16271.
 17. Mazmanian, S. K., H. Ton-That, and O. Schneewind. 2001. Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. *Mol. Microbiol.* **40**:1049–1057.
 18. Mazmanian, S. K., H. Ton-That, K. Su, and O. Schneewind. 2002. An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. *Proc. Natl. Acad. Sci. USA* **99**:2293–2298.
 19. McAleese, F., S. W. Wu, K. Sieradzki, P. Dunman, E. Murphy, S. Projan, and A. Tomasz. 2006. Overexpression of genes of the cell wall stimulon in clinical isolates of *Staphylococcus aureus* exhibiting vancomycin-intermediate-*S. aureus*-type resistance to vancomycin. *J. Bacteriol.* **188**:1120–1133.
 20. McCallum, N., G. Spehar, M. Bischoff, and B. Berger-Bachi. 2006. Strain dependence of the cell wall-damage induced stimulon in *Staphylococcus aureus*. *Biochim. Biophys. Acta* **1760**:1475–1481.
 21. Mongodin, E., J. Finan, M. W. Climo, A. Rosato, S. Gill, and G. L. Archer. 2003. Microarray transcription analysis of clinical *Staphylococcus aureus* isolates resistant to vancomycin. *J. Bacteriol.* **185**:4638–4643.
 22. Mychajlonka, M., T. D. McDowell, and G. D. Shockman. 1980. Inhibition of peptidoglycan, ribonucleic acid, and protein synthesis in tolerant strains of *Streptococcus mutans*. *Antimicrob. Agents Chemother.* **17**:572–582.
 23. Pichon, C., and B. Felden. 2005. Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. *Proc. Natl. Acad. Sci. USA* **102**:14249–14254.
 24. Rice, K. C., B. A. Firek, J. B. Nelson, S. J. Yang, T. G. Patton, and K. W. Bayles. 2003. The *Staphylococcus aureus* *cidAB* operon: evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance. *J. Bacteriol.* **185**:2635–2643.
 25. Roche, F. M., R. Massey, S. J. Peacock, N. P. Day, L. Visai, P. Speziale, A. Lam, M. Pallen, and T. J. Foster. 2003. Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. *Microbiology* **149**:643–654.
 26. Ruzin, A., A. Severin, F. Ritacco, K. Tabei, G. Singh, P. A. Bradford, M. M. Siegel, S. J. Projan, and D. M. Shlaes. 2002. Further evidence that a cell wall precursor [C₅₅-MurNAC-(peptide)-GlcNAc] serves as an acceptor in a sorting reaction. *J. Bacteriol.* **184**:2141–2147.
 27. Sowers, G., C. Hesslinger, N. Muller, and M. Kaiser. 1998. The glycyl radical enzyme TdcE can replace pyruvate formate-lyase in glucose fermentation. *J. Bacteriol.* **180**:3509–3516.
 28. Sieradzki, K., and A. Tomasz. 2003. Alterations of cell wall structure and metabolism accompany reduced susceptibility to vancomycin in an isogenic series of clinical isolates of *Staphylococcus aureus*. *J. Bacteriol.* **185**:7103–7110.
 29. Sieradzki, K., and A. Tomasz. 1997. Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of *Staphylococcus aureus*. *J. Bacteriol.* **179**:2557–2566.
 30. Sobral, R. G., A. M. Ludovice, H. de Lencastre, and A. Tomasz. 2006. Role of *murF* in cell wall biosynthesis: isolation and characterization of a *murF* conditional mutant of *Staphylococcus aureus*. *J. Bacteriol.* **188**:2543–2553.
 31. Sobral, R. G., A. M. Ludovice, S. Gardete, K. Tabei, H. De Lencastre, and A. Tomasz. 2003. Normally functioning *murF* is essential for the optimal expression of methicillin resistance in *Staphylococcus aureus*. *Microb. Drug Resist.* **9**:231–241.
 32. Ton-That, H., H. Labischinski, B. Berger-Bachi, and O. Schneewind. 1998. Anchor structure of staphylococcal surface proteins. III. Role of the FemA, FemB, and FemX factors in anchoring surface proteins to the bacterial cell wall. *J. Biol. Chem.* **273**:29143–29149.
 33. Ton-That, H., G. Liu, S. K. Mazmanian, K. F. Faull, and O. Schneewind. 1999. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proc. Natl. Acad. Sci. USA* **96**:12424–12429.
 34. Utaida, S., P. M. Dunman, D. Macapagal, E. Murphy, S. J. Projan, V. K. Singh, R. K. Jayaswal, and B. J. Wilkinson. 2003. Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology* **149**:2719–2732.
 35. Wilkinson, B. J., A. Muthaiyan, and R. K. Jayaswal. 2005. The cell wall stress stimulon of *Staphylococcus aureus* and other Gram-positive bacteria. *Curr. Med. Chem. Anti-Infect. Agents* **4**:259–276.