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Role of VraSR in Antibiotic Resistance and Antibiotic-Induced Stress Response in *Staphylococcus aureus*

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Exposure of *Staphylococcus aureus* to cell wall inhibitors induces massive overexpression of a number of genes, provided that the VraSR two-component sensory regulatory system is intact. Inactivation of *vraS* blocks this transcriptional response and also causes a drastic reduction in the levels of resistance to beta-lactam antibiotics and vancomycin. We used an experimental system in which the essential cell wall synthesis gene of *S. aureus*, *pbpB*, was put under the control of an isopropyl-β-D-thiogalactopyranoside-inducible promoter in order to induce reversible perturbations in cell wall synthesis without the use of any cell wall-active inhibitor. Changes in the level of transcription of *pbpB* were rapidly followed by parallel changes in the *vraSR* signal, and the abundance of the *pbpB* transcript was precisely mirrored by the abundance of the transcripts of *vraSR* and some additional genes that belong to the VraSR regulon. Beta-lactam resistance in *S. aureus* appears to involve a complex stress response in which VraSR performs the critical role of a sentinel system capable of sensing the perturbation of cell wall synthesis and allowing mobilization of genes that are essential for the generation of a highly resistant phenotype. One of the sites in cell wall synthesis "sensed" by the VraSR system appears to be a step catalyzed by PBP 2.

Brief exposure of *Staphylococcus aureus* to inhibitors of cell wall synthesis invoke an immediate and massive change in the transcription of a unique set of genes, some of which—such as *mgtB*, *murZ*, and *pbpB*—are clearly involved with wall biosynthesis, while others have as yet undefined functions (14, 16, 31). It was proposed that these genes be referred to as members of a coordinately regulated "cell wall stimulon" (31). A particularly interesting member of this group of genes is *vraSR*, the DNA sequence of which shows features typical of a two-component sensory regulatory system (14). A careful study by Kuroda and colleagues (14) has demonstrated that transcription of *vraSR* is a specific response to inhibitors of various stages in wall synthesis: inhibitors of other cellular polymers and/or conditions of stress, such as shifts in temperature, pH, or osmotic pressure, did not alter the transcription of *vraSR.* Furthermore, and most importantly, the burst of transcription of genes following exposure to cell wall inhibitors was greatly reduced and/or annulled in bacteria in which *vraSR* was inactivated (14), indicating the essentiality of an intact *vraSR* system for this response. On the basis of these observations, it was proposed that VraSR functions as a sentinel system capable of detecting conditions that threaten to interrupt the synthesis of the bacterial cell wall. Nevertheless, the precise site(s) of perturbation of wall synthesis "sensed" by this system has just begun to be explored. Recent studies by Boyle-Vavra, Daum, and colleagues have allowed a better definition of the *vraSR* operon and its mode of induction by cell wall-active antibiotics (2, 3, 32).

Kuroda and colleagues (14) also examined the role of *vraSR* in antibiotic resistance: inactivation of *vraSR* caused a substantial reduction in the beta-lactam antibiotic resistance of some but not all derivatives of the clinical methicillin-resistant *S. aureus* (MRSA) strain N315 carrying the resistance gene *mecA.*

The purposes of the experiments described in this communication were twofold: (i) to determine the general importance of *vraSR* in the *mecA*-based antibiotic resistance of MRSA isolates with different genetic backgrounds and also in bacteria in which beta-lactam and vancomycin resistance are based on different mechanisms and (ii) to identify more precisely the site(s) of perturbation of peptidoglycan synthesis that can trigger overexpression of *vraSR*. For the latter, we used an experimental system that allows interruption of the enzymatic machinery of staphylococcal peptidoglycan synthesis without the use of any cell wall-active inhibitor.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. The *Staphylococcus aureus* strains were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) with aeration at 37°C or on tryptic soy agar (TSA; Difco Laboratories) plates at 37°C. The *Escherichia coli* strains were grown in Luria-Bertani broth (Difco Laboratories) with aeration at 37°C. Erythromycin (10 μg/ml), chloramphenicol (10 μg/ml), and ampicillin (100 μg/ml) were used as recommended by the manufacturer (Sigma, St. Louis, Mo.) for the selection and maintenance of the *S. aureus* and *E. coli* transformants, respectively.

DNA methods. DNA manipulations were performed by standard methods. Restriction enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, Mass.). Routine PCR amplification was performed with *Tth* DNA polymerase (HT Biotechnology, Cambridge, United Kingdom). Wizard Plus Miniprep and Midiprep (Promega, Madison, Wis.) purification systems were used for plasmid extraction. PCR and digestion products were purified with Wizard PCR Preps and Wizard DNA clean-up systems (Promega). Ligation reactions were performed with T4 ligase (New England Biolabs). DNA sequencing was done at the Rockefeller University Protein/DNA Technology Center by the BigDye Terminator cycle sequencing method with either a 3700 DNA analyzer for capillary electrophoresis or an ABI Prism 377 DNA sequencers for slab gel electrophoresis.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)[c]	Source or reference
S. aureus		
COL	Homogeneous Mc^r Em^s; MIC,[a] 400 μg/ml	RU collection
RN4220	Mcs, restriction negative	R. Novick
COLVA$_{200}$	Strain COL carrying a plasmid-borne Tn*1546 vanA* gene complex	26
COL$_{spac::pbpB}$	COL with P$_{spac}$-*pbpB* fusion in the chromosome transformed with pMGPII	24
COL Δ*vraS*	COL/pSG4 inserted into the chromosome, Em^r	This study
COLVA$_{200}$Δ*vraS*	COLVA200/pSG4 inserted into the chromosome, Em^r	This study
301	Homogeneous Mc^r Em^s; MIC,[a] 200 μg/ml	United States
301Δ*vraS*	301/pSG4 inserted into the chromosome, Erm^r	This study
DU4916	Homogeneous Mc^r Em^s; MIC,[a] 200 μg/ml	8
DU4916Δ*vraS*	DU4916/pSG4 inserted into the chromosome, Erm^r	This study
319	Homogeneous Mc^r Em^s; MIC,[a] 100 μg/ml	Italy, 1990
319Δ*vraS*	319/pSG4 inserted into the chromosome, Erm^r	This study
404	Homogeneous Mc^r Em^s; MIC,[a] 100 μg/ml	Italy, 1990
404Δ*vraS*	404/pSG4 inserted into the chromosome, Erm^r	This study
E3971	Homogeneous Mc^r Em^s; MIC,[a] 100 μg/ml	6
E3971Δ*vraS*	E3971/pSG4 inserted into the chromosome, Erm^r	This study
RUSA11	RN2667 transformed with SCC*mec* type I from strain COL	RU collection (17)
RUSA11Δ*vraS*	RUSA11/pSG4 inserted into the chromosome, Erm^r	This study
ZOX3	Laboratory step mutant, Mc^s Cz^r; MIC,[b] 6 μg/ml,	15
ZOX12	Laboratory step mutant, Mc^s Cz^r; MIC,[b] 24 μg/ml	15
ZOX3Δ*vraS*	ZOX3/pSG4 inserted into the chromosome, Erm^r	This study
ZOX12Δ*vraS*	ZOX12/pSG4 inserted into the chromosome, Erm^r	This study
Sanger 476	Mc^s, hypervirulent strain	United Kingdom (11)
Sanger 476Δ*vraS*	476/pSG4 inserted into the chromosome, Erm^r	This study
Sanger 476 *mecA*	Sanger 476 containing plasmid pSTP181-borne *mecA* gene from strain COL; MIC,[a] 0.75 μg/ml; MIC,[b] 24 μg/ml	Unpublished data
Sanger 476 *mecA*Δ*vraS*	Sanger 476 *mecA*/pSG4 inserted into the chromosome, Erm^r	This study
E. coli DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* ϕ80 Δ*lacZ*ΔM15	Bethesda Research Laboratories
Plasmids		
pSP64E	Integrational vector for *S. aureus*, Ap^r Em^r	23
pSG4	pSP64E/637pb internal DNA fragment of *vraS* gene	This study

[a] Oxacillin MIC.
[b] Ceftizoxime MIC.
[c] Ap^r, ampicillin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant, Cz^r, ceftizoxime resistant.

Construction of plasmid pSG4. A 637-bp internal *vraS* fragment was amplified from the strain COL chromosomal DNA by PCR with *Pfu*Turbo DNA polymerase (Stratagene, Heidelberg, Germany) and primers VraS2UP (5′-CCCAAGCTTGTTGGTTCGGTACTCGCTTA–3′) and VraS2lower (5′-TGCGCAGCTGCTGTCACTTTTGTACCGTTT-3′). The primers were engineered to carry the HindIII (VraS2UP) and the SalI (VraS2lower) restriction sites (underlined in the primer sequences). The following PCR conditions were used: 94°C for 4 min; 40 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; and one final extension step of 72°C for 10 min. The purified PCR product was cleaned with a Wizard PCR Preps DNA purification system, digested with HindIII and SalI, and cloned into plasmid pSP64E, which was also digested with HindIII and SalI. The resultant plasmid, pSG4, was used to transform *E. coli* DH5α (Invitrogen, Carlsbad, Calif.) competent cells.

Inactivation of *vraS* gene in different MRSA and MSSA genetic backgrounds. Plasmid pSG4 was introduced into *S. aureus* RN4220 electrocompetent cells by electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, Calif.), essentially as described previously (13). The transformation mixture was plated on TSA containing erythromycin (10 μg/ml). The correct sequence insertion of the plasmid into the chromosome was confirmed by PCR and sequencing. The construct was transduced from RN4220 into the background of strain COL by phage 80α, as described previously (21), and was subsequently introduced into different MRSA and methicillin-susceptible *S. aureus* (MSSA) genetic backgrounds by phage 80α. Insertional inactivation of *vraS* caused the disappearance of the entire *vraSR* Northern signal, consistent with the cotranscription of the whole *vraSR* operon (14, 32). Thus, insertional inactivation of *vraS* has led to the disruption of the entire *vraSR* operon.

Multilocus sequence typing and *spa* typing. Multilocus sequence typing was performed as described previously by Enright et al. (9), and *spa* typing was performed by the procedure of Shopsin et al. (27).

Staphylococcal chromosomal cassette *mec* (SCC*mec*) typing. The *mec* element type assignment was performed by the procedure of Oliveira and de Lencastre (19).

Population analysis profiles. Population analysis profiles were determined as described previously (29). Overnight cultures were plated at various dilutions (10^0, 10^{-1}, 10^{-3}, and 10^{-5}) on TSA plates containing a series of concentrations of oxacillin (Sigma), and the bacterial colonies were counted after incubation of the plates at 37°C for 48 h.

Determination of antibiotic susceptibility. The levels of resistance to oxacillin and ceftizoxime were determined by the Etest, following the recommendations of the manufacturer (AB Biodisk, Solna, Sweden). Vancomycin MICs were determined by agar dilution: the bacteria were plated on TSA containing different concentrations of the antibiotic.

Transcription of *pbpB* gene and induction of *vraSR* genes. COL$_{spac::pbpB}$ (24) was grown overnight in the presence of 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) and the appropriate antibiotics. This starting culture was back-diluted in TSB supplemented with 100 μM IPTG to an optical density at 620 nm (OD_{620}) of 0.05 and incubated at 37°C until it reached an OD_{620} of 0.4. At this time point, the mutant culture was centrifuged, washed twice in TSB (to remove traces of the inducer), and back-diluted in fresh TSB with no IPTG. Ten-milliliter samples of the conditional mutant were collected for RNA extraction after 0, 10, and 60 min of growth in the absence of IPTG. The mutant strain was further incubated in the absence of IPTG for 30 min, at which time 100 μM

TABLE 2. Oxacillin, ceftizoxime, and vancomycin susceptibilities of *Staphylococcus aureus* strains used in this study

Strain	*spa* type[a]	ST[b]	SCC*mec* type	MIC (μg/ml)[d]		
				Oxacillin	Ceftizoxime	Vancomycin
COL	t008	250	I	>256	>256	
COL Δ*vraS*	t008	250	I	6	4	
301	t037	239	III	>256	>256	
301Δ*vraS*	t037	239	III	6	1	
DU4916	t303	759[c]	I	>256	>256	
DU4916Δ*vraS*	t303	759	I	6	3	
319	t051	247	I	96	>256	
319Δ*vraS*	t051	247	I	3	1	
404	t051	247	I	96	>256	
404Δ*vraS*	t051	247	I	6	0.75	
E3971	t562	247	I	96	>256	
E3971Δ*vraS*	t562	247	I	6	0.50	
RUSA11	t303	8	I	2	16	
RUSA11Δ*vraS*	t303	8	I	0.25	0.50	
ZOX3	t303	8		0.064	6	
ZOX3Δ*vraS*	t303	8		0.064	1.5	
ZOX12	t303	8		0.125	16	
ZOX12Δ*vraS*	t303	1		0.125	4	
Sanger 476	t607	1		0.38	1.5	
Sanger 476Δ*vraS*	t607	1		0.25	0.38	
Sanger 476 *mecA*	t607	1		0.75	24	
Sanger 476 *mecA*Δ*vraS*	t607	1		0.5	0.75	
$COLVA_{200}$	t008	250	I	>256		1,024
$COLVA_{200}$Δ*vraS*	t008	250	I	3		256

[a] *spa* type nomenclature in accordance with http://www.spaServer.ridom.de.
[b] ST, sequence type; nomenclature in accordance with http://saureus.mlst.net.
[c] New single-locus variant of ST250.
[d] Determined by Etest.

IPTG was added back to the growth medium. Three samples were taken at 0, 30, and 60 min after readdition of the inducer. Growth of the mutant culture (OD_{620}) was monitored during the experiment. $COL_{spac::pbpB}$ grown in a medium supplemented with 100 μM IPTG was used as a control.

The relationship between the abundance of the *pbpB* transcript and the induction of the *vraSR* genes was further tested by growing the COL conditional mutant with increasing concentrations of IPTG. $COL_{spac::pbpB}$, grown in the presence of 100 μM IPTG to an OD_{620} of 0.4, was centrifuged, washed, and resuspended in fresh TSB. The culture was next incubated in the absence of IPTG for 1 h, after which the culture was divided into five portions, which received 0, 12.5, 25, 50, and 100 μM IPTG, respectively. Ten milliliters of each culture was collected for total RNA extraction after 1 h of growth. RNA samples were probed with DNA fragments of *vraS*, *mgtB*, *prsA*, and open reading frame (ORF) SACOL2302.

Hybridization with the *vraS* probe consistently produced a "smear" in which diffuse bands within the size range of 1.6 up to 3 kb could be resolved. Washing and retesting of the same membranes with several different DNA probes produced the expected sharp bands, excluding the possibility of technical problems with the *vraS* probe. Identical observations were reported by Yin et al. (32), who characterized the *vraSR* operon and identified the major transcriptional start point.

Northern blotting. Total RNA was extracted as described previously (28). Next, 10 μg of each RNA sample was analyzed by electrophoresis in a 1.2% agarose gel containing 0.66 M formaldehyde and morpholinepropanesulfonic acid (Sigma). The RNA was blotted onto Hybond N^+ membranes (Amersham, Little Chalfont, United Kingdom) with a turbo blotter alkaline transfer system (Schleicher & Schuell, Inc., Keene, N.H.) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The PCR-amplified DNA probes were labeled with [α-^{32}P]dCTP (Amersham LifeSciences, Piscataway, N.J.) with a Ready to Go labeling kit (Amersham) and hybridized under high-stringency conditions. The blots were subsequently washed and autoradiographed.

Cell wall analysis. Cell walls were isolated from exponential-phase cells; the peptidoglycan was purified and hydrolyzed with the M1 muramidase. The resulting muropeptides were reduced with borohydride and separated by reverse-phase high-performance liquid chromatography (HPLC), as described previously (4).

Luciferin-luciferase assay. $COL_{spac::pbpB}$, grown in the presence of 100 μM IPTG to an OD_{620} of 0.4, was centrifuged, washed, resuspended in fresh TSB, and divided into two portions, one of which was supplemented with 100 μM IPTG. After 2 h 30 min of incubation at 37°C, the cultures were back-diluted in the same medium (without and with IPTG); and the relative bioluminescence units (RLUs), indicative of the total ATP content, were measured. Mid-log-phase cultures of RN4220 and COL exposed to 5 μg/ml and 800 μg/ml of oxacillin, respectively, were used as controls. The ATP released by each strain was evaluated indirectly by using the luciferin-luciferase assay adapted from O'Neill et al. (20). Briefly, 100 μl of each culture was added to a filtravette (New Horizons Diagnostics, Columbia, Md.), followed immediately by addition of 20 μl of luciferin-luciferase reagent (New Horizons Diagnostics). The fluid was mixed, the drawer of the model 3550i microluminometer (New Horizons Diagnostics) was closed, and light emission was integrated over 10 s. The microbial ATP level was recorded as the RLUs, which were taken directly from the luminometer's digital readout. The microluminometer internal calibration was checked before each measurement by performing a blank count with the filtravette with only the culture without the reagents. The relative luminescence units produced by each strain were normalized to the OD_{620}.

Microarray transcriptional profiling of strain COL treated with different concentrations of oxacillin. Microarray transcriptional profiling was carried out with a spotted DNA microarray containing 2,688 unique PCR products (from 50 to 1,200 bp) representing 98.7% of the 2,723 ORFs contained within the *S. aureus* COL genome (10). The entire protocol for printing the DNA microarray slides, probe preparation, and hybridization is described in detail elsewhere (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). Total RNA was extracted, as described previously (28), from strain COL that had been grown to mid-log phase and subsequently treated with 0, 5, and 800 μg/ml of oxacillin for 30 min. cDNA probes were produced by reverse transcription of RNA (2 μg) and indirect labeling with either Cy3 or Cy5 dye (Amersham Biosciences, Piscataway, N.J.). All hybridizations were done with cDNA probes containing a minimum of 170 pmol of dye molecule incorporated per microgram of cDNA. TIFF images of the hybridized arrays were captured with TIGR-Spotfinder (www.tigr.org/software/), the data set was normalized by using TIGR-MIDAS (www.tigr.org/software/) and by applying the LOWESS algorithm (block mode; smooth parameter, 0.33), and signifi-

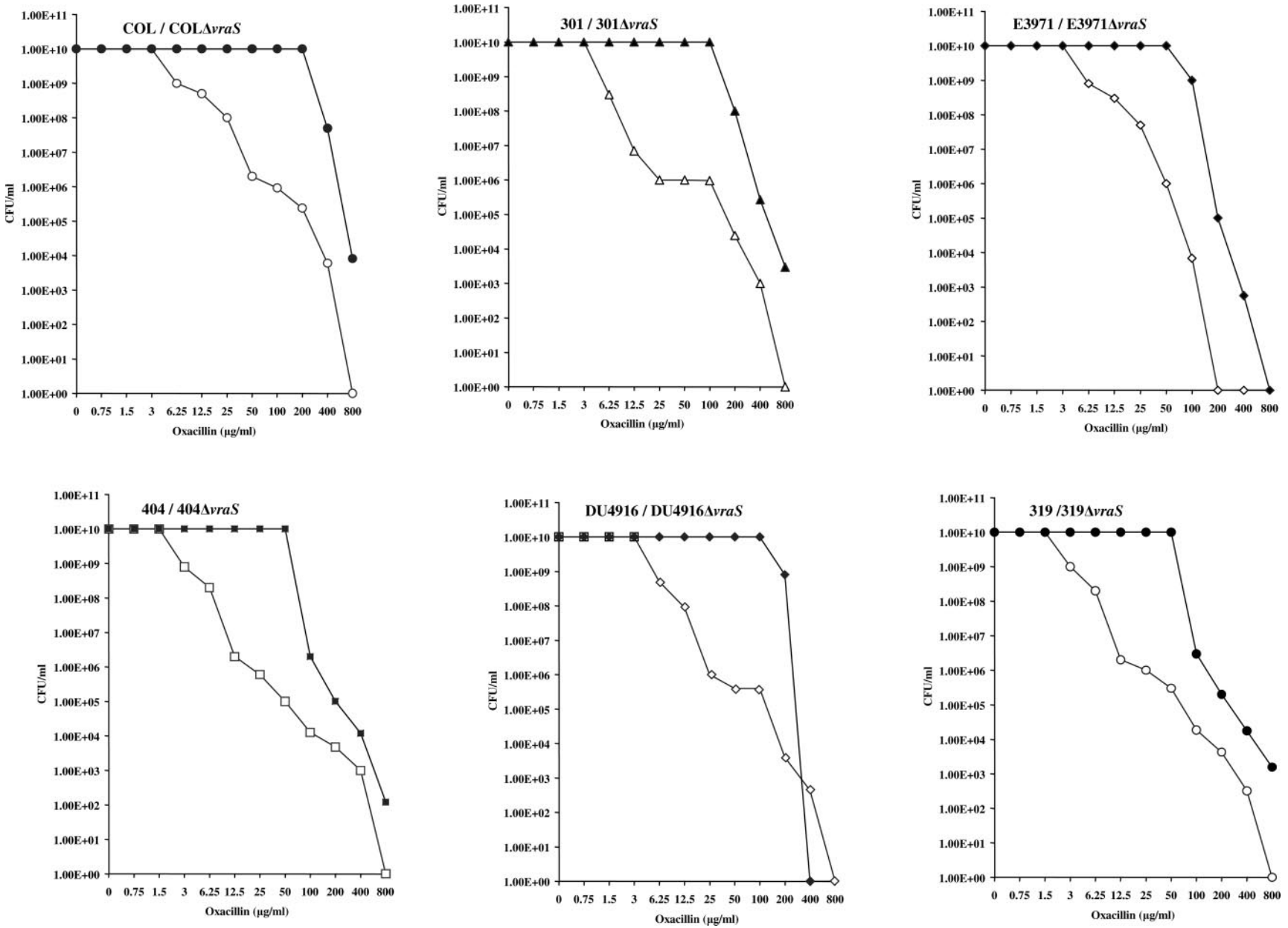


FIG. 1. Effect of disruption of *vraSR* on the resistance level and phenotype of MRSA isolates with high-level and homogeneous or nearly homogeneous resistance to oxacillin. Population analysis profiles were obtained as described in Materials and Methods. Strains with an active *vraS* gene are represented by the solid symbols, and strains with an inactive *vraS* gene are represented by the empty symbols. For the origins and properties of the isolates, see Tables 1 and 2.

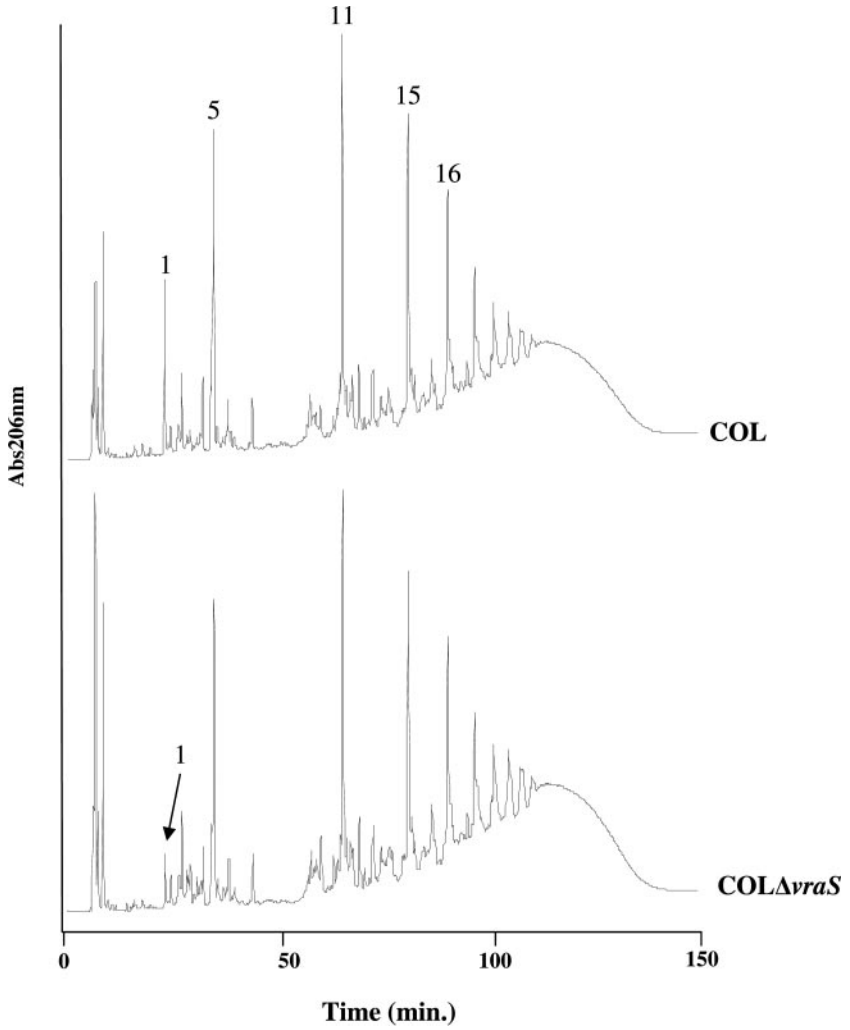


FIG. 2. Effect of disruption of *vraSR* on the composition of peptidoglycan. Cell wall peptidoglycan was prepared from strains COL and COLΔ*vraS* as described in Materials and Methods. Numbers above the peaks in the HPLC elution profile identify the structure of the particular muropeptide, as described earlier (4). Abs206nm, absorbance at 206 nm.

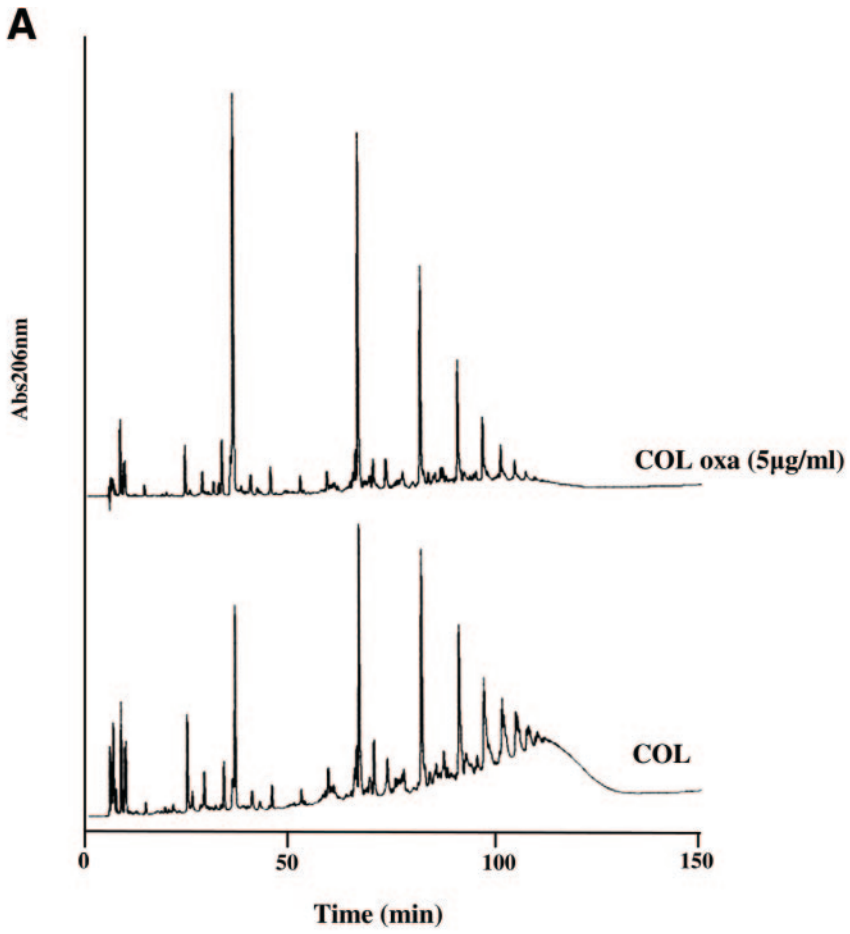
cant changes were identified by the use of SAM (significance analysis of microarrays; www-stat.stanford.edu/_tibs/SAM/index.html) (number of permutations, 1,000; median number of falsely called significant genes, 4.6; median false discovery rate, 3%) (30). Several controls were used to ensure that the data obtained were reproducible and of good quality. First, each ORF was present in duplicate on the array. Second, three independent RNA batches were used for each strain. Third, the quality of the RNA samples was checked in self-hybridization experiments by using the criterion of a Cy3/Cy5 value equal to ~1. Finally, all hybridizations for each RNA preparation were done as dye flips, with the incorporated dye reversed on two separate arrays.

RESULTS

Impact of inactivation of *vraS* on resistance to beta-lactam antibiotics and vancomycin. Insertional inactivation of *vraS* has led to the disruption of the entire *vraSR* operon (14, 32). The *vraSR* genes were disrupted in six clinical MRSA isolates that each exhibited high-level and more or less homogeneous resistance to beta-lactam antibiotics. The sequence types, *spa* types, and SCC*mec* types of these strains are shown in Table 2. Also included among the test strains was RUSA11, a heteroresistant MRSA strain constructed by introducing the SCC*mec* type I cassette from strain COL by chromosomal transformation into susceptible strain RN2677 (17).

Disruption of *vraSR* caused two kinds of changes in each of the MRSA isolates: extensive reduction in the oxacillin MIC and a change from a homogeneous to a highly heterogeneous resistance phenotype (Fig. 1). The degree of reduction in the oxacillin MIC was substantial: 16- up to 64-fold. Interestingly, the change in the MIC for ceftizoxime, a beta-lactam antibiotic with a highly selective affinity for the native *S. aureus* PBP 2 (18), was reduced even more extensively in each of the isolates (Table 2).

We extended testing of the impact of disruption of *vraSR* to several additional *S. aureus* strains: fully beta-lactam-susceptible strain 476 (11), strain 476 carrying a plasmid-borne *mecA* (476 *mecA*), ceftizoxime-resistant laboratory mutants ZOX3 and ZOX12 (15), and a MRSA strain ($COLVA_{200}$) carrying the Tn*1546*-based vancomycin resistance mechanism (26). The



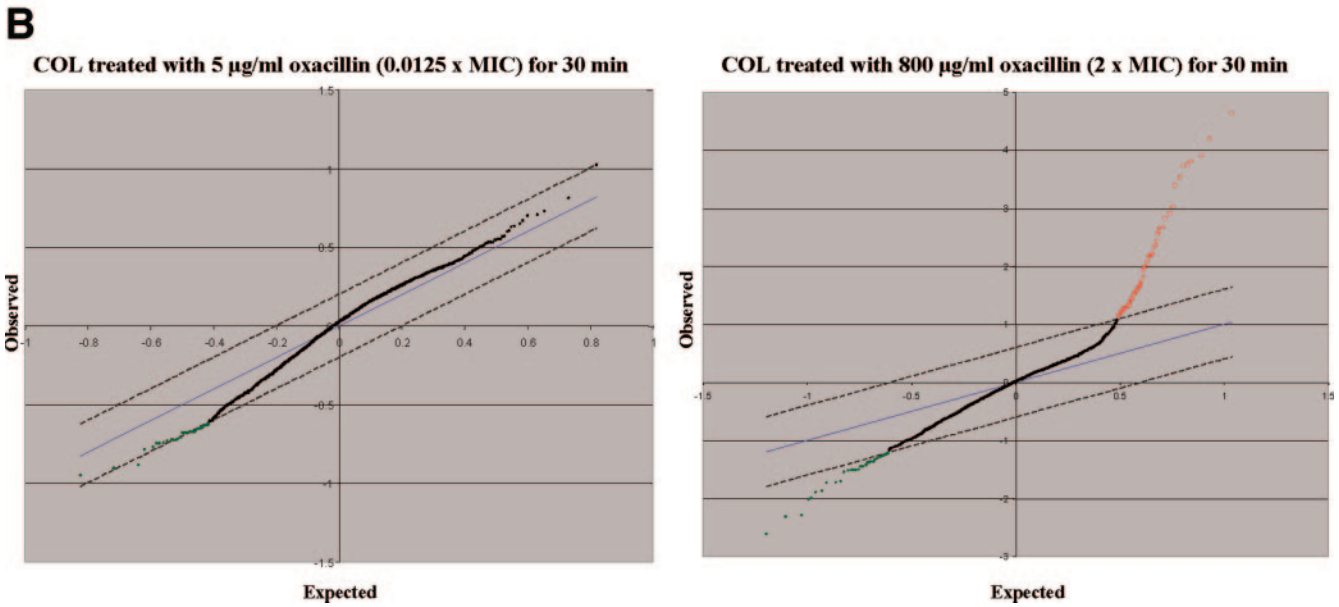


FIG. 3. (A) Changes in muropeptide compositions of the peptidoglycan of strain COL and strain COL grown with 5 μg/ml of oxacillin. The muropeptide compositions of peptidoglycans isolated from strain COL grown in either TSB or TSB containing oxacillin (oxa) at 5 μg/ml were determined. Peptidoglycan was isolated and hydrolyzed with muramidase, and the resulting muropeptides were separated by HPLC, as described in Materials and Methods. Abs206nm, absorbance at 206 nm. (B) Significant alterations in transcription of strain COL treated with 5 μg/ml (0.0125× the MIC) and 800 μg/ml (2× the MIC) of oxacillin by microarray analysis. Microarray analysis was used to compare the transcription profiles of strain COL exposed to 5 μg/ml or 800 μg/ml of oxacillin for 30 min with that of COL grown in TSB. The spots drawn in red in the scatter plots represent the genes up-regulated greater than 1.5-fold in response to oxacillin, while the spots drawn in green represent the genes whose transcription was repressed more than 1.5-fold after treatment with this beta-lactam antibiotic. Values within the two parallel dashed lines indicate variations that are not statistically significant.

TABLE 3. Genes overexpressed at least 1.5-fold in different *S. aureus* strains after treatment with super-MICs of cell wall inhibitors

N315 ORF	Gene	Product or putative function	Fold change (reference)[a]			
			COL (2× MIC of oxacillin) (present study)	JH1 (8× MIC of vancomycin) (16)	N315 (10× MIC of vancomycin) (14)	RN450 (super-MICs of oxacillin, bacitracin, and D-cycloserine) (31)
SA1712[b]		Conserved hypothetical protein	4.64	Up	4.73	Up
SA2221[b]		Conserved hypothetical protein	4.19	9.5	5.37	6.2–18.7
SA2113[b]		Conserved hypothetical protein	3.90	10.4	2.65	
SA1659[b]	*prsA*	Parvuli-like peptidyl-prolyl isomerase	3.80	12	5.74	2.7–7.7
SA1701[b]	*vraR*	Two-component response regulator	3.73	8.7	5.18	Up
SA1700[b]	*vraS*	Two-component sensor histidine kinase	3.75	8.7	7.41	
SA2103[b]		Similar to *lyt* divergon expression attenuator LytR	3.54	3.4	4.06	4.6–14
SA0914[b]		Similar to chitinase B	3.39	10.6	1.94	4.9–7.6
SA1702[b]	*yvqF*	Conserved hypothetical protein	2.91	8.1	7.27	3.4–7.8
SA0591		Conserved hypothetical protein	2.82	Up		Up
SA1549[b]	*htrA*	Heat shock protein homolog, similar to serine protease HtrA	2.67	6.8	3.62	4.2–11
SA1255[b]		PTS system, glucose-specific enzyme II, A component	2.66		2.95	5.1–9.2
SA1253[b]	*ctpA*	Carboxyl-terminal processing proteinase	2.60		2.64	
SA1195	*msrR*	Peptide methionine sulfoxide reductase regulator MsrR	2.56	4.8		2.5–4.3
SA1283[b]	*pbpB*	Penicillin binding protein 2	2.42		1.87	2.8–4.9
SA2220[b]		Glycerate kinase	2.36	Up	3.33	9.2–16.5
SA2146[b]	*tcaA*	TcaA protein	2.32	4.1	2.40	Up
SA2481		Conserved hypothetical protein	2.22	3.4		2.6–5.7
SA2296[b]		Hypothetical protein, similar to transcriptional regulator MerR	2.17	Up	3.87	
SA0824		Conserved hypothetical protein	2.17	5.8		
SA2343[b]		Hypothetical protein	2.09	Up	6.55	Up
SA1925		Conserved hypothetical protein	2.00	3.2		
SA1703[b]		Hypothetical protein	1.99	6.5	7.27	Up
SA0826	*spsB*	Type 1 signal peptidase 1B	1.81	4.7		Up
SA0222	*coa*	Staphylococcal coagulase precursor	1.74		3.17	
SA1691[b]	*mgtB*	Monofunctional glycosyltransferase	1.69	10.6	5.64	5.2–10.9
SA1926[b]	*murZ*	UDP-*N*-acetylglucosamine enolpyruvyl transferase	1.63	5.4	2.52	3.1–8.9
SA1711[b]		Hypothetical protein, similar to DNA damage-inducible protein P	1.56		3.07	
SA2297[b]		Hypothetical protein, similar to GTP-pyrophosphokinase	1.5	8.6	3.85	2.6–4.0

[a] Fold change refers to expression increases for up-regulated genes. Up, upregulated, numerical value not provided.
[b] Member genes of the *S. aureus* VraSR regulon.

levels of resistance to the relevant antibiotic were reduced 1.5- to 6-fold in each of these strains upon disruption of *vraSR* (Table 2).

Effect of disruption of *vraSR* on the composition of peptidoglycan. The muropeptide composition of MRSA strain COL was compared to that of the same strain in which *vraS* was insertionally inactivated. The cell wall peptidoglycans isolated from the parental strain and mutant were compared by HPLC analysis. Inactivation of *vraS* caused a substantial reduction in the muropeptide component number 1, which is the monomeric disaccharide pentapeptide carrying no oligoglycine branches on the epsilon amino group of lysine residues (4) (Fig. 2).

Testing of transcriptional changes in *S. aureus* producing cell walls of abnormal muropeptide composition. *S. aureus* strain COL was grown in TSB and in TSB containing 5 μg/ml oxacillin, and the muropeptide compositions of the bacteria were analyzed by HPLC. As expected (5), this concentration of oxacillin in the growth medium caused extensive changes in the composition of peptidoglycan: the proportion of highly cross-linked components (muropeptide numbers 18 and higher), representing over 60% of all muropeptides in the bacteria grown in drug-free medium, was decreased to less than 15% in the oxacillin-treated cells and was accompanied by parallel large increases in the proportions of monomeric, dimeric, and lower cross-linked muropeptides (Fig. 3A). This extensive alteration in the composition of peptidoglycan caused no increase in *vraSR* transcription detectable by Northern analysis (data not shown), and RNA extracts from the oxacillin-treated cells showed no transcriptional changes in any genes when they were analyzed by use of a DNA chip (Fig. 3B). In contrast, brief exposure (30 min) of a culture of COL to 800 μg/ml oxacillin (2× the MIC) triggered the transcription of *vraSR*, accompanied by extensive overexpression of a large number of determinants, many of which were identified as members of the cell wall stimulon (14, 16, 31) (Fig. 3B and Table 3). The COL strain in which *vraS* was inactivated, which caused a reduction of the oxacillin MIC from >256 to 6 μg/ml, produced neither a detectable *vraSR* signal nor overexpression of *pbpB* when it was treated with very high concentrations of oxacillin (up to 2 mg/ml) (data not shown).

Reversible induction of the transcription of *vraSR* during perturbation of cell wall synthesis without use of a cell wall-active inhibitor. Strain $COL_{spac::pbpB}$, in which transcription of

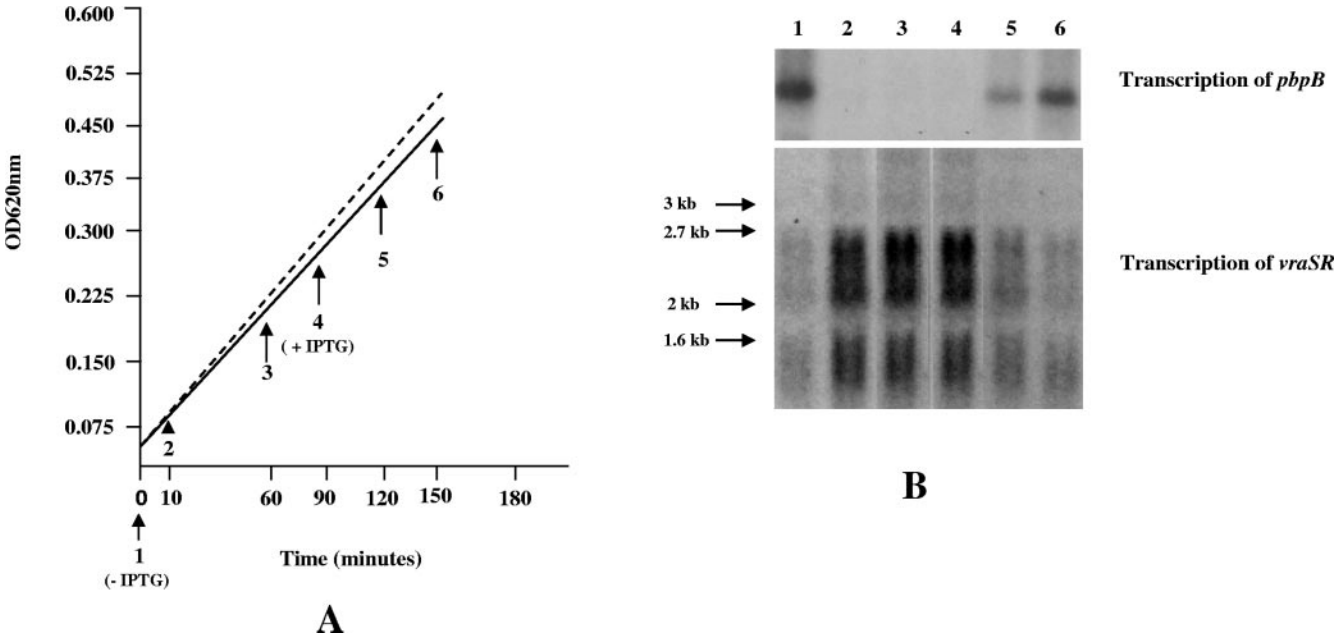


FIG. 4. Turning on and off of the transcription of *vraSR* mirrors closely in time the transcription of *pbpB*. (A) Schematic representation of the growth curve of $COL_{spac::pbpB}$ grown in the absence of IPTG (−IPTG; solid line) and in the presence of 100 μM IPTG (+IPTG; dashed line). Arrows and numbers indicate the times at which RNA extracts were prepared for Northern analysis. (B) Northern analysis of *pbpB* and *vraSR* transcription was done as described in Materials and Methods. Growth of cultures and the exact time structure of the experiment are described in detail in the Results. Lanes 1 through 6, the same time points indicated in Fig. 4A (0, 10, 60, 90, 120, and 150 min, respectively). IPTG was added back to the culture at time point 4 (90 min), followed by sampling 30 min (sample 5) and 60 min (sample 6) afterwards.

pbpB was under the control of an IPTG-inducible promoter, was grown in the presence of optimal concentrations of IPTG. Next, the culture was centrifuged, washed to remove IPTG, and resuspended in a medium free of the IPTG inducer. Such cultures grew for at least 2 h and had a doubling time of 51 min (Fig. 4A), which was only slightly less than the 47-min generation time of a culture supplemented with the optimal concentration of the inducer (Fig. 4A). Samples were removed from the culture at different times after the removal of IPTG from the medium (0, 10, and 60 min) (Fig. 4A), and the levels of expression of *vraSR* and *pbpB* were determined by Northern analysis (Fig. 4B). After 90 min of growth without IPTG, the inducer was readded to the culture and the levels of transcription of *vraSR* and *pbpB* were assayed at various times afterwards (90, 120, and 150 min) (Fig. 4A).

Removal of the inducer from the medium caused a rapid disappearance of the *pbpB* transcript (within 10 min) and the parallel appearance of the *vraSR* transcript in the bacteria. Conversely, repression of *vraSR* occurred equally rapidly (within 30 min) when *pbpB* transcription was turned on by the readdition of IPTG to the medium (Fig. 4B).

Induction of *vraSR* transcription by interruption of the transcription of *pbpB* caused virtually no release of ATP into the medium, indicating the full reversibility of this condition. This was in contrast to the experiment in which *vraSR* transcription was induced by treatment of the bacteria with inhibitory concentrations of oxacillin, which was accompanied by structural damage to the cells, as indicated by the large increase in the amount of ATP released into the growth medium (Fig. 5).

When cultures of strain COL carrying the *spac*-controlled *pbpB* construct were grown in the presence of different (suboptimal) concentrations of IPTG, the level of transcription of *pbpB* was mirrored by the level of transcription of *vraSR*: the higher the abundance of the *pbpB* transcript was, the lower the abundance of the *vraSR* transcript was (Fig. 6). The transcription of a number of other genetic determinants that were previously shown to be under the control of *vraSR* also behaved in a manner similar to that of *vraSR*. These determinants included *mgtB*, *prsA*, and ORF SACOL2302, which encodes a homolog of *lytR* (SA2103). In none of these experiments were cell wall inhibitors used, and the bacteria growing in the absence of the IPTG inducer from the medium underwent several apparently normal duplications in cell count.

DISCUSSION

The results of the experiments described in this communication confirm and extend the role of *vraSR* in antibiotic resistance and also allow the more precise identification of at least one of the sites of perturbation of cell wall synthesis, catalyzed by PBP 2, that triggers the rapid transcription of the *vraSR* system.

Disruption of *vraSR* reduces β-lactam and vancomycin resistance in both MRSA isolates and laboratory strains. In a previously described study, the impact of the deletion of *vraSR* on antibiotic resistance was not uniform (14). In MRSA strain N315 and its derivative from which the *mecI* gene was deleted, inactivation of *vraSR* caused a moderate decrease in the level of oxacillin resistance in the majority of the cells. The progeny of one of the highly resistant colonies picked from the cultures of N315 responded to inactivation of *vraSR* with an extremely large reduction in the oxacillin MICs. However, in a

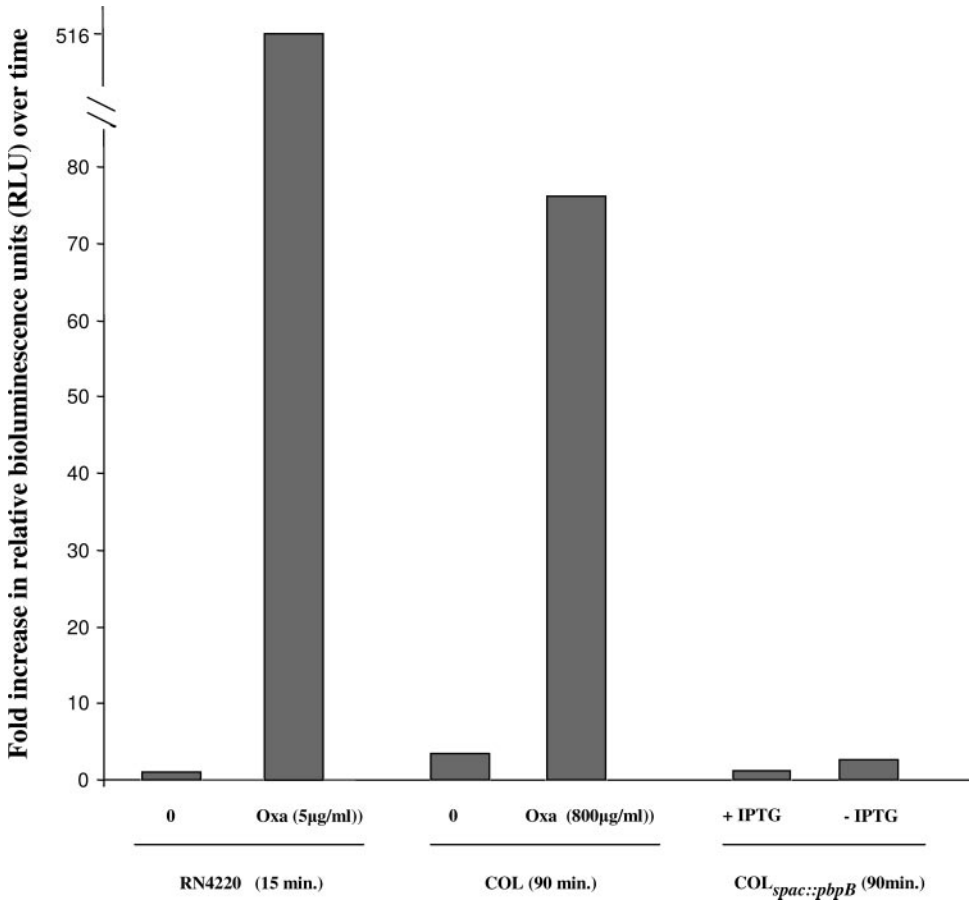


FIG. 5. ATP production in oxacillin-treated strains or the strain with interrupted transcription of *pbpB*. The ATP released by strains RN4220 exposed to 5 μg/ml of oxacillin (Oxa), COL treated with 800 μg/ml of oxacillin, and $COL_{spac::pbpB}$ grown in the absence and in the presence of 100 μM IPTG (−IPTG and +IPTG, respectively) were measured at different time points by using the luciferin-luciferase assay. The total ATP content was expressed as RLUs and was normalized to the OD_{620}.

second highly resistant colony, deletion of *vraSR* had only a relatively minor effect on oxacillin resistance and no effect at all on resistance to ceftizoxime (14). Both of these rare colonies were recovered from cultures of strain N315 after antibiotic selection in vitro and therefore cannot be considered representatives of clinical MRSA isolates.

We tested the effect of disruption of *vraSR* on the antibiotic susceptibility profiles of six clinical MRSA isolates; a heteroresistant laboratory construct in which the primary mechanism of beta-lactam resistance is a chromosomal *mecA* gene (17); beta-lactam-susceptible strain 476 and its oxacillin-resistant derivative carrying the plasmid-borne *mecA* gene (unpublished data); two laboratory mutants (ZOX3 and ZOX12) which were selected for ceftizoxime resistance and which carry a point mutation in the native *pbpB* gene (15); and the highly vancomycin-resistant strain $COLVA_{200}$, in which the key component of vancomycin resistance is the production of a depsipeptide cell wall precursor (26).

In each of these cases, the disruption of *vraSR* resulted in a reduction of beta-lactam and/or vancomycin MICs, despite the clearly different primary mechanisms of drug resistance involved.

Selective effect of *vraSR* inactivation on functioning of PBP 2. Each of the mechanisms tested appeared to have a common component, namely, the need for a functioning PBP 2. A special role of PBP 2 was already suggested by the data summarized in Table 2, which show that in each of the six MRSA strains and in the beta-lactam-resistant laboratory mutant as well, the disruption of *vraSR* always affected resistance to ceftizoxime—a beta-lactam with high selective affinity for PBP 2 (18)—more than resistance to the other beta-lactam antibiotic tested, oxacillin (Table 2). The essential role of PBP 2 in the expression of *mecA*-dependent beta-lactam resistance in MRSA has been documented (22), and the mechanism of resistance in strain ZOX3 is based on a point mutation in the transpeptidase domain of *pbpB* (15). It was also shown recently that a functioning PBP 2 is essential for the expression of high-level vancomycin resistance in a vancomycin-resistant *S. aureus* strain (26).

Triggering *vraSR* transcription by disruption of transcription of *pbpB*, the structural gene of PBP 2. In order to follow up on these observations, we designed experiments that allowed testing of the effect of a direct interference with the transcription of *pbpB* on the expression of *vraSR* without the use of cell wall inhibitors. In previous experiments described in the literature, the expression of *vraSR* was induced by exposing bacteria to inhibitory concentrations of various cell wall-active

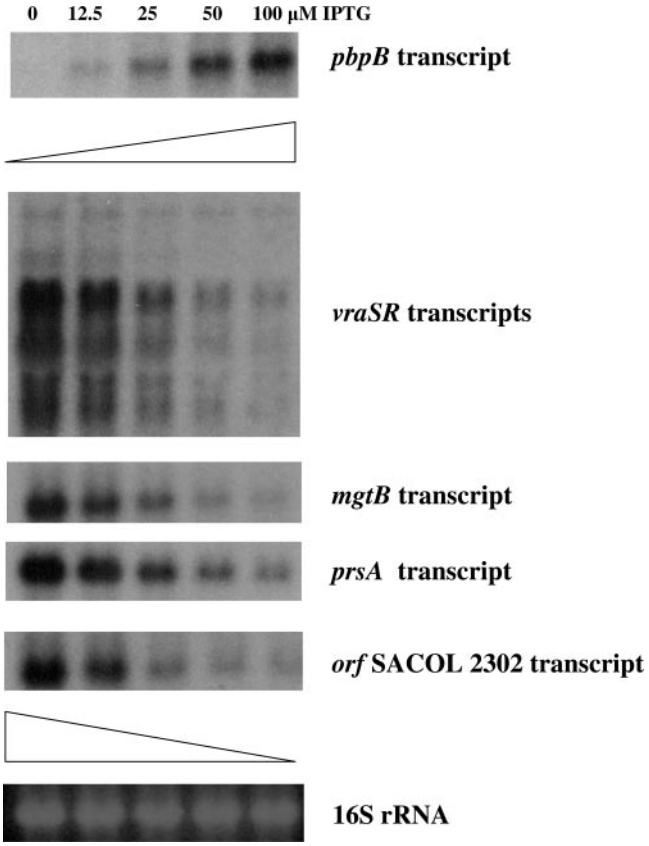


FIG. 6. Gradual decrease in the transcription of *vraSR* and genes *mgtB*, *prsA*, and a homologue of *lytR* in cells with gradually increasing levels of *pbpB* transcription. $COL_{spac::pbpB}$ was grown with different concentrations of IPTG, as indicated; and the levels of transcription of *pbpB*, *vraSR*, *mgtB*, *prsA*, and ORF SACOL2302 (*lytR* homologue) were determined by Northern blotting. An electrophoresis image of the 16S rRNA bands was used as an internal control.

agents. However, such treatments can rapidly lead to complex indirect effects, such as structural damage to the bacteria, as documented, for instance, by the release of ATP into the growth medium even after a short exposure of strain COL to oxacillin (Fig. 5).

Removal of IPTG from the medium of an *S. aureus* mutant in which the transcription of the native *pbpB* was under the control of an IPTG-inducible promoter (24) caused a rapid cessation of *pbpB* transcription which was accompanied by triggering of the transcription of the *vraSR* system. Readdition of the inducer to a culture growing in the absence of IPTG caused a similar rapid response: reappearance of the *pbpB* transcript followed by a quick turning down of *vraSR* transcription.

Most importantly, this rapid response of the *vraSR* system to perturbation of *pbpB* transcription did not influence the growth rate (Fig. 4A) or the cell wall composition (data not shown); was fully reversible; and occurred under conditions in which a sensitive assay for cellular damage, namely, secretion of ATP into the medium (20), was minimal, if it was detectable at all. This was in contrast to the large amounts of ATP which appeared in the growth medium upon treatment of the bacteria with oxacillin above the concentration needed to inhibit the growth and induction of *vraSR* (see Fig. 5). The results of these experiments suggest that one specific site in cell wall biosynthesis under close surveillance by the *vraSR* sentinel system is the transcription of the *S. aureus pbpB* gene, the structural determinant of the bifunctional protein penicillin binding protein 2. The sensitive connection between the transcription of *pbpB* and *vraSR* was already demonstrated in studies by Kuroda et al. (14) and Boyle-Vavra and colleagues (2, 3, 32), and the results of our experiments confirm and extend their conclusions.

The VraSR regulon and methicillin resistance. It was reported by several laboratories (14, 16, 31) that exposure of staphylococci to cell wall inhibitors caused the rapid and extensive up-regulation of a unique set of genes which had a wide range of functions, such as determinants of cell wall synthesis (*mgtB*, *murZ*, and *pbpB*); genes of intermediary metabolism (for instance, *thrB* and *thrC*, *dapA*, and *serA*); and genes for chaperones, heat shock proteins, and osmoprotectant transporters (*prsA*, ORF SA1549, *opuD*, and *proP*). A large proportion of the genes overexpressed were the same, independent of the particular bacterial strain and/or the specific cell wall inhibitor used (14, 16, 31). The data shown in Fig. 3 and Table 3 extend these findings to yet another system: strain COL treated with 2× its MIC of oxacillin. Importantly, disruption of *vraSR* has previously been shown to block overexpression of a subset of genes (see the genes labeled with footnote *b* in Table 3), suggesting that they represented the coordinately controlled "VraSR regulon" (14).

These observations strongly suggest that the reduction in the beta-lactam resistance level observed in *vraSR* mutants of MRSA strains is actually caused by the inhibition of this transcriptional response. We propose that the genes of the VraSR regulon are part of a stress response pathway which is mobilized in bacteria exposed to cell wall inhibitors; and together with the resistance gene *mecA*, the mobilization of this pathway is essential for generating a highly antibiotic resistant phenotype.

Supportive evidence for the stress response model comes from the experiment whose results are illustrated in Fig. 6, which shows that the different levels of abundance of the *pbpB* transcript in bacteria grown in the presence of various suboptimal concentrations of the IPTG inducer were precisely mirrored by the expression not only of *vraSR* but also of *mgtB*, *prsA*, and ORF SACOL2302, i.e., three genetic determinants that belong to the VraSR regulon. A gradual increase in the transcription of *fmtA*, another member of the VraSR regulon, was described during treatment of cells with increasing sub-MICs of oxacillin (12).

***vraSR* are auxiliary genes.** Disruption of *vraSR* in the highly resistant MRSA strain COL caused not only a reduction in the MIC but also the conversion of the homogeneous phenotype to a heterogeneous phenotype and a change in the muropeptide composition of the cell wall. Similar changes were observed in several previously described so-called auxiliary mutants of strain COL, in which these genetic determinants were identified through Tn*551* mutagenesis (1, 7). Interestingly, at least two auxiliary genes, *pbpB* and *fmtA*, are part of the VraSR regulon (12, 14, 25).

Our observations suggest that one may consider two sets of genetic determinants under the same conceptual umbrella:

auxiliary genes identified through transposon mutagenesis and members of the "VraSR regulon" identified with the help of DNA microarrays. In this model, VraSR has the critical role of a determinant without which the bacteria are unable to generate a type of stress response that is an essential component of the antibiotic resistance phenotype.

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