Role of a Sodium-Dependent Symporter Homologue in the Therosensitivity of β-Lactam Antibiotic Resistance and Cell Wall Composition in Staphylococcus aureus

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Expression of high-level β-lactam resistance is known to be thermosensitive in many methicillin-resistant Staphylococcus aureus (MRSA) strains, including strain COL, in which the high methicillin MIC for cultures grown at 37°C (800 µg/ml) was reduced to 12 µg/ml at 42°C. COL grew faster at 42°C than at 37°C and at the higher temperature produced cell walls of abnormal composition: there was an over-representation of the monomeric muropeptide without the oligoglycine chain and an increase in the representation of multimers that contained this wall component as the donor molecule. Screening of a Tn551 insertional library for mutants, in which the high and homogenous β-lactam antibiotic resistance of strain COL is retained at 42°C, identified mutant C245, which expressed high-level methicillin resistance and produced a cell wall of normal composition independent of the temperature. The Tn551 inactivated gene was found, by homology search, to encode for a sodium-dependent symporter, homologues of which are ubiquitous in both prokaryotic and eukaryotic genomes. Inactivation of this putative symporter in several heteroresistant clinical MRSA isolates caused striking increases in the level of their β-lactam resistance.

The β-lactam-resistant phenotype of methicillin-resistant Staphylococcus aureus (MRSA) strains is known to depend sensitively not only on genetic determinants of resistance (i.e., expression of the resistance gene mecA and the functionality of the so-called auxiliary genes) (10, 12, 17, 19) but also on the conditions of growth, such as the temperature, pH, and salt concentration during challenge with the antibiotics (3, 5, 24, 25). Reduced temperature of incubation is used routinely in most diagnostic microbiological laboratories in order to optimize detection of MRSA in clinical specimens (6). How these environmental factors affect the activity and/or expression of the genetic elements associated with β-lactam resistance is not known, although in the case of higher temperatures, it has been suggested that the observed reduced resistance levels may be related to decreased amounts of PBP2A in the cells (16, 22, 27).

Strain COL is one of the MRSA strains in which the level of β-lactam resistance is extremely temperature dependent: preliminary tests showed that growth of COL at 42°C, which caused a nearly 100-fold decrease in the methicillin MIC, caused only a minor reduction in the transcription of mecA. In the studies described here, we used strain COL as a model to better understand the mechanism of the temperature sensitivity of antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The laboratory S. aureus constructs and plasmids used in the present study are listed in Table 1. All S. aureus strains were grown in tryptic soy broth (TSB; Difco, Detroit, MI) at 37 and 42°C with aeration. E. coli transformants were grown in Luria-Bertani medium (Difco) at 37°C supplemented with 100 µg of ampicillin/ml. The growth medium of the transposon mutant C245 and its backcrosses and complementation mutants was supplemented with erythromycin (10 µg/ml) and chloramphenicol (25 µg/ml), respectively. Growth was followed by monitoring the absorbance (A620) using an LKB spectrophotometer (Pharmacia LKB Biotechnology, Inc., Sweden).

Determination of antibiotic susceptibility. Determination of antibiotic susceptibility was done by plating diluted overnight cultures on petri dishes containing tryptic soy agar (TSA; Difco) and serial (twofold) dilutions of the appropriate antibiotic according to a population analysis method described previously (26). Antibiotic MICs for the majority of cells were calculated as the lowest concentration during challenge with the antibiotics (3, 5, 24, 25).

Isolation of total RNA and Northern blot hybridization. Overnight cultures were inoculated into fresh TSB where they were grown to mid-log phase (A620 = 0.6). Before being harvested, bacterial cells were stabilized for 10 min with RNAProtect bacteria reagent (Qiagen GmbH, Hilden, Germany), and RNA was extracted by using a FastRNA Blue isolation kit (Bio 101, Vista, CA) according to the manufacturer's recommendations. After the concentration was adjusted with a GeneQuant spectrophotometer (Pharmacia), RNA samples (5 µg) were resolved by electrophoresis on 1.2% agarose–0.66 M formaldehyde gels in morpholinepropanesulfonic acid running buffer. Blotting of RNA onto a Hybond N+ membrane (Amersham, Arlington Heights, IL) was performed with the Turbo Blotter neutral transfer system (Schleicher & Schuell, Keene, NH). For detection of transcripts, DNA probes corresponding to internal fragments of particular gene, amplified by using the GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Perkin-Elmer) and purified by using the QIAquick PCR purification kit (Qiagen), were labeled with [α-32P]dCTP by the random prime method using a Ready-to-Go labeling kit (Amersham) and hybridized under high-stringency conditions. The blots were subsequently washed and autoradiographed.

Mutagenesis and selection of Tn551 mutants. The transposition experiment and selection of mutants were performed as described before (9). The parental strain COL harboring the thermosensitive plasmid pRN3208 (carrying Tn551 with an erythromycin resistance determinant) was grown overnight at 30°C and then diluted and plated at different concentrations on TSA containing erythromycin (100 µg/ml). The plates were incubated at 42°C for 76 h. The approximate frequency of transposon insertion was 3 × 10−4. In order to cure cells from residual plasmid, bacteria were cultured for 48 h at 42°C. Only erythromycin-resistant and cadmium-susceptible colonies were used for further study.
Screening for mutants with altered methicillin resistance. The erythromycin-resistant, cadmium-sensitive colonies were tested sequentially in three stages. In the first screen, all colonies were streaked onto TSA plates containing erythromycin (10 \( \mu \)g/ml) and different concentrations of methicillin (0, 25, 50 and 100 \( \mu \)g/ml). Strain COLpRN3208 was used as control. In the second screen, colonies that seemed to have altered methicillin resistance were grown overnight in TSB with erythromycin (10 \( \mu \)g/ml), diluted (10 \(^{-3}\)), and tested on TSA plates containing a 1.0 mg methicillin disk. After 24 h of incubation at 42°C the halos of inhibition were measured. After this preliminary testing for altered resistance, a third screen was used to more precisely determine the methicillin resistance phenotype of mutants by using population analysis (26).

Transductional crosses and analysis of transductants. Transductional crosses were performed with phage 80\( \lambda \) H9251 as a donor the newly isolated transposon mutant COL245. Transductants were selected on plates that contained erythromycin at a final concentration of 10 \( \mu \)g/ml. From the cross, 25 transductants were tested for increased levels of methicillin resistance by the 1.0 mg methicillin disk method. Eventually, two transductants were selected for testing by PAP analysis for their antibiotic resistance phenotypes.

Peptidoglycan preparation and analysis. Cell wall peptidoglycan was prepared, and muropeptide composition of peptidoglycan was analyzed by reversed-phase high-performance liquid chromatography (HPLC) as described previously (7), except that the alkaline phosphatase step was omitted.

Autolysis assay. Triton X-100-stimulated autolysis in glycine buffer (pH 8.0) was measured as previously described (8). Cells were grown exponentially to an absorbance at 620 nm of 1.0 in 50 mM glycine buffer supplemented with 0.01% Triton X-100. Autolysis was measured during incubation at 37°C as the decrease in \( A_{620} \) by using a model 340 spectrophotometer (Sequoia-Turner Corp.,Mountain View, CA).

DNA methods. Chromosomal DNA preparation and manipulations were performed by standard methods (4). Restriction enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, MA). 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 ABI Prism 377 DNA sequencers for slab gel electrophoresis.

Sequencing and identification of Tn551 inactivated region. Inverse PCR was performed to isolate DNA region flanking the Tn551 insertion site, as previously described (28).

The chromosomal DNA preparations were digested with HindIII to completion. The self-ligation of the HindIII digests was performed at a DNA concentration of 2 \( \mu \)g/ml overnight at 4°C, and the ligation mixture was then used as a recipient the parental strain COL (cured from the plasmid pRN3208) and as a donor the newly isolated transposon mutant COL245. Transductants were selected on plates that contained erythromycin at a final concentration of 10 \( \mu \)g/ml. From the cross, 25 transductants were tested for increased levels of methicillin resistance by the 1.0 mg methicillin disk method. Eventually, two transductants were selected for testing by PAP analysis for their antibiotic resistance phenotypes.

Table 1. Laboratory derivatives of strain COL and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>Homogeneous Me (^r) at 37°C, heterogeneous Me (^r) at 42°C, Em (^r) mecA</td>
<td>RU collection</td>
</tr>
<tr>
<td>COLmec-245</td>
<td>COL with removed SCmec cassette</td>
<td>21</td>
</tr>
<tr>
<td>COLmec::Tn551</td>
<td>COLmec, (mutagenized smr1::Tn551) Me (^r) Em (^r)</td>
<td>This study</td>
</tr>
<tr>
<td>COLpRN3208</td>
<td>COL with pRN3208 (Tn551) [Rep(Ts)]; Me (^r) Em (^r) Cd (^r)</td>
<td>15</td>
</tr>
<tr>
<td>C245</td>
<td>COL (mutagenized smr1::Tn551), homogeneous Me (^r) at 42°C, Em (^r)</td>
<td>This study</td>
</tr>
<tr>
<td>C245Tsd</td>
<td>COL (smr1::Tn551, backcross from C245), homogeneous Me (^r) at 42°C, Em (^r)</td>
<td>This study</td>
</tr>
<tr>
<td>C245KS20g</td>
<td>C245Tsd with pGCKS20; heterogeneous Me (^r) at 42°C, Em (^r)</td>
<td>This study</td>
</tr>
<tr>
<td>pGC2</td>
<td>pGC2 carrying XbaI-AvaI-restricted 2.5-kb smr1 insert</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Abbreviations: Me, methicillin; Em\(^r\), erythromycin; Cd, cadmium; Cm, chloramphenicol; Ap, ampicillin. Superscripts: r, resistant; s, sensitive.

**FIG. 1.** Effect of growth temperature on the phenotypic expression of methicillin resistance. (A) and growth rate (B) in strain COL. For the determination of methicillin resistance, cultures grown overnight in TSB were plated at different cell concentrations on agar plates containing twofold concentrations of methicillin. Colonies were counted after 48 h of incubation at 37 and 42°C. Growth rates were measured in TSB cultures, which were monitored by determining the \( A_{620} \) as previously described (8).
(5'-GTA TCA CTG TCT CTA GAG CAC CAA AGG AAA AG-3'; underlined letters indicate the introduced restriction site) and KS-smr-PR (5'-CTG ACG CGG ACC CTA TGCTG TAT TA-3') using a High-Fidelity PCR System (Roche Diagnostics, Indianapolis, IN). The amplified smr1 fragment and shuttle vector pGC2 were digested with AvaI and XbaI and ligated with T4 DNA ligase (Roche Diagnostics), generating pGCKS20. Cloning was performed in Escherichia coli XL1-Blue (Stratagene, La Jolla, CA), and the suitable plasmid was selected from among the ampicillin-resistant transformants by its molecular size verification and PCR analysis; one transformant was picked, and the amplified replicative plasmid pGCKS20 was then introduced into RN4220 by electroporation (29) and subsequently transferred to C245 by transduction, generating C245KS20. For selection, ampicillin (100 μg/ml) was used in E. coli and chloramphenicol (25 μg/ml) was used in S. aureus.

FIG. 2. HPLC elution muropeptide profiles of strain COL grown at 42°C (A) and 37°C (B). Separation of S. aureus muropeptides was carried out by reversed-phase HPLC. Muropeptides were prepared and analyzed after reduction, as described previously (7). The chemical structures assigned to the muropeptides have been published elsewhere (7).

FIG. 3. Schematic representation of the Tn551 insertion site in the disrupted open reading frame in strain C245. Genomic DNA fragments, containing the investigated gene, after self-ligation of the HindIII digests were amplified by the PCR and then sequenced. The lower part of the figure shows the amino acid sequence deduced from the nucleotide sequence. The orientation of the open reading frame is indicated by the arrowhead.
RESULTS AND DISCUSSION

Effect of temperature on β-lactam resistance, growth rate, and cell wall composition. Overnight cultures of strain COL were plated for population analysis and incubated at either 37 or 42°C to evaluate the effect of incubation temperature on the expression of methicillin resistance. The increase in incubation temperature caused major and multiple alterations in the properties of strain COL. The high-level methicillin resistance at 37°C (MIC = 800 µg/ml) was reduced to 12 µg/ml at 42°C and was accompanied by a change from a homogeneous to a heterogeneous population structure (Fig. 1A). The growth rate of the culture increased (Fig. 1B), and there were major alterations in the muropeptide composition of the cell wall peptidoglycan (Fig. 2).

Bacterial cells grown at 42°C overproduced muropeptide 1, a disaccharide pentapeptide monomer free of the pentaglycine branch, which is normally present in very limited relative amounts in most staphylococcal muropeptides. In addition, there was also an increase in the proportion of multimers, which contained this glycine-free muropeptide as the original donor molecule (see peaks 9, 11, and 14 through 18 in Fig. 2A).

The loss of resistance appeared to be specific for β-lactam inhibitors: cultures grown at 37°C versus 42°C had the same MICs of bacitracin (50 µg/ml), D-cycloserine (50 µg/ml), and tetracycline (100 µg/ml). There was slight increase in the vancomycin MIC (from 1.5 to 3.0 µg/ml) and decrease in the fosfomycin MIC (from 50 to 25 µg/ml) at the higher temperature.

These observations do not allow one to establish a causal relationship among the multiple changes that accompany the shift in the incubation temperature from 37 to 42°C. However, the increased growth rate at 42°C in parallel with the decreased level of resistance was reminiscent of the “fitness cost” (slower growth rate) described by several investigators in bacteria carrying genetic determinants of antibiotic resistance (2). This prompted us to search for transposon mutants in which the temperature dependence of methicillin resistance and growth rate was altered.
Selection for Tn551 mutants. Strain COL harboring the thermosensitive plasmid pRN3208 (carrying Tn551 with the erythromycin resistance determinant) was used to generate a shotgun library of Tn551 mutants as described in Materials and Methods. The library was then screened for mutants that were able to express high-level and homogeneous methicillin resistance at 42°C. Transposon mutant C245, exhibiting this phenotype, was identified among over a thousand initially screened Tn551 inserts. Mutant C245 grown at either 37 or 42°C produced homogeneously resistant cultures with methicillin MICs no lower than 400 μg/ml. Backcrossing of the Tn551-inactivated gene into the parental strain COL produced transductant C245Td, which expressed a resistance phenotype virtually identical to that of the original transposon mutant.

Sequencing and identification of the suppressor of methicillin resistance (smr1). The genetic determinant in mutant C245 was amplified and sequenced, and the obtained open reading frame and the deducted amino acid sequence (Fig. 3) were compared to sequences of known polypeptides in the TIGR and GenBank databases by using the BLAST algorithm. The homology search in the TIGR database for the S. aureus strain COL yielded (with 100% peptide similarity) identification of the locus SA0501. Further search in the GenBank peptide databases, based on the statistical significance of sequence similarity, showed that the protein in question showed significant homology with the large neurotransmitter sodium symporter family, homologues of which can be found among both eukaryotes and prokaryotes (13).

Since SA0501 seems to play a negative role in terms of methicillin resistance, we propose to name this previously uncharacterized gene suppressor of methicillin resistance 1 (smr1).

Phenotype of the C245 transposon mutant. The methicillin resistance profile of strain COL and its transductant derivative carrying the C245 mutation was tested at 37 and 42°C in parallel with the rates of autolysis and growth rates of these strains at the two temperatures. The methicillin resistance profiles of C245 and C245Td transductant showed virtually identical high-level and homogeneous resistance at both temperatures (Fig. 4A).

Figure 4B shows that autolysis—a property closely associated with the response of bacteria to β-lactam antibiotics—was also altered in the C245Td transductant. Cells of strain COL grown at 42°C (and tested at 37°C) autolyzed slower than cells grown at 37°C. Autolysis rates were relatively fast and were identical for cultures of C245Td mutant grown at both temperatures.

The C245 transductant showed identical slow growth rates at both temperatures (Fig. 4C). The slow growth of the transductant C245Td was also evidenced by the small size of its colonies on solid medium (Fig. 4D).

In contrast to the parental strain COL, C245Td produced cell walls of identical (i.e., normal) muropeptide profiles at both temperatures, and the representation of the glycine-free muropeptide monomer and its oligomeric derivatives was reduced to the levels seen in COL grown at 37°C (Fig. 5).

Thus, each of the phenotypes that were temperature dependent in the parental strain COL became temperature independent in the transposon mutant. Although these observations do not allow the sorting out of the pleomorphic phenotypes into a sequential order, the critical role of the smr1 gene is quite evident.

Effects of temperature shift on transcription levels of mecA, pbpB, mtgA, and femX. Several previous studies suggested that the reduced levels of methicillin resistance in cultures grown at elevated temperatures might be related to the lowered production of PBP2A and/or PBP2 at higher temperatures (16, 22, 27). We tested by Northern analysis the transcription of mecA, pbpB, mtgA, and femX—four genes that are involved with the β-lactam resistance phenotypes in S. aureus—in strain COL and its C245 transductant grown at either 37 or 42°C (Fig. 6). A modest reduction in the signal intensity for each gene was detectable in the COL samples grown at 42°C. However, the same difference was also seen in mutant C245 grown at the same temperatures, although the resistant phenotype in C245 is no longer temperature dependent.
Temperature-dependent changes in the muropeptide composition of an isogenic methicillin-susceptible derivative of strain COL.

The SCCmec type I cassette was removed from strain COL by the method of Katayama et al. (14) to generate COLmec-. Incubation of the strain at 37 and 42°C reproduced the same effects on growth rate and cell wall composition, as already documented for the methicillin-resistant parent strain COL. HPLC elution profiles of enzymatic peptidoglycan digests from COLmec- showed temperature-dependent differences in muropeptide composition identical to those shown in Fig. 2 for strain COL. Also, similar to what we observed with strain COL, the C245 transductant of COLmec- produced peptidoglycan of identical (normal) composition at both temperatures (Fig. 7). These observations make it even less likely that the temperature-dependent changes observed in resistance level involved changes in the transcription and/or translation of the resistance gene mecA.

Impact of smr1 inactivation on the expression of oxacillin resistance in clinical heteroresistant MRSA isolates with different genetic backgrounds. Inactivation of smr1 in the background of COL caused two prominent effects: (i) elimination of the temperature dependence of growth rate and (ii) an increase in the methicillin MIC. Table 2 summarizes the results of a screen in which several clinical MRSA strains with different genetic backgrounds were inactivated for symporter gene smr1 and tested for oxacillin resistance at 37°C and 42°C. These observations indicate that smr1 is involved in the regulation of oxacillin resistance in MRSA.

### Table 2. Effect of inactivation of the symporter gene smr1 on the expression of oxacillin resistance in MRSA strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MLST</th>
<th>SCCmec type</th>
<th>Clonal complex</th>
<th>37°C Growth</th>
<th>42°C Growth</th>
<th>37°C MIC</th>
<th>42°C MIC</th>
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<tbody>
<tr>
<td>COL</td>
<td>250</td>
<td>I</td>
<td>8</td>
<td>++</td>
<td>&gt;256</td>
<td>+++</td>
<td>6.0</td>
</tr>
<tr>
<td>C245Td</td>
<td>250</td>
<td>I</td>
<td>8</td>
<td>++</td>
<td>&gt;256</td>
<td>+</td>
<td>&gt;256</td>
</tr>
<tr>
<td>DJ9455</td>
<td>247</td>
<td>I</td>
<td>8</td>
<td>++</td>
<td>1.0</td>
<td>+++</td>
<td>0.25</td>
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<tr>
<td>DJ9455-T2</td>
<td>247</td>
<td>I</td>
<td>8</td>
<td>++</td>
<td>64</td>
<td>+</td>
<td>0.25</td>
</tr>
<tr>
<td>E2125</td>
<td>247</td>
<td>I</td>
<td>8</td>
<td>++</td>
<td>1.0</td>
<td>+++</td>
<td>0.25</td>
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<tr>
<td>E2125-T2</td>
<td>247</td>
<td>I</td>
<td>8</td>
<td>++</td>
<td>128</td>
<td>+</td>
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<td>E3005</td>
<td>247</td>
<td>I</td>
<td>8</td>
<td>++</td>
<td>1.5</td>
<td>+++</td>
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<td>E3005-T4</td>
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<tr>
<td>MW2</td>
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<td>IV</td>
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<td>+++</td>
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<tr>
<td>301</td>
<td>239</td>
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<td>319</td>
<td>247</td>
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<td>0.75</td>
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<td>&gt;256</td>
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<td>RUSA11</td>
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<td>RUSA11-T3</td>
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<td>4.0</td>
<td>+</td>
<td>0.25</td>
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</table>

*MICs were determined from the E-test results after 24 h of incubation at the indicated temperatures.*
ent geographic origins, isolation dates, and genetic back-
grounds were compared for their growth rates and oxacillin
MICs when grown at 37 or 42°C. All but one of the strains
tested belonged to clonal complex 8. In parallel, we analyzed
transductants of the same strains, in which \textit{smr1}
is inacti-
vated. Although the degrees of increase in the oxacillin MICs
of the transductants clearly varied from strain to strain, im-
proved resistance and elimination of the temperature depen-
dence of growth rates was evident in each transductant. The
HPLC analysis of strains 319 and 404 revealed that, as in COL,
inactivation of \textit{smr1} also caused a normalization of the cell wall
muropeptide composition produced by these strains at 42°C
(data not shown).

Our results show that the central genetic determinant of
methicillin resistance—the \textit{mecA} gene—is only modestly
downregulated when COL cultures are grown at 42°C. A sim-
lar drop in the \textit{mecA} transcription level could also be observed
in the resistant mutant C245. Thus, in this case, the slightly
decreased cellular amount of PBP2A is not likely to account
for the drastic reduction of the methicillin resistance in strain
COL. The same is true for the other tested genes that poten-
tially contribute to methicillin resistance (\textit{ppbB}, \textit{femX}, and
\textit{mtgA}): they remain unaltered, and their transcription levels are
comparable for both the parental strain COL and the resistant
mutant C245.

When trying to answer the question of what is the mecha-
nism of the reduction of methicillin resistance in \textit{Staphylococ-
cus aureus} at 42°C, one has to remember that this is a tem-
perature that already triggers the heat shock response, inducing
multiple transcriptional changes, which may, in turn, cause
extensive metabolic alterations in the bacterial cells. Anderson
et al. (1) found that heat-shocked \textit{S. aureus} cells (at 42°C)
overexpressed 98 genes at the expense of 42 other genes that
were downregulated.

As for the role of the inactivated putative symporter in
antibiotic resistance, one may consider two alternatives. (i)
The putative symporter may catalyze the uptake of an impor-
tant amino acid, or related nitrogenous compounds, utilized by
the bacterial cells. Inactivation of that gene, vital for the inter-
mediary metabolism, would slow down bacterial growth, which
may allow bacterial cells to take full advantage of their slow-
paced resistance protein PBP2A. Otherwise, in fast-growing
cells, PBP2A, as a low-affinity penicillin-binding protein/poor
catalyst for the transpeptidation reaction, may lag behind other
enzymes participating in cell wall biosynthesis. This possibility
is suggested by the fact that the substantially improved resis-
tance levels of the symporter transductants (originating from
the fast-growing and only modestly resistant clinical MRSA
isolates) could be achieved even at low temperatures.

(ii) Alternatively, the putative symporter may be involved in
the sensing and global transcriptional response to the temper-
ature shifts and, as such, when inactivated, it would not be
possible for bacterial cells to switch their metabolism in re-
response to the changing temperature. In this case, the expres-
sion of factors involved in cell wall biosynthesis (\(\beta\)-lactam resis-
tance) would continue undisturbed. The “corrected” features of
the symporter mutants, i.e., methicillin resistance, cell wall compo-
nition, and growth and autolysis rates, which are
phenotypic manifestations of complex interactions between
numerous genes involved in cell wall synthesis and metabolism,
seem to confirm this hypothesis. This scenario seems to be
quite possible since some sodium-dependent symporters are
known components of the regulatory feedback control systems.
For instance, an Na\textsuperscript{+}/glycine betaine symporter was found to

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Effect of \textit{smr1} complementation on oxacillin resistance of MRSA strain C245Td. Bacterial cultures grown overnight in TSB (COL), TSB supplemented with 10 \(\mu\)g of erythromycin/ml (C245Td), or 10 \(\mu\)g of chloramphenicol/ml (C245KS20) were diluted to approximately \(5 \times 10^7\) CFU/ml, swabbed onto TSA plates, and allowed to dry. Oxacillin E-test strips were then applied to the agar surface. The inhibitory effects of oxacillin on the growth of COL (A), C245Td (B), and C245KS20 (C) were determined after 18 h of incubation at 42°C.}
\end{figure}
be involved with the protection of Listeria monocytogenes against osmotic and thermal shock (18). The accumulation of glycine betaine may lead to a regulatory feedback: the Na+/H+ antiporter homologue may turn out to be in at least in part responsible for the methicillin resistance phenotype in MRSA strains. This conclusion is confirmed by the results of the complementation experiment illustrated in Fig. 8. Complementation of the C245Tld mutant with plasmid-encoded smr1 literally abolished its high level of β-lactam resistance at 42°C and restored the parent-like (COL) phenotype. Experiments are currently in progress to clarify the mechanism of action of the symporter in β-lactam resistance and cell wall synthesis.

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