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Transcriptomic and Functional Analysis of an Autolysis-Deficient, Teicoplanin-Resistant Derivative of Methicillin-Resistant *Staphylococcus aureus*†

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The molecular basis of glycopeptide-intermediate *S. aureus* (GISA) isolates is not well defined though frequently involves phenotypes such as thickened cell walls and decreased autolysis. We have exploited an isogenic pair of teicoplanin-susceptible (strain MRGR3) and teicoplanin-resistant (strain 14-4) methicillin-resistant *S. aureus* strains for detailed transcriptomic profiling and analysis of altered autolytic properties. Strain 14-4 displayed markedly deficient Triton X-100-triggered autolysis compared to its teicoplanin-susceptible parent, although microarray analysis paradoxically did not reveal significant reductions in expression levels of major autolytic genes *atl*, *lytM*, and *lytN*, except for *sle1*, which showed a slight decrease. The most important paradox was a more-than-twofold increase in expression of the *cidABC* operon in 14-4 compared to MRGR3, which was correlated with decreased expression of autolysis negative regulators *lytSR* and *lrgAB*. In contrast, the autolysis-deficient phenotype of 14-4 was correlated with both increased expression of negative autolysis regulators (*arlRS*, *mgrA*, and *sarA*) and decreased expression of positive regulators (*agr* RNAII and RNAIII). Quantitative bacteriolytic assays and zymographic analysis of concentrated culture supernatants showed a striking reduction in Atl-derived, extracellular bacteriolytic hydrolase activities in 14-4 compared to MRGR3. This observed difference was independent of the source of cell wall substrate (MRGR3 or 14-4) used for analysis. Collectively, our results suggest that altered autolytic properties in 14-4 are apparently not driven by significant changes in the transcription of key autolytic effectors. Instead, our analysis points to alternate regulatory mechanisms that impact autolysis effectors which may include changes in posttranscriptional processing or export.

The intensive use of vancomycin, which has for decades been the only drug uniformly active for treatment of multiresistant nosocomial isolates of *Staphylococcus aureus*, exerts a high selective pressure for emergence of glycopeptide resistance (52, 53). Since their first discovery in 1997 (32, 33), clinical isolates of glycopeptide-intermediate *S. aureus* (GISA) have been recovered worldwide (31, 52, 105). A major problem in evaluating the clinical and epidemiological significance of GISA isolates is the limited sensitivity and specificity of glycopeptide resistance phenotypic assays combined with the absence of specific molecular resistance markers (31, 52, 105). This situation is even more problematic when dealing with *S. aureus* isolates displaying heterogeneous expression of intermediate glycopeptide susceptibility (hGISA) (31, 52, 105). Emergence of GISA seems to result from multifactorial, endogenous changes in contrast to the few vancomycin-resistant *S. aureus* isolates that have exogenously acquired the *vanA* gene from enterococci (107).

Numerous investigations were carried out to understand the molecular basis and discover reliable phenotypic markers of GISA isolates (5, 8, 17, 18, 21, 26, 29, 31, 43, 46, 66, 67, 78, 89, 91–93, 105, 108). Several factors have been proposed as contributors to endogenous acquisition of resistance (5, 8), including a thickened cell wall (15, 17, 18, 78, 89), the presence of increased D-Ala-D-Ala residues in cell walls preventing glycopeptides from reaching their true target (31, 91), and other alterations in the composition or regulation of cell wall biosynthesis and turnover (21, 29, 43, 46, 63, 67, 92–94, 102). Some of these studies also mentioned fitness changes characterized by lower in vitro growth rates for GISA compared to non-GISA isolates (63, 66, 71, 89, 90). The functional implication of each of these metabolic changes for decreased glycopeptide susceptibility is not well understood. Furthermore, there is some debate whether these changes occur in all GISA/hGISA isolates or a subset of them (8, 26, 78, 90, 105).

Despite detailed genetic and DNA microarray studies, no comprehensive molecular model that explains the GISA phenotype has emerged. While the list of genes exhibiting altered expression (*vraSR*, *tcaAB*, *sigB*, and *msrA2*) in GISA compared to non-GISA isolates has been considerably expanded by DNA microarray studies (16, 48, 63, 66), the mutual interactions of these genes and their respective roles in resistance are still elusive (3, 5, 9, 16, 48, 49, 58). A major problem that complicated interpretation of most previous molecular studies has been the difficulty in identifying isogenic non-GISA parents of

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clinical GISA strains for true comparison. Instead, all reported studies except a recent one (63) were performed with in vitro-selected GISA, derived from multipassaged non-GISA parents (16–18, 43, 67) or isogenic in vitro-derived revertants from clinical GISA, whose fitness properties and virulence could be significantly altered by multiple subcultures (6, 16–18, 66).

Among factors controlling cell wall expansion, remodeling, and daughter cell separation, peptidoglycan hydrolases, referred to as autolysins, that participate in peptidoglycan turnover play an essential role (27, 42, 65). Autolysins that cleave the cell wall in a tightly controlled manner to maintain cell wall integrity during cell division are classified according to their specific cleavage types, e.g., *N*-acetylmuramidases, *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases (27). The major autolysis gene (*atl*) of *S. aureus* yields a 63-kDa amidase and a 54-kDa glucosaminidase after processing (2, 22, 69, 97–99, 111). Other autolytic genes include *sle1*, which encodes an additional *N*-acetylmuramyl-L-alanine amidase that is distinct from *atl* (39); *lytM*, which encodes glycylglycine endopeptidase (75, 76); and *lytN*, which possibly encodes muramidase activity (96).

Several regulators of autolysis have been described. First, the two-component regulator *lytS-lytR* is a negative regulator of extracellular peptidoglycan hydrolases via *lrgA* and *lrgB*, which in turn control the *cidABC* operon (10, 11, 25, 28, 81–83). Additional global regulators such as *agr*, *sarA*, *arlRS*, *mgrA*, and *sarV* can also influence autolytic activity, thus revealing the complex regulation of autolysis (2, 11, 23, 24, 37, 62, 98, 99).

The decreased autolytic activity of a substantial proportion of clinical or laboratory GISA isolates has been previously reported (7, 35, 43, 70, 71, 85, 91, 102, 108). Two alternative mechanisms were proposed for explaining the autolysis-deficient phenotypes of GISA, one being the reduced expression of *atl* (43, 108) and the other the occurrence of cell wall alterations that would decrease GISA susceptibility to autolysis (7). While there is a growing consensus that decreased autolysis of GISA strains may, directly or indirectly, contribute to their reduced susceptibility to glycopeptides, the molecular pathways linking defective autolysis to glycopeptide resistance are still undefined (43, 94, 102).

We previously described altered expression of some virulence factors in the teicoplanin-resistant (Tei^r) strain 14-4 compared to its teicoplanin-susceptible (Tei^s) parental strain, MRGR3, a methicillin-resistant *S. aureus* (MRSA) clinical isolate that shows a high degree of virulence in a tissue cage rat model of chronic foreign body MRSA infection (54, 79, 103). In this report, we explored the molecular basis of the markedly reduced autolytic phenotype of the Tei^r strain compared to its isogenic Tei^s parent by transcriptomic analysis of a subset of autolysis- and cell wall-associated genes (13).

MATERIALS AND METHODS

Bacterial strains. MRSA strain MRGR3 is a Tei^s and vancomycin-susceptible isolate from a patient with catheter-related sepsis in 1979 (54, 103). The average MICs of vancomycin and teicoplanin in cation-adjusted Mueller-Hinton broth (MHB; Difco, Detroit, Mich.) for strain MRGR3 are 1 and 1 to 2 μg/ml, respectively. Strain 14-4 is a stable Tei^r derivative of MRGR3 that was isolated in a rat model of chronic tissue cage infection followed by two in vitro passages on teicoplanin-containing agar (103). The average MICs of vancomycin and teicoplanin for strain 14-4 are 4 and 16 to 32 μg/ml, respectively (79). The glycopeptide susceptibility or resistance phenotypes of strains MRGR3 and 14-4 were homogeneous, as assessed by population analysis profiles of teicoplanin and vancomycin (data not shown).

Several criteria confirmed the isogenic link of strains MRGR3 and 14-4. First, besides their different susceptibilities to glycopeptides, strains 14-4 and MRGR3 exhibit identical patterns of resistance to penicillin, gentamicin, cholamphenicol, erythromycin, tetracycline and polymyxin B, while remaining susceptible to rifampin, cotrimoxazole, mupirocin, fosfomycin, and all fluoroquinolones. Pulsed-field gel electrophoresis of strains 14-4 and MRGR3 yielded similar patterns except for a single-band difference (57), and their isogenic links were confirmed by a previously described comparative genomic hybridization technique (13), which showed >99.98% identity (data not shown).

In some experiments, parental strain 8325-4 and its *atl* mutant, SH108, (kindly provided by S. J. Foster) were used as control strains for autolysin production.

Triton X-100-induced autolysis assays. Autolysis was assayed essentially as described previously (37, 60) on *S. aureus* cultures grown under three different conditions. In initial experiments (37, 60), overnight cultures of *S. aureus* were diluted to an optical density at 600 nm (OD_{600}) of 0.05 and grown for 3 h with shaking at 37°C in MHB containing 1 M NaCl until the OD_{600} reached 0.7 (37). In further experiments, *S. aureus* cultures were preferably grown in MHB without NaCl supplementation, either with shaking for 3 h or without shaking for 5 h.

Thereafter, cells were harvested, washed twice with ice-cold water, and then resuspended in the same volume of 0.05 M Tris-HCl (pH 7.2) containing 0.05% Triton X-100. Cells were incubated at 30°C with shaking and checked for lysis by measuring the progressive decrease in absorbance (OD_{600}). Autolysis was quantified as a percentage of the initial OD_{600} remaining at each sampling point.

Zymographic analysis. Measurement of extracellular bacteriolytic hydrolases was performed essentially as described previously (28, 60, 62). Supernatants from overnight cultures of strains MRGR3 and 14-4 or 8325-4 and its *atl* mutant, SH108, used as controls, were grown in 10 ml MHB, filter sterilized, and then concentrated 20-fold using Centricon-3 concentrators (Millipore). Various loading amounts (from 1.25 to 20 μg) of extracellular proteins, determined by Bradford assay (Bio-Rad), were separated on sodium dodecyl sulfate–7.5% polyacrylamide gels containing autoclaved and lyophilized cell preparations (1 mg of dry weight per ml) of either strain MRGR3 or 14-4. After electrophoresis, proteins were renatured by overnight incubation in 1% (vol/vol) Triton X-100 in 25 mM Tris-HCl (pH 8.0), and then gels were stained with 1% (wt/vol) methylene blue in 0.05% KOH prior to photography (28, 60, 62).

Quantitative bacteriolytic hydrolase assays. Bacteriolysis of lyophilized *S. aureus* by extracellular hydrolases was quantified as previously described (28). Equivalent amounts (100 μg) of concentrated supernatants from either 5-h or overnight cultures of strain MRGR3, 14-4, 8325-4, or its *atl* mutant, SH108, were added to a suspension of autoclaved and lyophilized *S. aureus* MRGR3 or 14-4 (1 mg/ml) in 100 mM Tris-HCl (pH 8.0) and incubated at 37°C with shaking. Lytic activity was recorded by measuring the progressive decrease in absorbance (OD_{600}).

Total RNA extraction and labeling. Cultures of MRGR3 and 14-4 were grown at 37°C for 5 h in MHB without shaking. Then, bacteria were harvested and RNA extraction was performed as previously described (30, 50). Briefly, bacteria were recovered, fixed in acetone-ethanol (1:1), and washed in Tris-sucrose buffer. Samples were treated with ice-cold lysostaphin, and RNA was purified as described previously (50, 79). The absence of contaminating DNA was verified by PCR (50, 79). Purified RNA samples were analyzed using the RNA NanoLab chip on the 2100 Bioanalyser (Agilent, Palo Alto, CA).

DNA microarray preparation, hybridization, and data analysis. The customized StaphChip oligoarray was designed in our laboratory and manufactured by Agilent Technologies (Palo Alto, CA) as described previously (13). A set of 5,337 *S. aureus* 40- to 60-mer probes, recognizing 97.5, 93, and 81% of N315, Mu50, and COL open reading frames (ORFs), respectively, were represented in each oligoarray. More than 95% of ORFs evaluated in this study were recognized by two or three nonoverlapping specific probes.

Total RNAs (12 μg) from strains MRGR3 and 14-4 were labeled in parallel with Cy3-dCTP and Cy5-dCTP (New England Nuclear), respectively, using the SuperScript kit (Invitrogen) by following the manufacturer's instructions. Labeled cDNAs were then purified using QIAQuick (QIAGEN) columns.

For competitive hybridization using a dual-label experimental approach, equivalent amounts of Cy3-labeled and Cy5-labeled cDNAs were diluted in 250 μl Agilent hybridization buffer and cohybridized for 17 h at 60°C. Slides were washed, dried under nitrogen flow, and scanned (Agilent) as described previously (13) using 100% photomultiplier power tube power for both wavelengths. All positive and significant-local-background-subtracted signals, obtained using Feature Extraction software (version A6.1.1; Agilent), were corrected for unequal dye incorporation or unequal load of labeled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS (locally

TABLE 1. TaqMan primers and probes used in this study

Gene	Forward primer	Reverse primer	Probe (5′–3′)[a]
arlR	304F-CCGTTTGATATTGAAGAACTTTTAGCA	386R-CCGTTGACATCGATAATATCCTTTT	333T-AATTCGTGCAATTTTACGTCGTCAGCCA
atl	878F-AAACAGCACCAACGGATTACTTATC	977R-AATGAAGCATAGTCGTGTGTGTGTAC	911T-TCGGTGCAGTCGGTAACCCTAGATTCA
lytR	475F-GGGATTGGCACACATAATGGT	576R-AGTGGGATTCAATCGTTTTTCATAA	500T-CAACCATACATACAACGAATCATAAATACGA AACAACAGA
lrgB	525F-CCGAATTACTAACCCTATTGCCC	602R-TCTTTGGCTGGTGCTACACCT	549T-AGGATTAGCACTTGGAACAAGTGGTCACACA
cidB	517F-TTTGAATCTTCTATCGCCAAAGG	591R-TTCTAGTGCTTTAGCTGTGCCAA	541T-TTAACGTATGGGAATGCGTCACATGCA
mgrA	142F-TGGGATGAATCTCCTGTAAACGT	233R-TGTTCCATTCGTTTTAATAATGGTG	166T-AAGAAAGTCGTAACTGAATTAGCACTCGATA CTGGTACA
pbp4	90F-CCCTGTACAAGCAGCAAATCAA	159R-AACAGCACTCGTCGGTTCGT	113T-ATGGTTATGCAGGTTTGTCGGCTGCA
pbp2	407F-AAAGACGCAGTACTTGCAACTGAAG	490R-ACCAATTGCACCGAATAAACGT	437T-CGTTTCTACGAACATGGCGCACTTGATT
tcaA	428F-GTAAAAATGGCACGCGTTATATCTT	597R-CTTACCACCAGATTTAAACTCATATTTTGT	532T-CCTACCAAGCAACCAATTGTTAAGCCGAAA

[a] Probes were labeled with 5′-6-carboxyfluorescein and 3′-6-carboxy tetramethylrhodamine.

weighted linear regression) method. Irregular or saturated spots were excluded from subsequent analysis. Spots showing a reference signal lower than background plus 2 standard deviations were also excluded from subsequent analysis. Average background values were 328 ± 17 and 363 ± 24 arbitrary units for green and red signals, respectively.

To provide estimates of expression levels of the subset of genes analyzed in our study with respect to the whole transcriptome, all Feature Extraction-processed dye-normalized signals from the oligoarray were subdivided into four categories according to their intensities in the Tei^s control strain MRGR3: the 25th percentile of probes, yielding the lower-intensity signals (<126 arbitrary units), followed by the 25th to 50th percentile (127 to 924 units), the 50th to 75th percentile (925 to 6,612 units), and the 75th to 100th percentile, yielding the highest-intensity signals (>6,612 units).

Data analysis. The oligoarray-generated signals across three independent experiments were expressed as mean (± standard error of the mean [SEM]) processed green or red signals separately recorded from each individual probe, or from two or more nonoverlapping probes recognizing common gene transcripts. For all genes whose signal intensities and strain-specific signal ratios originating from multiple probe subsets were equivalent, statistical differences were evaluated by *t* test on the basis of pooled signals from multiple gene-associated probes (see Table S1 in the supplemental material).

Real-time RT-PCR. mRNA levels were determined by quantitative reverse transcriptase PCR (RT-PCR) using the one-step reverse transcriptase qPCR Master Mix kit (Eurogentec, Seraing, Belgium), as described previously (79). All primers and probes that were not described previously are listed in Table 1; they were designed using PrimerExpress software (version 1.5; Applied Biosystems) and obtained from Eurogentec or Applied Biosystems. Conditions for reverse transcription, PCR, detection of fluorescence emission, and normalization of the levels of mRNA of the target genes extracted from the different strains on the basis of their 16S rRNA levels were described previously (50, 79, 104). The statistical significance of strain-specific differences in normalized cycle threshold values for each transcript was evaluated by paired *t* test, and data were considered significant when P was <0.05.

Microarray data accession number. Microarray data analyzed in this study have been deposited in the ArrayExpress database with accession number E-MEXP-378 (http://www.ebi.ac.uk/arrayexpress).

RESULTS

Major differences in Triton X-100-induced autolysis of Tei^r and Tei^s strains. The Tei^r strain 14-4 exhibited markedly reduced autolysis compared to its isogenic Tei^s parent, MRGR3 (Fig. 1). Changing the preculture conditions did not lessen the strongly reduced autolytic response of the Tei^r strain compared to its Tei^s parent. In initial experiments, we used previously recommended conditions by growing cells incubated with shaking in MHB containing 1 M NaCl (37, 60), which led to autolytic rates of nearly 80% at 3 h for Tei^s strain MRGR3 compared to ca. 20% for Tei^r strain 14-4 (Fig. 1A). In further experiments, the autolytic response of Tei^r and Tei^s strains was preferably tested on cells that were grown in MHB without NaCl supplementation to be more consistent with conditions used for assaying transcript levels or extracellular bacteriolytic activities. Comparative growth characteristics of Tei^r strain 14-4 and Tei^s strain MRGR3, grown in MHB without NaCl supplementation in either shaking or nonshaking conditions, are shown on Fig. 2.

After growth in shaking conditions, autolysis at 9 h was approximately 40% for Tei^s MRGR3 and <20% for Tei^r 14-4 (Fig. 1B). In similar conditions autolytic rates of strain 8325-4 and its *atl* mutant, SH108, at 9 h were approximately 50% and 20%, respectively, indicating the involvement of Atl-derived products in the Triton X-100-induced autolytic assay.

Finally, after growth in nonshaking conditions, autolytic rates of strains MRGR3 and 14-4 at 9 h were approximately 40% and <10%, respectively (Fig. 1C). Importantly, the auto-

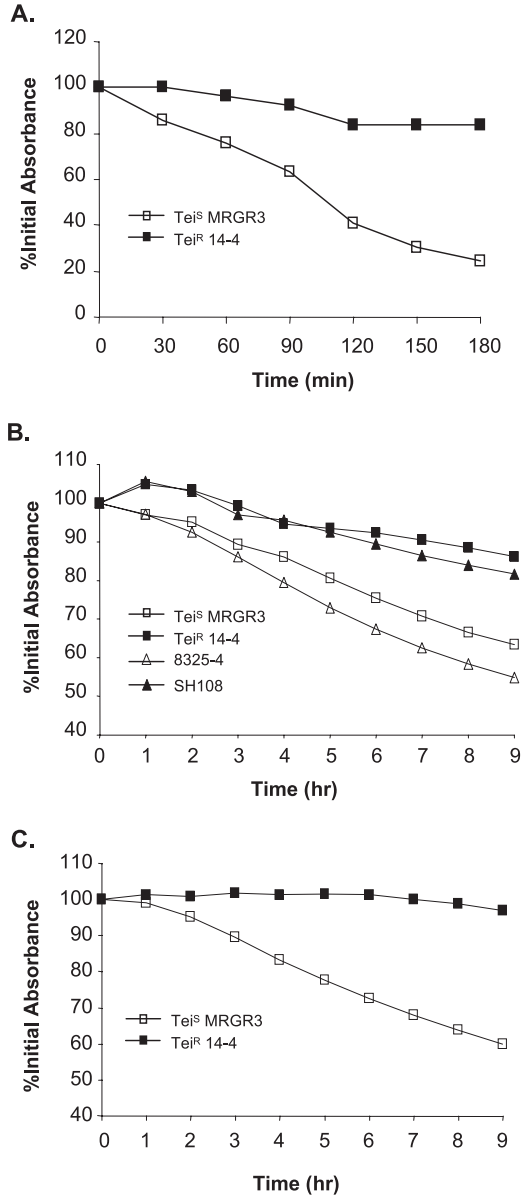

FIG. 1. Differences in Triton X-100-induced autolysis of Tei^r strain 14-4 compared to its isogenic Tei^s parent, MRGR3. Exponential cultures were grown either in shaking conditions for 3 h at 37°C in MHB supplemented with 1 M NaCl (A) or unsupplemented (B) or for 5 h in MHB in nonshaking conditions without NaCl supplement (C). When indicated, strain 8325-4 and its *atl* mutant, SH108, were used as controls (B). These data are from a single representative experiment and were reproduced several times.

lytic differences recorded on bacterial cultures grown in MHB without shaking and without NaCl supplementation were particularly relevant since identical growth conditions were used for assaying transcript levels of strain 14-4 and MRGR3 by DNA oligoarray or real-time reverse transcription-PCR (RT-PCR).

Reliability of the DNA oligoarray for studying differential gene expression between Tei^r and Tei^s strains. The reliability of the StaphChip oligoarray-generated strain-specific signals was established across three independent experiments by (i) the high interexperiment reproducibility of processed intensity signals recorded on each individual probe for Cy3-labeled or Cy5-labeled hybridized cDNAs from Tei^s or Tei^r strains, respectively, and (ii) the equivalent processed intensity signals recorded on multiple, nonoverlapping probes of common gene transcripts (see Table S1 in the supplemental material). As an example, Fig. 3A shows mean ± SEM Cy3-generated signals for a subset of 23 different probes recognizing 23 nonoverlapping regions of 11 different gene transcripts from parental Tei^s strain MRGR3.

For most assayed genes, changes in transcript levels, expressed as ratios of Tei^r to Tei^s signal intensities, were highly reproducible, not only on individual probes but also on multiple probes recognizing nonoverlapping regions of each transcript (Fig. 3B and Table 2; see Table S1 in the supplemental material). Collectively, these results provided strong evidence that multiple probes recognizing different regions of each transcript provided reliable data allowing detection of minor, but statistically significant, strain-specific changes in gene expression (Table 2).

Comparison of expression levels of autolysis- and cell wall-associated genes between Tei^r and Tei^s strains. Expression levels of 117 genes involved in autolysis or cell wall biosynthesis or known as related regulators were analyzed by DNA oligoarray (Table 2; Fig. 3B), and a small subset of those transcript levels were also evaluated by real-time RT-PCR (Fig. 4). Overall, 85% of those analyzed genes ranged among the most highly expressed ones of the *S. aureus* transcriptome, and those with expression levels lower than the 50% percentile were predominantly found within the category of extracellular proteases (Table 2).

Surprisingly, transcript levels of most autolysin genes either showed no significant decrease, except for *sle1*, whose expression slightly decreased, in the autolysis-deficient Tei^r strain 14-4 compared to its autolysis-proficient Tei^s parent. Instead, a number of those transcripts were even slightly (*atl*) or markedly (*cidA*, *cidB*, and *cidC*) increased. Of note, expression of *cidA* was very weak, compared to the more intense signals of *cidB* and *cidC* (Table 2; see Table S1 in the supplemental material). Increased *cidABC* transcript levels were consistent with the reduced expression of their major autolytic repressors *lytSR* and *lrgAB* in the Tei^r strain compared to the Tei^s strain, according to previous studies (10, 11, 25, 28, 81, 81, 83), but failed to explain the autolysis-deficient phenotype of the Tei^r strain. Real-time RT-PCR confirmed increased *atl* and *cidB* levels and reduced *lrgB* levels but showed no significant change in *lytR* levels (Fig. 4).

In contrast, significantly increased expression levels were recorded for three additional negative regulators of autolysis, namely, *mgrA*, *arlRS*, and *sarA*, which individually or collectively may contribute to the autolysis-deficient phenotype of Tei^r strain 14-4 (23, 37, 56). An additional explanation for the deficient autolytic phenotype of the Tei^r strain compared to its Tei^s parent might be the loss of *agr* global regulator function, previously reported to promote autolysis, as evidenced by the strong reduction in its RNAII and RNAIII levels (24, 85). The oligoarray-detected changes in *mgrA*, *arlR*, *sarA*, and RNAIII transcript levels in Tei^r strain 14-4 compared to MRGR3 were all confirmed by real-time RT-PCR (Fig. 4).

The potential influence of additional cell wall-associated

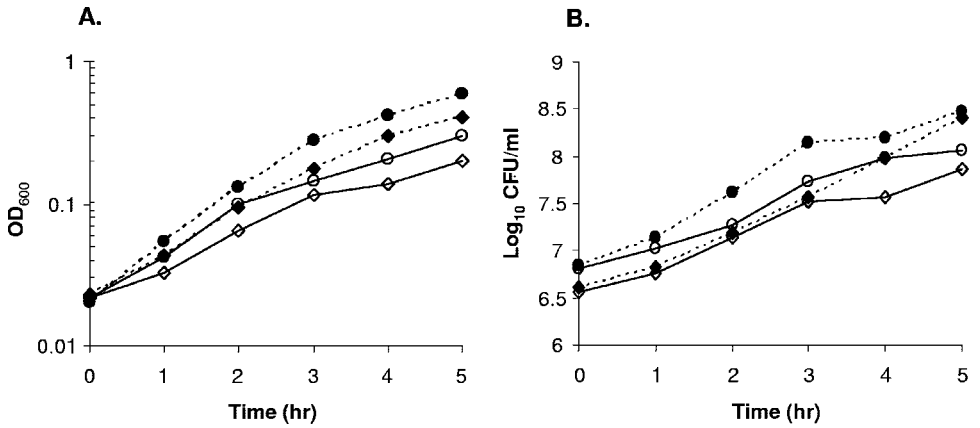

FIG. 2. Growth curves in MHB of Tei^r strain 14-4 (◇) compared to its isogenic Tei^s parent, MRGR3 (○), in nonshaking (open symbols) or shaking (solid symbols) conditions, recorded as OD_{600} (A) or viable counts (B).

factors or global regulators of autolysis was also considered. Whereas transcript levels of *llm* (59), also referred to as *tagO* (106), *fmtA* (47), and the weakly expressed *sarV*, recently described as a positive regulator of autolysis (62), were slightly increased in strain 14-4 compared to MRGR3, this was not the case for *fmtB*, *ftsA*, *ftsZ*, and *scdA* (12, 45, 73, 74), which showed marginal (<30%) changes in transcript levels (Table 2). The significance of the twofold decline in *fmtC* (also called *mprF*) transcript levels is unclear because of its questionable impact on autolysis (44) and controversial effect on glycopeptide resistance (110).

Differential expression of major cell wall biosynthesis genes by Tei^r and Tei^s strains was also studied. No consistent changes were recorded in transcript levels of the cytoplasmic enzymes MurA to MurF, involved in the formation of the monomeric building block *N*-acetylglucosamine-*N*-acetylmuramyl pentapeptide or MurI (glutamate racemase) in Tei^r strain 14-4 compared to MRGR3 (Table 2). The only notable exception was the more-than-twofold increase in *murZ* transcript levels, which contrasted with the weak increase of its *murA* functional homolog (19) (Table 2). The molecular basis and biological significance of the differential expression of UDP-*N*-acetylglucosamine enolpyruvyl transferase isozymes 1 (MurA) and 2 (MurZ) (16) remain unexplained.

Transcript levels of enzymes catalyzing further steps of peptidoglycan synthesis were either decreased by twofold (for MraY) or similar (for MurG, a translocase) in Tei^r strain 14-4 compared to Tei^s strain MRGR3 (Table 2). Furthermore, expression levels of FemX, FemA, and FemB factors, catalyzing the assembly of pentaglycine interpeptide bridges (87), remained constant in strain 14-4 compared to MRGR3 (Table 2). Finally, transcript levels of major penicillin-binding proteins (PBPs) involved in the final stages of peptidoglycan synthesis were either similar (PBP1 and PBP3) or significantly increased (PBP2 and PBP4) in the Tei^r strain compared to the Tei^s strain (Table 2), as confirmed by real-time RT-PCR (Fig. 4). In line with these findings, MICs for Tei^r strain 14-4 of oxacillin and imipenem showed a slight twofold increase compared to its Tei^s parent (data not shown).

These data are compatible with previous observations showing increased production and penicillin-binding activity of PBP2 in some laboratory-derived teicoplanin- and vancomycin-resistant derivatives of methicillin-susceptible *S. aureus* (67, 88). While the increased expression levels of PBP4 are compatible with previous data recorded on teicoplanin-resistant laboratory isolates (67), an opposite situation was recorded in some clinical isolates displaying intermediate susceptibility to vancomycin (VISA) and exhibiting a lower degree of cell wall cross-linking, which demonstrated decreased expression of PBP4 potentially linked with acquisition of the VISA phenotype (21, 63, 93). As opposed to PBP2 and PBP4, transcript levels for *mecA*, coding for PBP2A, involved in expression of methicillin resistance, were decreased by more than twofold in strain 14-4 compared to MRGR3 (Table 2).

Transcript levels of the six contiguous genes (*tagA*, *tagH*, *tagG*, *tagB*, *tagX*, and *tagD*) of the cell wall teichoic acid biosynthetic pathway operon were virtually identical in strains 14-4 and MRGR3 (Table 2). In contrast, *dltABCD* transcript levels were consistently reduced by ca. twofold in the Tei^r strain compared to the Tei^s strain (Table 2). While the complete absence of D-alanine esters in teichoic acids was reported to increase simultaneously the autolytic rate and susceptibility to vancomycin of the glycopeptide-susceptible *S. aureus* strain SA113 (70), a significant contribution of partially decreased *dltABCD* transcript levels to the autolysis-deficient phenotype of Tei^r strain 14-4 is uncertain because such a reduction would rather predict an increased susceptibility of *S. aureus* to teicoplanin (70).

Overall, gene expression levels of most cell wall-building components were not significantly altered in Tei^r strain 14-4 compared to its Tei^s isogenic parent, MRGR3. While gene expression levels of the capsular polysaccharide biosynthetic pathway were upregulated in Tei^r strain 14-4 compared to its Tei^s parent, this was not the case for the *icaABCD* operon (data not shown).

Differential expression of additional global or antibiotic-inducible cell wall regulators in Tei^r and Tei^s strains. Expression of several previously identified antibiotic-inducible cell wall regulators was found to be increased in Tei^r strain 14-4 compared to its Tei^s parent. With the exception of *tcaB* transcript levels, which remained constant, those of *drp35* and *msrA2* were increased by >50%, those of *msrR*, *msrA1*, *msrB*,

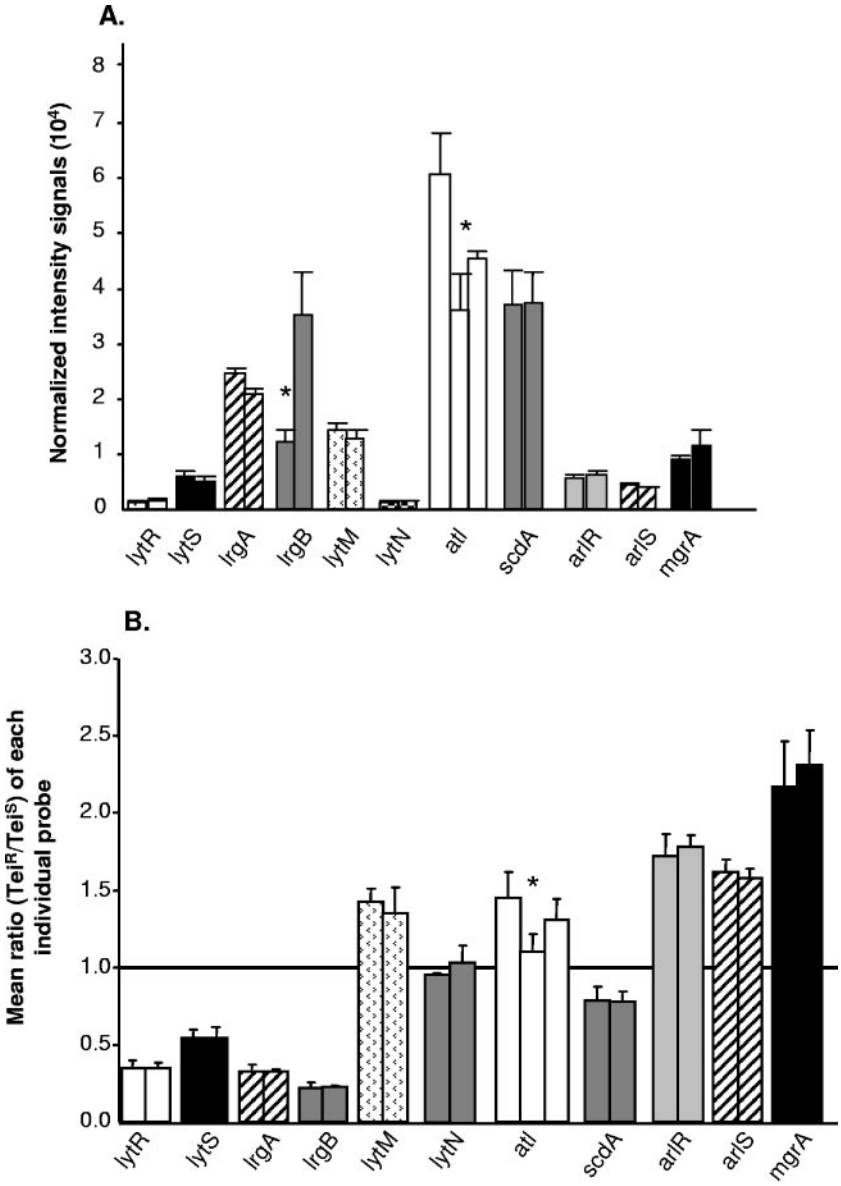

FIG. 3. (A) Reproducibility of intensity signals generated from nonoverlapping probes covering individual gene transcripts. Shown are normalized intensity signals (mean plus SEM; $n = 3$) from 23 probes covering 11 different genes from Tei^s strain MRGR3. *, significantly different intensities. (B) Reproducibility of transcript level changes of Tei^r strain 14-4 compared to Tei^s strain MRGR3, expressed as mean (plus SEM; $n = 3$) ratios (Tei^r/Tei^s), for multiple probes recognizing common transcripts. *, significantly different ratios.

tcaA, and *tcaR* by >2-fold, and those of *vraR* and *vraS* by 3.5-fold and 5-fold, respectively (Table 2). Two additional genes whose expression levels were highly increased were the putative monofunctional glycosyltransferase gene *sgtB* (9-fold) (16, 48, 63) and the peptidyl-prolyl *cis/trans* isomerase homolog *prsA* (6.5-fold) (48, 63, 101), in contrast to *sgtA* and *abcA*, which showed only marginal changes (Table 2).

Differential expression of the SigB regulon, known to influence glycopeptide resistance (3–5) and peptidoglycan hydrolase regulation (83), was also studied. While *rsbU*, *rsbV*, *rsbW*, and *sigB* transcript levels were similar in Tei^r and Tei^s strains, those of their target genes, for instance, *asp23* and *sarS* (4), were increased by ca. 80%. These results were quite similar to those previously recorded by real-time RT-PCR for *rsbU*, *sigB*, and *asp23* transcript levels of strains 14-4 compared to MRGR3 (79). It should be mentioned that increased expression of *tcaR* may also contribute to increased *sarS* transcript levels (64).

In contrast, other strongly expressed global regulators, namely, *yycFG*, *srrAB*, and *saeRS*, remained at nearly identical levels in Tei^r and Tei^s strains (Table 2). Among members of the *sarA* protein family (14), only *rot* was significantly expressed but at similar levels in both Tei^r and Tei^s strains, whereas expression levels of *sarU* and *sarT* were too low, as expected from previous studies (61, 86), to allow detection of significant strain-specific differences.

Finally, we also evaluated additional sets of genes reported to be upregulated in two microarray studies comparing vancomycin-intermediate with vancomycin-susceptible

TABLE 2. Ratios of transcript levels of Tei^r 14-4 to Tei^s MRGR3

N315 SA no.	Gene	Description	Probe intensity	Ratio of signal intensity (14-4/MRGR3)		*P*
				Mean[a]	SEM	
Autolysis genes						
SA0905	*atl*	Bifunctional precursor autolysin (Atl)	****	1.29	0.06	<0.05
SA0423	*sle1*	*N*-Acetylmuramyl-L-alanine amidase	****	0.76	0.03	<0.05
SA0265	*lytM*	Peptidoglycan hydrolase	****	1.39	0.05	<0.001
SA1090	*lytN*	LytN protein	***	0.99	0.03	NS[c]
SA2329	*cidA*	Hypothetical protein, similar to transcription regulator	**	2.24	0.14	<0.001
SA2328	*cidB*	Conserved hypothetical protein	****	3.25	0.05	<0.001
SA2327	*cidC*	Pyruvate oxidase	****	2.57	0.15	<0.001
SA0251	*lytR*	Two-component response regulator	***	0.34	0.02	<0.001
SA0250	*lytS*	Two-component sensor histidine kinase	***	0.55	0.02	<0.001
SA0252	*lrgA*	Murein hydrolase regulator LrgA	****	0.33	0.01	<0.001
SA0253	*lrgB*	Antiholin-like protein LrgB	****	0.22	0.01	<0.001
SA0641	*mgrA*	Transcriptional regulator MgrA	****	2.24	0.10	<0.001
SA0650	*norA*	Quinolone resistance protein	***	1.47	0.04	<0.001
SA1248	*arlR*	Truncated (putative response regulator ArlR [S	***	1.75	0.04	<0.001
SA1246	*arlS*	Sensor histidine kinase ArlS	***	1.60	0.03	<0.001
SA0573	*sarA*	Staphylococcal accessory regulator A	***	3.53	0.13	<0.001
SAS065	RNAIII	Delta hemolysin	****	0.02	0.00	<0.001
SA1844	*agrA*	Accessory gene regulator A	****	0.08	0.01	<0.001
Cell wall-associated genes						
SA0702	*llm*/*tagO*	Lipophilic protein affecting bacterial lysis rate and methicillin resistance level	***	1.60	0.04	<0.001
SA0909	*fmtA*	Autolysis and methicillin resistant-related protein	***	1.62	0.04	<0.001
SA2062	*sarV*	Staphylococcal accessory regulator A homolog	**	1.45	0.15	NS
SA1964	*fmtB*	FmtB protein	***	1.28	0.13	NS
SA1028	*ftsA*	Cell division protein	****	1.36	0.04	<0.001
SA1029	*ftsZ*	Cell division protein	****	1.10	0.04	<0.05
SA0249	*scdA*	Cell division protein and morphogenesis-related protein	****	0.78	0.03	<0.001
SA1193	*fmtC*/*mprF*	Oxacillin resistance-related FmtC protein	****	0.46	0.03	<0.001
SA1902	*murA*	UDP-*N*-acetylglucosamine 1-carboxyvinyl transferase 1	****	1.35	0.08	<0.01
SA0693	*murB*	UDP-*N*-acetylenolpyruvoylglucosamine reductase	****	0.99	0.03	NS
SA1562	*murC*	DNA translocase stage III sporulation protein homolog	****	0.85	0.02	<0.05
SA1026	*murD*	UDP-*N*-acetylmuramoylalanine–D-glutamate ligase	****	0.75	0.02	<0.001
SA0876	*murE*	UDP-*N*-acetylmuramoylalanyl-D-glutamate–2,6-diaminopimelate ligase	***	0.84	0.07	NS
SA1886	*murF*	UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanyl ligase	****	1.23	0.04	<0.01
SA1926	*murZ*	UDP-*N*-acetylglucosamine 1-carboxylvinyl transferase 2	****	2.34	0.09	<0.001
SA1251	*murG*	Undecaprenyl-PP-MurNAc-pentapeptide-UDPGlcNAc GlcNAc transferase	****	1.03	0.03	NS
SA0997	*murI*	Glutamate racemase	****	0.80	0.02	<0.001
SA1025	*mraY*	Phospho-*N*-muramic acid-pentapeptide translocase	****	0.52	0.02	<0.001
SA2057	*femX*/*fmhB*	FmhB protein	****	1.29	0.05	NS
SA1206	*femA*	Essential factor for expression of methicillin resistance	****	1.39	0.08	<0.01
SA1207	*femB*	FemB protein	****	1.03	0.05	NS
SA1024	*pbp1*	Penicillin-binding protein 1	****	0.91	0.03	<0.05
SA1283	*pbp2*	Penicillin-binding protein 2	****	1.61	0.06	<0.001
SA1381	*pbp3*	Penicillin-binding protein 3	****	0.91	0.04	<0.05
SA0598	*pbp4*	Penicillin binding protein 4	***	1.44	0.05	<0.001
SA0038	*mecA*	Penicillin binding protein 2A	****	0.39	0.02	<0.001
SA0592	*tagA*	Teichoic acid biosynthesis protein	***	0.80	0.02	<0.001
SA0595	*tagB*	Teichoic acid biosynthesis protein B	****	1.18	0.04	<0.05
SA0597	*tagD*	Teichoic acid biosynthesis protein D	***	1.03	0.02	NS
SA0594	*tagG*	Teichoic acid translocation permease protein	****	1.06	0.03	NS
SA0593	*tagH*	Teichoic acid translocation ATP-binding protein	****	0.98	0.03	NS
SA0596	*tagX*	Teichoic acid biosynthesis protein X	****	1.01	0.05	NS
SA0793	*dltA*	D-Alanine-D-alanyl carrier protein ligase	****	0.48	0.01	<0.001
SA0794	*dltB*	DltB membrane protein	****	0.57	0.03	<0.001
SA0795	*dltC*	D-Alanine-poly(phosphoribitol) ligase subunit 2	****	0.43	0.03	<0.001
SA0796	*dltD*	Poly D-alanine transfer protein	****	0.54	0.02	<0.001
Global or antibiotic cell wall regulators						
SA2145	*tcaB*	TcaB protein	***	0.93	0.04	NS
SA2480	*drp35*	Drp35	***	1.63	0.10	<0.05
SA1194	*msrA2*	Methionine sulfoxide reductase A	***	1.53	0.03	<0.001
SA1195	*msrR*	Peptide methionine sulfoxide reductase regulator MsrR	***	2.03	0.06	<0.001
SA1257	*msrA1*	Peptide methionine sulfoxide reductase A	****	2.05	0.06	<0.001
SA1256	*msrB*	Methionine sulfoxide reductase B	****	2.13	0.05	<0.001
SA2146	*tcaA*	TcaA protein	***	2.57	0.13	<0.001
SA2147	*tcaR*	TcaR transcription regulator	***	2.35	0.15	<0.001
SA1700	*vraR*	Two-component response regulator	****	3.54	0.14	<0.001

Continued on following page

TABLE 2—*Continued*

N315 SA no.	Gene	Description	Probe intensity	Ratio of signal intensity (14-4/MRGR3)		*P*
				Mean[a]	SEM	
SA1701	*vraS*	Two-component sensor histidine kinase	****	5.01	0.16	<0.001
SA1691	*sgtB*	Monofunctional glycosyltransferase	***	8.94	0.29	<0.001
SA1659	*prsA*	Peptidyl-prolyl *cis*/*trans* isomerase homolog	****	6.53	0.13	<0.001
SA1551	*sgtA*	Monofunctional glycosyltransferase	***	1.21	0.06	<0.05
SA0599	*abcA*	ATP-binding cassette transporter A	****	0.69	0.02	<0.001
SA1869	*sigB*	Sigma factor B	****	0.93	0.03	<0.05
SA1872	*rsbU*	Sigma B regulation protein RsbU	****	0.91	0.04	NS
SA1871	*rsbV*	Anti-sigma B factor antagonist	****	0.98	0.05	NS
SA1870	*rsbW*	Serine-protein kinase RsbW	****	0.93	0.05	NS
SA1984	*asp23*	Alkaline shock protein 23, Asp23	****	1.79	0.03	<0.01
SA0108	*sarS*	Staphylococcal accessory regulator A homologue	****	1.85	0.07	<0.001
SA0018	*yycG*	Two-component sensor histidine kinase	****	1.12	0.03	<0.05
SA0017	*yycF*	Response regulator	****	0.99	0.04	NS
SA1323	*srrA*	Staphylococcal respiratory response protein SrrA	****	1.18	0.01	<0.001
SA1322	*srrB*	Staphylococcal respiratory response protein SrrB	****	1.19	0.05	<0.05
SA0661	*saeR*	Response regulator	****	1.02	0.03	NS
SA0660	*saeS*	Histidine protein kinase	****	0.99	0.01	NS
SA1583	*rot*	Repressor of toxins Rot	***	1.14	0.05	NS
SA2287	*sarU*	Staphylococcal accessory regulator A homolog	*	0.92	0.06	NS
SA2286	*sarT*	Staphylococcal accessory regulator A homolog	**	1.21	0.08	<0.05
Other genes						
SA0614	*graR*	Hypothetical protein, similar to two-component response regulator	***	1.17	0.1	NS
SA0615	*graS*	Hypothetical protein, similar to two-component sensor histidine kinase	***	1.04	0.05	NS
SA0639	*graA*	Hypothetical protein, similar to ABC transporter for expression of cytochrome bd	****	0.97	0.04	NS
SA0743	*graB*	Hypothetical protein, similar to staphylocoagulase precursor	*	1.39	0.25	NS
SAS044	*graC*	4-Oxalocrotonate tautomerase	**	1.08	0.04	NS
SA1318	*graD*	Hypothetical protein	*	0.57	0.05	<0.05
SA1337	*graE*	Transcription regulator AraC/XylS family homolog	****	0.72	0.02	<0.001
SA1339	*malR*	Maltose operon transcriptional repressor	***	2.14	0.12	<0.001
SAS030	*graF*	Hypothetical protein	**	2.8	0.17	<0.05
SA0016	*purA*	Adenylosuccinate synthase	***	0.72	0.04	<0.05
SA1724	*purB*	Adenylosuccinate lyase	****	1.04	0.06	NS
SA0918	*purC*	Phosphoribosylaminoimidazolesuccinocarboxamide synthetase homolog	***	1.52	0.08	<0.001
SA0926	*purD*	Phosphoribosylamine–glycine ligase PurD	****	1.35	0.06	<0.05
SA0916	*purE*	Hypothetical protein, similar to phosphoribosylaminoimidazole carboxylase PurE	***	1.48	0.04	<0.001
SA0922	*purF*	Phosphoribosylpyrophosphate amidotransferase PurF	****	1.57	0.07	<0.001
SA0925	*purH*	Bifunctional purine biosynthesis protein PurH	****	1.4	0.08	<0.05
SA0917	*purK*	Phosphoribosylaminoimidazole carboxylase carbon dioxide fixation chain	***	1.46	0.12	<0.05
SA0921	*purL*	Phosphoribosylformylglycinamidine synthetase PurL	****	1.42	0.06	<0.05
SA0923	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase PurM	****	1.44	0.04	<0.001
SA0924	*purN*	Phosphoribosylglycinamide formyltransferase	****	1.29	0.04	<0.001
SA0920	*purQ*	Phosphoribosylformylglycinamidine synthase I PurQ	****	1.36	0.07	<0.05
SA0454	*purR*	*pur* operon repressor homologue	****	2.23	0.18	<0.05
Proteases						
SA1631	*splA*	Serine protease SplA	**	0.68	0.02	<0.001
SA1630	*splB*	Serine protease SplB	**	0.74	0.06	<0.05
SA1629	*splC*	Serine protease SplC	**	0.68	0.03	<0.001
SA1628	*splD*	Serine protease SplD	**	0.86	0.04	<0.01
SA1627	*splF*	Serine protease SplF	**	1.68	0.19	<0.01
SA0901	*sspA*	Cysteine protease/V8 protease	***	1.17	0.04	NS
SA0900	*sspB*	Cysteine protease precursor	**	1.70	0.06	<0.001
SA0899	*sspC*	Cysteine protease	*	0.94	0.07	NS
SA2430	*aur*	Zinc metalloproteinase aureolysin	**	0.92	0.06	NS
SA1725	*scpA*	Staphopain, cysteine proteinase	**	1.36	0.15	NS
SA1726	*scpB*	Hypothetical protein	**	1.50	0.03	<0.001
SA0879	*htrA*	Serine protease HtrA	***	0.50	0.03	<0.001

[a] Recorded in three independent experiments; see text for details.
[b] ****, 75th to 100th percentile; ***, 50th to 75th percentile; **, 25th to 50th percentile; *, <25th percentile (see Materials and Methods for details).
[c] NS, not significant.

isolates (16, 66). In contrast to those studies, we found no significant difference in transcript levels of *graR*, *graS*, *graA*, *graB*, and *graC* genes (1) and only minor changes in expression of 13 genes belonging to the purine biosynthetic operon compared to those previously reported apart from a twofold increase of the *pur* operon repressor *purR* (66) (Table 2). Interestingly, both previous microarray studies, but not a more recent one (102), demonstrated increased autolytic activity in vancomycin-intermediate isolates (16, 66) in contrast to our study.

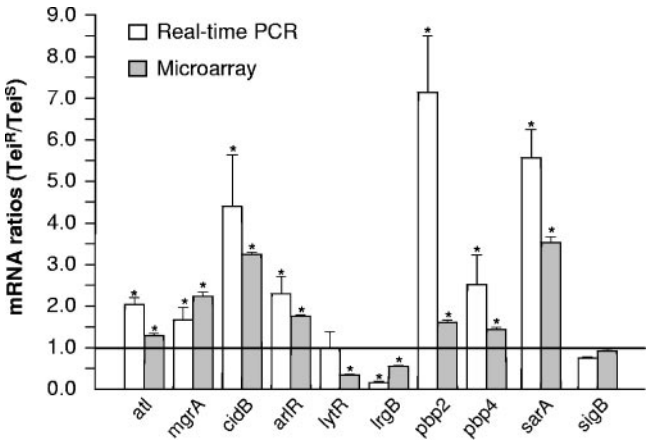

FIG. 4. Comparison of transcript level ratios recorded for 10 different genes in Tei^r strain 14-4 compared to Tei^s strain MRGR3 by real-time RT-PCR (white boxes) and DNA microarray (gray boxes). Results are presented as means plus SEM for three experiments. Comparative microarray and RT-PCR values (<5%) for RNAIII are too small to be shown on the graph. In most microarray data, error bars are too small to be shown on the graph. *, significant differences between strains MRGR3 and 14-4.

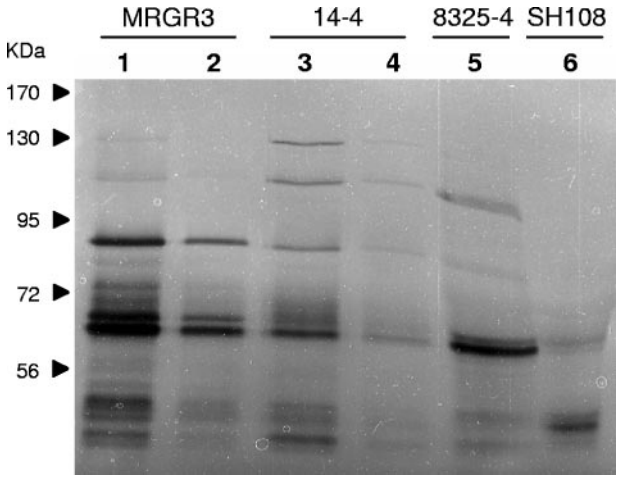

FIG. 5. Zymographic analysis of bacteriolytic hydrolase activities of concentrated supernatants of cultures of strains MRGR3 (Tei^s), 14-4 (Tei^r), 8325-4, and its *atl* mutant, SH108, against lyophilized *S. aureus* MRGR3 cells. Lanes 1 and 3, 5 μg of protein; lanes 2 and 4, 1.25 μg of protein; lanes 5 and 6, 20 μg of protein. Molecular size markers are indicated on the left. The data shown are from a single representative experiment and were reproduced several times.

Expression of extracellular proteases. Previous studies indicated a role for extracellular proteases in the processing of cell wall hydrolases (40, 77, 80). Extracellular protease activities are thought to be required for converting the preprotein *atl* gene product into a bifunctional enzyme (2, 69, 99) and may yield multiple autolytic cleavage products in the extracellular medium (22, 95). Another postulated function of extracellular proteases might be to attenuate or inactivate cell wall hydrolase activities (23, 108).

Transcript levels of 11 protease genes assayed by DNA oligoarray were quite low in both Tei^r and Tei^s strains except for *sspA*, which was more highly expressed (Table 2). The low expression of these proteases in either strain grown for 5 h fits with recent data recorded in our laboratory showing nearly undetectable levels of extracellular proteases in *S. aureus* grown in similar conditions (50). Previous reports indicate that production of extracellular proteases mainly occurs during later phases of bacterial growth (34, 40, 41). Altogether, the overall low transcript levels of protease-encoding genes, despite minor changes recorded in the Tei^r strain compared to Tei^s strain (Table 2), provided marginal evidence for their significant involvement in the autolytic changes of Tei^r strain 14-4.

Evaluation of extracellular bacteriolytic hydrolase activities. Since Tei^r strain 14-4 displays an autolysis-deficient phenotype without showing any significant decline in the expression of its autolysis-associated genes compared to its autolysis-proficient Tei^s parent, MRGR3, we investigated by zymographic analyses and quantitative bacterial hydrolytic assays whether this could result from any potential defect in posttranscriptional expression and/or processing of cell wall hydrolases by the Tei^r strain compared to its Tei^s parent. Figure 5 presents bacteriolytic hydrolase profiles of concentrated culture supernatants of strains MRGR3 and 14-4, compared to 8325-4 and its *atl* mutant, SH108, used as controls, assayed at different protein concentrations against lyophilized MRGR3 cells. The most prominent hydrolytic bands, with estimated molecular sizes of 86 kDa and 62 kDa in concentrated supernatants of strains MRGR3 and 14-4, and the concomitant presence of equivalent hydrolytic bands in strain 8325-4 but not its *atl* mutant, SH108, led to their tentative identification as Atl-derived products (2). The 86-kDa bacteriolytic band likely represented an intermediate Atl cleavage product, composed of *N*-acetylmuramyl-L-alanine amidase and two C-terminal (R1 and R2) repeat domains, and the 62-kDa band *N*-acetylmuramyl-L-alanine amidase (2). When compared at equivalent loading amounts, both 86-kDa and 62-kDa bacteriolytic bands were consistently qualitatively less intense in the Tei^r strain compared to the Tei^s parent, MRGR3, especially at the smaller loading amount (1.25 μg) of extracellular proteins (lanes 2 and 4). Besides the 86-kDa and 62-kDa cell wall-hydrolytic bands, there were also two higher-molecular-size hydrolytic bands that likely represented native pro-Atl (134 kDa) and an early 113-kDa Atl-processed product (2, 22). The fact that the 134-kDa and 113-kDa bands were more easily detected in strain 14-4 compared to MRGR3 suggested a less extensive rate of Atl processing by the Tei^r strain compared to the Tei^s strain. Nearly identical profiles and strain-specific differences were recorded in zymographic analyses performed in parallel against lyophilized 14-4 cells (not shown).

It is noteworthy that larger amounts (≥10 μg) of extracellular proteins of strain 8325-4 had to be loaded on gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis-based zymographic analysis, compared to MRGR3 to reveal closely matching Atl-derived bacteriolytic bands. Similar findings were also repeatedly observed with strain RN6390, another member of the NCTC8325 family (data not shown).

To extend the zymographic analysis, quantitative bacteriolytic assays of extracellular proteins of strains MRGR3 and 14-4 against lyophilized *S. aureus* suspensions were performed. Nearly identical results were obtained with concentrated su-

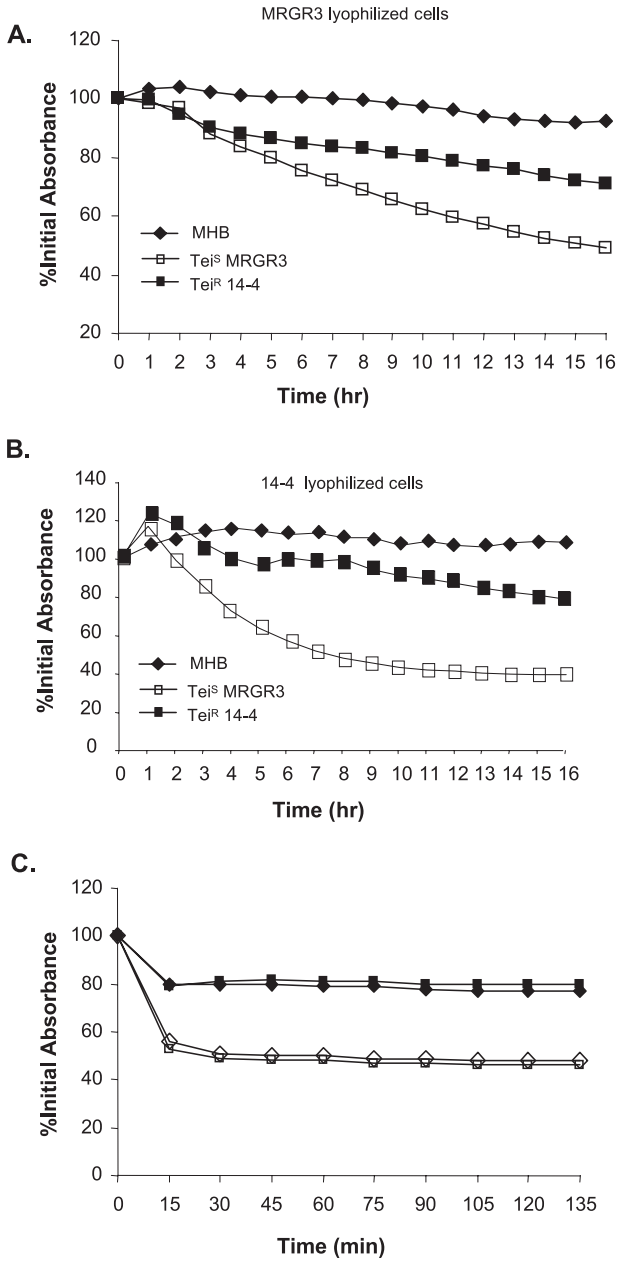

FIG. 6. A and B. Quantitative analysis of extracellular bacteriolytic hydrolase activities in concentrated supernatants of cultures of Tei^r strain 14-4 and Tei^s strain MRGR3. Extracellular proteins (100 μg) from either strain or MHB alone (used as a negative control) were added to heat-killed and lyophilized suspensions (1 mg/ml) of either strain MRGR3 (A) or 14-4 (B), and turbidity was monitored for 16 h. C. Lysostaphin susceptibilities of lyophilized (solid symbols) and viable (open symbols) cells of *S. aureus* MRGR3 (◇) and 14-4 (□) are shown. The data are from a single representative experiment and were reproduced several times.

pernatants (100 μg/ml) from either 5-h (not shown) or overnight cultures (Fig. 6). Extracellular proteins of strain MRGR3 caused a more significant decrease, compared to 14-4, in the turbidity of lyophilized *S. aureus* MRGR3 (Fig. 6A). Similar data were recorded with lyophilized *S. aureus* 14-4 (Fig. 6B). As anticipated from zymographic analyses of strains 8325-4 and SH108 (Fig. 5), the decrease of turbidity of lyophilized *S. aureus* by 100 μg/ml extracellular proteins from strain 8325-4 was low (<20%) compared to MRGR3, thus preventing detection of any significant difference from its *atl* mutant (data not shown). The identical susceptibilities of lyophilized substrates of 14-4 and MRGR3 to extracellular bacteriolytic hydrolase fractions from the same strains clearly indicated the absence of any significant change in their respective cell wall compositions that would contribute to the strain-specific autolytic differences. This finding is further supported by recently described observations showing identical muropeptide compositions in strains MRGR3 and 14-4 (57) and by the similar lysostaphin susceptibilities of Tei^r and Tei^s strains, whether tested in a nonviable lyophilized or viable state (Fig. 5C). Of note, significantly greater lytic effects were obtained against viable compared to nonviable cell suspensions of strains 14-4 and MRGR3.

DISCUSSION

GISA/hGISA clinical and laboratory isolates frequently exhibit a decreased autolytic activity compared to non-GISA isolates, thus potentially providing a selective advantage to drug-exposed bacterial subclones during emergence of glycopeptide resistance (7, 35, 43, 70, 71, 85, 91, 102, 108). Despite this repeated observation, the molecular links between increased glycopeptide tolerance, glycopeptide resistance, and autolysis are not yet elucidated (7, 108).

A majority of clinical and GISA/hGISA laboratory-derived isolates exhibit cell wall thickening (17, 18, 78, 89), which may significantly contribute to glycopeptide resistance (15–17, 31). Other properties of the cell wall thickening model are increased D-Ala-D-Ala residues in cell walls, forming false targets trapping glycopeptides, and a decrease in the level of muropeptide glutamine amidation (8, 18, 21, 29, 31, 43, 46, 67, 91–93), but these characteristics are not shared by all GISA clinical isolates, thus pointing to the multifactorial origin of GISA (8, 78). Furthermore, it is unclear whether a majority of clinical or laboratory strains of Tei^r *S. aureus* exhibit cell wall thickening as extensive as that seen in some VISA isolates.

The molecular basis of cell wall thickening is not well understood (16, 43, 109) and might involve complex regulatory mechanisms (63, 72, 93, 94, 102). Morphological characteristics of clinical and laboratory-derived GISA strains (29, 43, 46, 67, 78, 92, 93) are frequently shared with some autolysin-defective mutants of *S. aureus*, in particular the presence of cell surface roughness, cell thickening, and sometimes abnormal septum formation (22, 39, 46, 68, 99). Thus, cell wall thickening in GISA might not only result from activated cell wall synthesis as initially suggested (29) but also potentially reflect an imbalance between cell wall synthesis and impaired cell wall remodeling by cell wall hydrolases (8, 43, 93, 94, 102, 108).

Despite intensive investigations, a comprehensive model of cell wall remodeling and autolytic control by peptidoglycan hydrolases, including a complete list of genes and gene pathways controlling cell wall expansion and cell septum and daughter cell separation (27, 42, 65, 100), is not available. A number of genetic loci are reported to influence autolysis (*atl*, *sle1*, *lytM*, *lytN*, *lytSR*, *lrgAB*, *cidABC*, *alrRS*, *mgrA*, *agr*, *sarA*, *sarV*, and RNAIII) (2, 10, 11, 11, 23–25, 28, 37, 62, 81–83, 98,

99), but their mutual interaction and respective contributions to cell wall- and autolysis-controlling molecular processes are still poorly understood and may also be significantly altered by different strain backgrounds.

DNA microarray methods have been shown to be powerful tools for evaluating the impact of individual global regulator (*agr*, *sarA*, *sigB*, *rot*, *vraSR*, *arlRS*, and *mgrA*) mutations on expression levels of *S. aureus* genes (4, 20, 48, 51, 55, 84). While recent expression studies explored the molecular mechanisms of glycopeptide resistance (16, 63) and stress responses to cell wall-active antibiotics (48, 101), no extensive functional and transcriptomic study evaluated the regulation and global expression of cell wall hydrolases and autolytic factors in *S. aureus*.

The development and validation in one of our laboratories of a custom-designed oligoarray for gene expression studies (13) were instrumental for accurate evaluation of gene expression changes in isogenic Tei^r and Tei^s strains. Statistical analysis demonstrated significantly different expression levels for all genes whose transcript ratios were either >1.3 or <0.80 in the Tei^r strain compared to the Tei^s strain when more than one probe was available for each transcript. Moreover, the choice of appropriate isogenic strains, namely, Tei^r strain 14-4, which was initially selected in a rat tissue cage infection model and displayed a remarkably stable resistance level after only two in vitro passages, and its Tei^s MRSA parent, MRGR3, whose virulence has been repeatedly demonstrated in animal studies (103), was essential for recording reliable expression changes even when these strains were grown in antibiotic-free media. We previously demonstrated altered expression of virulence factors including upregulation of fibronectin-binding proteins by Tei^r strain 14-4 compared to its Tei^s parent, MRGR3 (79), and ongoing studies indicate that strain 14-4 can also induce experimental infection in a rat tissue cage model (unpublished data).

An interesting and provocative aspect of our gene expression study was the observation of undiminished (*atl*, *lytM*, and *lytN*) or even significantly (more than twofold) increased (*cidA*, *cidB*, and *cidC*) expression of major autolytic genes in the autolysis-deficient strain 14-4 compared to its autolytic parent, MRGR3. These data contrasted with two recent reports that stressed the reduced expression of the *atl* gene in laboratory or clinical GISA strains displaying autolysis-deficient phenotypes (43, 108). While the increased levels of some autolytic gene transcripts (*cidA*, *cidB*, and *cidC*) recorded in Tei^r strain 14-4 compared to Tei^s strain MRGR3 were consistent with the reduced expression of their negative regulators *lytSR* and *lrgAB* (10, 11, 25, 28, 81, 81, 83), their mechanisms of autolytic regulation are currently unknown (82). At least, our transcriptomic and RT-PCR data seem to rule out a significant role of the *cidABC* operon in the autolysis-deficient phenotype of Tei^r strain 14-4.

In contrast to the above-mentioned results, other global regulators such as *arlRS*, *mgrA*, and *sarA*, known as negative regulators of autolysis, may individually or in concert contribute to the autolysis-deficient phenotype of strain 14-4 compared to MRGR3. Furthermore, the strongly reduced expression of the positive autolytic regulator *agr*, known to be frequently downregulated in GISA (85) as well in strain 14-4 (79), may also play a significant role in the deficient autolysis of our Tei^r strain. Further work is required to understand the mutual interactions and respective contribution of those global regulators to the cell wall- and autolysis-controlling pathways (23, 24, 36, 37).

The potential contribution of the *sigB* operon was also carefully examined in our study since this regulon was previously described as one of the preferred mutation sites associated with first-step teicoplanin resistance in SigB-deficient *S. aureus* strains (3, 5). In view of the modest increase found in expression of the SigB-dependent *asp23* gene in Tei^r strain 14-4 compared to Tei^s strain MRGR3, and in line with a previous report (79), our data do not support a major role for this regulon in the expression of teicoplanin resistance and deficient autolysis by strain 14-4.

In contrast to the similar transcript levels of several global regulators (*yycFG*, *srrAB*, *saeRS*, *rot*, *sarU*, and *sarT*) recorded in Tei^r and Tei^s strains, other antibiotic-inducible regulators reported in previous microarray studies of antibiotic-exposed *S. aureus* cells (16, 48, 101), such as *drp35*, *msrA2*, *msrR*, *msrA1*, *msrB*, *tcaA*, *tcaR*, and especially *vraR* and *vraS*, were found upregulated in Tei^r 14-4 compared to Tei^s MRGR3. It should be stressed that overexpression of those antibiotic-inducible regulators, recently identified as a cell wall stress stimulon (101), occurred constitutively in Tei^r strain 14-4 compared to its Tei^s parent, especially in the absence of any antibiotic exposure. A recent microarray study comparing isogenic vancomycin-susceptible and VISA MRSA clinical isolates also described the constitutive overexpression of the cell wall stress stimulon in a VISA derivative that was selected after a 2-month period of extensive chemotherapy and whose vancomycin MIC reached 8 μg/ml (63). Nevertheless, the specific contribution of the cell wall stress stimulon to decreased glycopeptide susceptibility is difficult to evaluate due to the fact that this stimulon is also triggered in glycopeptide-susceptible strains exposed to vancomycin or other cell wall antimicrobial agents (16, 48, 63, 101). Site-specific mutagenesis combined with transcriptomic studies may help to more precisely define the individual as well as combined contributions of major stress cell wall stimulon components to the emergence of the GISA phenotype, as done for *vraRS* (49).

It should be mentioned that, despite some similarities in gene expression changes, the phenotypic properties of our isogenic Tei^r strain 14-4 differ in several aspects from the above-mentioned, late-stage VISA isolate (63, 89, 93), which was shown to display striking alterations in cell wall composition and morphology, including abnormally thick cell walls, markedly decreased growth rate, and formation of multicellular aggregates during growth in antibiotic-free liquid medium (93). In contrast, the vancomycin-susceptible (MIC: 4 μg/ml) but Tei^r strain 14-4 used for our study showed minor differences in growth rate and no evidence of multicellular aggregates, which fit with its identical peptidoglycan cell wall composition compared to its Tei^s parent (57). Furthermore, ongoing transmission electron microscopy studies failed to detect any major alteration in either cell wall thickness or septum formation for strain 14-4 compared to MRGR3 (unpublished data).

Our study also revealed the lack of significant differences in expression of major genes (*ftsA*, *ftsZ*, and *scdA*) coding for regulators of cell wall septum and daughter cell formation (12,

45, 73, 74). Thus, despite the likely occurrence of common molecular pathways contributing to decreased susceptibility to both teicoplanin and vancomycin, some glycopeptide-specific resistance phenotypes should also be considered. One argument for glycopeptide-specific resistance mechanisms is provided by the relatively easy in vitro selection of teicoplanin-resistant derivatives by one or two passages (38), while selection of stable VISA subclones seem to require a larger number of in vitro passages (38) and a potentially higher fitness cost (63, 89, 93). This may indicate that a larger number of mutational and regulatory changes are required for emergence of isolates displaying decreased susceptibility to vancomycin compared to teicoplanin.

The mechanisms of the autolysis-deficient phenotype of strain 14-4 were found to be linked with a decreased content of extracellular bacteriolytic hydrolase activities, as shown by zymographic analyses and quantitative bacteriolytic activities of concentrated supernatants from the Tei^r strain compared to its Tei^s parent against lyophilized suspensions of *S. aureus*. Further studies are required to elucidate the molecular components of the decreased autolytic bacteriolytic hydrolases and the altered rate of Atl processing by the Tei^r strain compared to the Tei^s strain. The relatively low extracellular bacteriolytic activity of strain 8325-4 and other members of the 8325 family such as RN6390 (not shown) compared to Tei^s strain MRGR3 remains unexplained and would deserve further studies.

In conclusion, our results suggest that altered autolytic properties in 14-4 are apparently not driven by significant changes in the transcription of key autolytic effectors. Instead, our analysis points to alternate regulatory mechanisms that impact autolysis effectors, which may include changes in posttranscriptional processing or export. Microarray studies offer a unique opportunity not only to evaluate global gene expression changes associated with resistance phenotype but also to compare such changes in different backgrounds or lineages of susceptible and resistant *S. aureus* isogenic pairs. Detailed comparison of global gene regulation in isolates from different clinically and epidemiologically relevant clonotypes of susceptible and resistant *S. aureus* strains may also bring key information for a deeper understanding of the molecular basis of virulence expression in this highly important nosocomial and community-acquired pathogen.

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