Cation-Induced Transcriptional Regulation of the *dlt* Operon of *Staphylococcus aureus*

Tomaz Koprivnjak,^{1,3} Vid Mlakar,⁵ Lindsey Swanson,^{1,2} Benedicte Fournier,⁶ Andreas Peschel,⁷
and Jerrold P. Weiss^{1,2,3,4*}

Inflammation Program¹ and Departments of Microbiology² and Internal Medicine,³ Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, and Veterans Administration Medical Center,⁴ Iowa City, Iowa 52242; The University of Ljubljana, 1000 Ljubljana, Slovenia⁵; Laboratoire des Listeria, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France⁶; and Cellular and Molecular Microbiology, Department of Medical Microbiology and Hygiene, University Hospitals Tübingen, 72076 Tübingen, Germany⁷

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Lipoteichoic and wall teichoic acids (TA) are highly anionic cell envelope-associated polymers containing repeating polyglycerol/ribitol phosphate moieties. Substitution of TA with D-alanine is important for modulation of many cell envelope-dependent processes, such as activity of autolytic enzymes, binding of divalent cations, and susceptibility to innate host defenses. D-Alanylation of TA is diminished when bacteria are grown in medium containing increased NaCl concentrations, but the effects of increased salt concentration on expression of the *dlt* operon encoding proteins mediating D-alanylation of TA are unknown. We demonstrate that *Staphylococcus aureus* transcriptionally represses *dlt* expression in response to high concentrations of Na⁺ and moderate concentrations of Mg²⁺ and Ca²⁺ but not sucrose. Changes in *dlt* mRNA are induced within 15 min and sustained for several generations of growth. Mg²⁺-induced *dlt* repression depends on the ArlSR two-component system. Northern blotting, reverse transcription-PCR, and SMART-RACE analyses suggest that the *dlt* transcript begins 250 bp upstream of the *dltA* start codon and includes an open reading frame immediately upstream of *dltA*. Chloramphenicol transacetylase transcriptional fusions indicate that a region encompassing the 171 to 325 bp upstream of *dltA* is required for expression and Mg²⁺-induced repression of the *dlt* operon in *S. aureus*.

The cell envelope provides a protective barrier and regulates communication between the inside and outside of the cell. In nonencapsulated gram-positive bacteria, the cell envelope consists of the cytoplasmic membrane and cell wall peptidoglycan layers, to which proteins and teichoic acids (TA) are covalently linked. The covalently linked wall teichoic acid (WTA) and cell membrane-anchored lipoteichoic acid (LTA) contain repeating units of ribitol or glycerol-phosphate (29). Together, TA represent the most abundant polyanions of the gram-positive bacterial cell envelope (8). The charge properties of these polymers can be modified by substitution for the ribitol or glycerol-phosphate units by glycosyl substituents or D-alanine esters (29). As judged by comparison of wild-type and mutant strains, esterification of D-alanine to TA has pleiotropic effects, including effects on regulation of the activity of autolytic enzymes (33, 42), binding of cations (e.g., Na⁺, Mg²⁺, and Ca²⁺) to the cell envelope (16, 26, 35), resistance to many antimicrobial cationic peptides/proteins (23, 32), and virulence (4). D-Alanylation of TA also promotes coaggregation, biofilm formation, and adhesion of bacteria on artificial surfaces (3, 13). In addition, D-alanylation increases the proinflammatory activity of LTA (5).

An operon, including *dltABCD*, is necessary for D-alanylation of both LTA and WTA in *Staphylococcus aureus*. D-Alanyl carrier protein ligase (Dcl; *dltA*) activates D-alanine

using ATP. With assistance of DltD (*dltD*), this activated complex is delivered to the D-alanine carrier protein (Dcp) encoded by *dltC*. DltB (*dltB*) is predicted to be a transmembrane protein and is thought to be involved in passage of the D-alanyl-Dcp complex across the cytoplasmic membrane, where D-alanine is transferred to the glycerol phosphate backbone of LTA (29). D-Alanine esterified to LTA is the precursor for D-alanylation of WTA (15).

Relatively little is known about the regulation of D-alanylation of TA in *S. aureus*. It is known that the degree of D-alanylation of TA varies depending on several different environmental factors such as pH, temperature, and salt (e.g., NaCl) concentration (16, 19, 28). An increase in pH, temperature, or NaCl concentration lowers the degree of D-alanylation of TA. For example, the degree of D-alanylation of LTA is 0.77 mol D-alanine/mol glycerol-phosphate when bacteria are grown under low-salt conditions (0.2% NaCl) but decreases to only 0.3 mol D-alanine/mol glycerol-phosphate when bacteria are grown in medium containing much higher (7.5% NaCl) salt concentration (22). The effect of NaCl on D-alanylation of LTA is reversible (22). These findings indicate that the bacteria can respond to changes in the environment by adjusting the amount of D-alanine esterified to TA.

D-Alanylation of TA could be modulated by regulating the abundance or activity of the proteins encoded by the *dlt* operon. In vitro regulation by salt of the carrier protein (Dcp) has been demonstrated, with increasing concentrations of NaCl favoring hydrolysis of D-alanine from LTA and Dcp rather than D-alanylation of LTA (21). The properties of Dcp in vitro are consistent with the changes in D-alanylation of LTA

* Corresponding author. Mailing address: Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Oakdale Research Campus, 2501 Crosspark Road, Coralville, IA 52241. Phone: (319) 335-4268. Fax: (319) 335-4194. E-mail: jerrold-weiss@uiowa.edu.

TABLE 1. Primers used in this study

Primer	Forward sequence	Reverse sequence
<i>cat</i> constructs ^a		
–599 (BamHI)	CGAGGATCCCGCACATACTCCAGTAAA	
–518 (HindIII)	CGATACAAGCTTTTGACTTGCGAAT	
–363 (HindIII)	CGATACAAGCTTGATGTTCTCAACA	
–325 (HindIII)	CGATACAAGCTTTGACAACCAA	
–217 (HindIII)	CGATACAAGCTTGAACGGTCTTTAT	
–105 (HindIII)	CCGTCCAAGCTTACACTATTGTAT	
–465 (HindIII)	AAGCTTCTCGTCATATTAATG	
+137 (EcoRI)		CGGAATTCCCTTGTAATCGATGTGCT
–12 (EcoRI)		CGAGACGAATTCTCATTAGAACTC
–49 (EcoRI)		GAATTCTCGCCACTGCCATAAATTA
–88 (EcoRI)		GGAATTCGCCAAATACAATAGTGTTA
–171 (EcoRI)		GAATTCGCACCTCTTAAAGTTCCTAGT
–393 pdlT _{Sx} -US	GCAAAATTATCTAAGAATAAACAAATGTTTTGG	
–3 pdlT _{Sx} -DS		CCTTCATAGAATTCTCCTCGACTAAAATTC
Real-time RT-PCR		
<i>dltA</i>	CACAGAGCAGCAAAAGCGTTAG	ACATATGGTCCAACCTGAAGCTACG
<i>dltD</i>	TGACCCATTTAATCCTGCAATTG	TCTGTAGAACCACCAGCACCTAATAA
<i>gapdh</i>	TACACAAGACGCACCTCACAGA	ACCTGTTGAGTTAGGGATGATGGT
16S rRNA	AGCCGACCTGAGAGGGTGA	TCTGGACCGTGTCTCAGTTCC
<i>cat</i>	GAAATTTATCCTTCTTTGATTTATGCA	CCTGTTCTAAACACTTTATTTTATTACAACCTTC
Northern blot probes		
1 (–599/–341)	CACATACTCCAGTAAATTGAAAATAAG	GATACACTTTGTTGAGAACATCATTCTAT
2 (–217/–31)	TATATTGAACGGTCTTTATAAAGGCG	ATGAAGTTATTGTGTGTGTGCGCC
3 (+178/+876)	ATATGATTGTTGGGATGATTGGTGCCA	ACATATGGTCCAACCTGAAGCTACG
4 (+2603/+3227)	AGCATTGTGATTACATTCCACTTTGT	TCTGTAGAACCACCAGCACCTAATAA
SMART RACE		
analysis		
TK42 (+959)		GCGCTTGGTCTTTCAACGCCAACAGGT
TK43 (+204)		TGGCACCAATCATCCCAACAATCATAT

^a DNA region (in bp), relative to *dltA* translation start site that is fused to *cat* gene. See also Fig. 6. Restriction sites are underlined.

that are observed in bacteria grown under increasing NaCl concentration.

In addition, transcriptional regulation of *dlt* has been shown. In *Bacillus subtilis*, the *dlt* operon is part of the σ^x regulon and is regulated by the global transcriptional regulators AbrB and Spo0A (31). In *Streptococcus agalactiae* a two-component system, *dltRS*, that is part of the *dlt* operon is presumably involved in transcriptional regulation of *dlt* expression (34). In *S. aureus*, transcription profiling studies have demonstrated increased *dlt* mRNA in an accessory gene regulator (*agr*) mutant and decreased *dlt* mRNA in a *rot* (repressor of toxins) mutant, suggesting a role for the *agr* and *rot* global regulators in negative and positive regulation of *dlt*, respectively (7, 36). However, the ability of *S. aureus* to modify transcription of *dlt* in response to changes in salt (cation) concentration has not been previously examined.

In the present study, we show that expression of the *dlt* operon in *S. aureus* is acutely repressed by increases in monovalent and, especially, divalent cations. Transcriptional regulation is dependent on *cis* elements located between 325 and 171 bp upstream of the first codon of *dltA* and is partly dependent on the ArlSR two-component system. Our findings indicate that the initiation of transcription begins at 250 bp upstream of the *dltA* ATG and that the transcript produced includes a conserved open reading frame (ORF) immediately upstream of *dltA* that is part of the *dlt* operon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Staphylococcus aureus* strains used in this study were as follows: SA113, SA113 *dltA::spec* (32), RN6390, BF21 (RN6390 *arlR::cat*), BF22 (RN6390 *arlR::cat* complemented) (10), and SH1000 (8325-4 *rsbU*⁺) (18). *Escherichia coli* DH5 α T1^R (Invitrogen) as well as stationary-phase overnight cultures of *S. aureus* strains were grown in LB broth with appropriate antibiotics. *S. aureus* bacteria were subcultured in basal medium (RPMI medium [Gibco] supplemented with 10 mM HEPES [Cellgro], 1 mM CaCl₂, and 1% bovine serum albumin) with a starting optical density at 550 nm (OD₅₅₀) of 0.05.

DNA preparation techniques. PCR was performed using *Taq* Gold polymerase (Roche) or the BD Advantage PCR 2 enzyme system (BD Biosciences). The primers used are listed in Table 1. PCR products were cloned into pCR-4 TOPO linearized vector (Invitrogen), digested with appropriate restriction endonucleases (New England Biolabs), and ligated into linearized pRB 594 (30) shuttle vector using a quick ligation kit (New England Biolabs). Plasmids were transformed into the CaCl₂-competent *E. coli* according to TOPO TA cloning kit instructions (Invitrogen). Plasmid DNA was isolated with the QIAGEN miniprep kit. *S. aureus* SA113 was transformed with plasmid DNA by electroporation (37).

Real-time reverse transcription (RT)-PCR. *S. aureus* at 5×10^7 bacteria/ml was incubated at 37°C in basal medium or basal medium supplemented with NaCl, CaCl₂, MgCl₂, or sucrose. Two volumes of RNAProtect bacterial reagent (QIAGEN) were added prior to isolation of total RNA using an RNeasy minikit (QIAGEN) according to the manufacturer's instructions with a modified bacterial lysis step. *S. aureus* bacteria were lysed by the addition of 50 μ g/ml of lysostaphin (Sigma) in Tris-EDTA buffer, pH 8. Contaminating DNA in each sample was eliminated by incubation with 30 U of on-column DNase I treatment (QIAGEN). Total RNA was reverse transcribed using AMV-RT (Roche), 20 ng/ μ l random hexamers (Roche), and 20 U RNase inhibitor (Roche) at 40°C for 1 h. The PCR was set up with SYBR green (Applied Biosystems), 200 nM of each

FIG. 1. Effects of MgCl_2 , CaCl_2 , NaCl , or sucrose on *dlt* mRNA. *S. aureus* organisms at 5×10^7 bacteria/ml were incubated in basal medium with or without indicated supplements for 15 min. Levels of *dlt* mRNA were measured by real-time RT-PCR as described in Materials and Methods, expressed relative to 16S rRNA, and normalized to the control sample incubated in basal medium alone. Data shown represent means $\pm$ standard errors of the means (SEM) of triplicate determinations from one (0.325 M and 0.65 M NaCl supplements [A]), two (sucrose [A] and Ca^{2+} [C] supplements), or five independent experiments.

forward and reverse primer (Table 1), and cDNA equivalent to 4 ng of input RNA. The reactions were performed with an ABI PRISM 7000 detection system (Applied Biosystems). In each run, standard curves for each primer pair were run simultaneously with samples and used to determine relative numbers of amplicons.

Chloramphenicol acetyltransferase assay. *S. aureus* bacteria bearing pRB 594 (30), encoding transcriptional fusions of promoterless *cat* and different lengths of the putative *S. aureus dlt* regulatory region, were grown in basal medium or basal medium supplemented with 10 mM or 50 mM MgCl_2 until reaching an OD_{550} of ~ 0.3 to 0.5 . Bacterial pellets were washed with 20 mM Tris-HCl, pH 7.8. Cell extracts were obtained from 5×10^8 bacteria disrupted by 0.1-mm zirconia-silica beads (Biospec Products). The beads were removed by centrifugation ($80 \times g$ for 2 min). The activity of chloramphenicol acetyltransferase (CAT) in cell extracts was measured as previously described (30). Units of CAT activity were calculated from a standard curve with purified CAT (Sigma) and normalized per microgram protein in each sample as determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

Northern blotting. Total RNA was isolated from *S. aureus* grown in basal medium until reaching an OD_{550} of ~ 0.5 . RNA (15 μg) was denatured, separated by electrophoresis, and transferred to positively charged nylon membranes (Roche) according to the NorthernMax-Gly protocol (Ambion). Hybridization of the blots with digoxigenin (DIG)-labeled probes and subsequent washes were performed using solutions provided with the NorthernMax-Gly kit (Ambion). DIG probes were synthesized by PCR using the reagents and cycling conditions supplied with the PCR-based DIG probe synthesis kit (Roche) and the primers listed in Table 1. Hybridized probes were detected using the buffers and reagents of the DIG luminescent detection kit (Roche).

Determination of initiation of transcription. SMART-RACE technology (BD Biosciences) was used to determine the initiation site of *dlt* transcription as described previously (39), with some modifications. Total RNA isolated from *S. aureus* grown in basal medium was reverse transcribed using random hexamers and BD PowerScript RT according to the manufacturer's recommendations. BD PowerScript RT exhibits terminal transferase activity by adding three to five residues of predominately dC to the 3' end of the first-strand cDNA. This allows annealing of the BD SMART oligonucleotide that contains a terminal stretch of G residues and serves as an extended template for the RT. BD PowerScript RT switches templates from RNA to BD SMART oligonucleotides, generating a complete cDNA copy of the original RNA with BD SMART sequences at the end. Since the dC tailing activity of RT is most efficient if the enzyme has reached the end of the RNA template, the BD SMART sequence is principally found at the end of complete first-strand cDNAs. RACE-ready *dlt* cDNA was amplified using primer TK42 and universal primer mix (BD Biosciences) or nested primer TK43 and nested universal primer mix by the BD Advantage 2 PCR system (Table 1) (BD Biosciences). To increase the specificity of amplification, touchdown PCR was performed as recommended by the manufacturers. PCR products were separated by electrophoresis on a 1% agarose (wt/vol) gel and purified by a NucleoTrap gel extraction kit (BD Biosciences) before cloning into pCR-4 TOPO linearized vector (Invitrogen) for sequencing. Plasmids were isolated from 16 transformants that contained DNA inserts. The sequences and the lengths of the inserts from *dltA* ATG were determined by sequencing using primer TK42 (Table 1).

RESULTS

Level of *dlt* mRNA changes in response to increasing concentration of Na^+ , Mg^{2+} , and Ca^{2+} . Because growth of *S. aureus* in medium containing high concentrations of NaCl results in synthesis of LTA with reduced D-alanine content (16, 22), we hypothesized that this effect of NaCl might include repression of expression of the *dlt* operon. As an initial test of this hypothesis, we used real-time PCR to measure mRNA from both 5' (*dltA*) and 3' (*dltD*) ends of the *dlt* operon. *S. aureus* acutely (within 15 min) responded to increased concentrations of NaCl by decreasing the level of *dltD* (Fig. 1A) and *dltA* (not shown) mRNA. In contrast, 0.6 M sucrose had little effect on *dltD* mRNA levels (Fig. 1A, last bar), indicating that the effects of NaCl were due to electrolytic and not osmotic effects. Inhibitory effects on *dltD* mRNA levels were also induced by MgCl_2 (Fig. 1B) and CaCl_2 (Fig. 1C) but at 10- to 30-fold lower concentrations than that needed for NaCl, indicating that the effects were due to the added presence of cations and not chloride. Effects of Mg^{2+} on *dltD* mRNA levels were sustained for at least several generations (data not shown).

Mg^{2+} -induced reduction of *dlt* mRNA reflects repression of transcription. The cation-induced reduction in levels of *dlt* mRNA could reflect repression of *dlt* expression or a decrease in *dlt* mRNA stability. To distinguish between these two possibilities, we made use of an available plasmid (pRB 594dlt_{sc}) encoding a transcriptional fusion between a promoterless chloramphenicol acetyltransferase gene (*cat*) and the putative *dlt* regulatory region of *Staphylococcus xylosus* (i.e., -3 to -393 bp from the *dltA* start codon). A plasmid pRBdlt1 (32) with cloned *S. xylosus dltABCD*, including 393 bp upstream of the *dltA* start codon and 185 bp downstream of the *dltD* stop, can complement the *S. aureus dlt* operon, suggesting that this region in *S. xylosus* contains all the necessary elements for transcription and function of the *dlt* operon (32). In addition to CAT activity, we measured both endogenous *dltD* and plasmid-encoded *cat* mRNA after incubation of bacteria in basal medium (~ 0.5 mM Mg^{2+} , ~ 0.5 mM Ca^{2+} , ~ 5 mM K^+ , ~ 130 mM Na^+) or medium supplemented with 10 mM or 50 mM MgCl_2 (Fig. 2). Addition of MgCl_2 induced an acute (15-min) reduction in endogenous *dltD* mRNA levels that was virtually the same in the wild-type and plasmid-bearing (pRB 594dlt_{sc}) *S. aureus* (Fig. 2A), indicating that regulation of the endoge-

FIG. 2. Mg^{2+} -induced repression of *dltD* mRNA, *cat* mRNA, and CAT activity. (A) *dltD* mRNA and *cat* mRNA were measured by real-time RT-PCR after incubation of 5×10^7 *S. aureus* (with or without pRB 594dlt_{Sx}) bacteria/ml for 15 min in basal medium with or without $MgCl_2$ as indicated. mRNA levels are expressed as described in the legend to Fig. 1. Note that pRB 594dlt_{Sx} contains the region -3 to -393 bp from the *dltA* structural gene and *dltD* is present only in chromosomal genomic DNA. (B) CAT activity was measured after growth of *S. aureus* SA113 pRB 594dlt_{Sx} in basal medium with or without $MgCl_2$ as indicated. CAT activity is expressed in units of enzyme activity/microgram protein and normalized to control. Data shown represent the means $\pm$ SEM of two or three experiments, each done in triplicate.

nous *dlt* operon was not affected by the presence of pRB 594dlt_{Sx}, *cat* mRNA levels were similarly reduced by Mg^{2+} treatment, indicating that Mg^{2+} -induced effects were most likely mediated by repression of *dlt*-directed transcription and not alteration of *dlt* mRNA stability. Parallel Mg^{2+} -induced changes in CAT activity were observed (Fig. 2B) consistent with effects of Mg^{2+} on *dlt*-directed gene expression.

The amount of D-alanine in TA does not serve as a signal for *dlt* regulation. The ability of increased concentrations of NaCl to promote net release of D-alanine from LTA (21) raised the possibility that the signal for decreased *dlt* transcription could be reduced amounts of esterified D-alanine in TA. If so, we would expect that *dlt* mRNA levels would be constitutively low in *dltA* strains unable to D-alanylate TA and hence not further repressed by added cations (Na^+ or Mg^{2+}). To test this hypothesis we measured *dltD* mRNA in wild-type and *dltA* *S. aureus* after incubation of the bacteria in the basal medium with or without added NaCl or $MgCl_2$. Because an insertion of the spectinomycin resistance cassette within *dltA* was nonpolar, we were able to measure *dltD* mRNA in *dltA* *S. aureus* (Fig. 3A). As shown in Fig. 3, levels of *dltD* mRNA in wild-type and *dltA* *S. aureus* incubated in basal medium were similar. Incubation with an increased concentration of Na^+ (Fig. 3B) or Mg^{2+} (Fig. 3A) repressed levels of *dltD* mRNA at least as much in the mutant strain. These findings indicate that esterification or release of D-alanine in LTA/WTa has little or no effect on the transcription of the *dlt* operon.

ArlSR is needed for maximal repression of the *dlt* operon. The ability of Mg^{2+} to repress expression of the *dlt* operon suggested that an Mg^{2+} sensor/ Mg^{2+} -regulating system could be important in the transcriptional regulation of the *dlt* operon in *S. aureus*. To date, such a regulatory system has not been described for *S. aureus*. In *Streptococcus pyogenes*, the CsrRS two-component system has been shown to negatively regulate expression of capsular genes in response to Mg^{2+} (14). We therefore performed a BLAST search to search for CsrRS

FIG. 3. Effects of $MgCl_2$ and NaCl on *dlt* mRNA of wild-type and *dltA* *S. aureus*. Bacteria were incubated for 60 min at 37°C in basal medium alone or supplemented with $MgCl_2$ (A) or NaCl (B) before harvesting mRNA for real-time RT-PCR analyses. Levels of *dlt* mRNA are expressed as described in the legend to Fig. 1. Note that in panel A, the *dlt* mRNA levels are not normalized to the sample grown in basal medium alone but, in each sample, expressed relative to 16S rRNA. Data shown represent the means $\pm$ SEM of two experiments, each in triplicate.

homologues in *S. aureus*. We found that in *S. aureus*, ArlR is most closely related to CsrR, exhibiting 52% identity and 68% similarity, and that ArlS exhibits 31% identity and 51% similarity to CsrS. In addition, it has been recently shown that ArlSR is involved in regulation of protein A expression in response to high NaCl concentration (9). In order to test the hypothesis that ArlSR is involved in the negative regulation of the *dlt* operon, we measured changes of *dltD* mRNA in response to Na^+ , Mg^{2+} , and Ca^{2+} in wild-type, *arlRS*, and complemented strains (10). Mg^{2+} -, Ca^{2+} -, and Na^+ -induced repression of *dlt* transcription (i.e., reduced levels of *dltD* mRNA) was diminished in *arlRS* compared to the wild-type and complemented strains (Fig. 4). These data demonstrate a role for ArlSR in cation-induced repression of the *dlt* operon. However, there was still some Na^+ -/ Mg^{2+} -/ Ca^{2+} -induced repression of the *dlt* operon in *arlRS* *S. aureus* (Fig. 4) suggesting involvement of an additional, yet unidentified, regulatory factor(s).

Normal σ^B function is not needed for Mg^{2+} -induced repression of *dlt* expression. Bacteria respond to many extreme environmental stimuli through alternative sigma factors (2, 17). In *S. aureus*, σ^B influences expression of genes involved in general stress responses as well as genes involved in virulence (12, 24, 25, 38). Each of the strains used in the experiments

FIG. 4. Effects of $MgCl_2$, $CaCl_2$, and NaCl on *dlt* mRNA levels in wild-type, *arlRS*, and complemented *S. aureus*. Bacteria were incubated as indicated at 37°C for 15 min. Levels of *dlt* mRNA were measured by real-time RT-PCR assays. Levels of *dlt* mRNA are expressed as described in the legend to Fig. 1. Data shown represent the means $\pm$ SEM of one to three experiments, each in triplicate. wt, wild type; ND, not determined.

FIG. 5. Effect of MgCl₂ on *dlt* mRNA in *S. aureus* SH1000 (8325-4 *rsbU*⁺). Bacteria were incubated as indicated at 37°C for 15 min. Levels of *dlt* mRNA were measured by real-time RT-PCR assay. Levels of *dlt* mRNA are expressed as described in the legend to Fig. 1. Data shown represent the means $\pm$ SEM of two experiments, each in triplicate.

described above was derived from *S. aureus* NCTC 8325. Thus, each strain has a deletion in *rsbU* and is, therefore, σ^B defective (24). To test whether cation-induced *dlt* repression is influenced by σ^B , we repeated selected experiments with the *rsbU*-repaired strain, SH1000. Similar Mg²⁺-induced *dlt* repression was observed in SH1000 (Fig. 5) as in *S. aureus* 8325 (Fig. 1B), suggesting that Mg²⁺-induced *dlt* repression is independent of this alternative σ^B factor.

Identification of DNA region needed for expression and repression of the *dlt* operon. Mg²⁺-induced repression of *dlt* expression suggests the involvement of an Mg²⁺-activated

repressor and/or an Mg²⁺-inactivated activator. Nothing is known about the promoter or *cis* elements (activator or repressor binding site[s]) in the *S. aureus dlt* operon. To better define the molecular basis of transcriptional regulation of the *dlt* operon in *S. aureus*, we constructed a series of plasmid-carried reporter *cat* constructs containing different portions of the *S. aureus* genomic region upstream of *dltA* (Fig. 6) extending from +137 bp to -599 bp from the *dltA* start codon (all numbers are relative to the *dltA* start codon). We measured CAT activities of *S. aureus* strains bearing different constructs after growth under low (basal medium) or high (50 mM) Mg²⁺ conditions. Wild-type *S. aureus* without a plasmid construct served as the negative control while the strain bearing the largest DNA insert (-599 to +137) served as the positive control for *dlt*-driven CAT expression. The inhibitory effect of Mg²⁺ on CAT activity expressed by this insert was closely similar to the magnitude of Mg²⁺-induced repression of *dlt* expression in wild-type *S. aureus*, suggesting that this DNA region contains the *cis* elements needed for cation-induced transcriptional regulation of *dlt* expression. Truncations of the DNA insert from the 5' end (-599) to -325 or from the 3' end (+137) to -171 bp from the *dltA* ATG had little effect on either CAT expression or Mg²⁺-induced repression compared to the -599- to +137-bp DNA insert (Fig. 6). However, further trimming from the 5' end to 217 bp upstream of the *dltA* ATG almost completely eliminated CAT expression under low Mg²⁺ conditions (Fig. 6). This precluded meaningful assay of Mg²⁺-induced repression of CAT expression in this plasmid. However, the retention of Mg²⁺-induced repression in the other plasmid constructs indicates that the DNA region be-

FIG. 6. CAT activity of transcriptional fusions between the promoterless *cat* reporter gene and putative *S. aureus dlt* regulatory regions. The DNA region (in base pairs) that is fused to the *cat* gene, relative to the *dltA* translation start site, is schematically represented on the left. CAT activity (units/microgram protein of bacterial lysate) was measured under low- or high-Mg²⁺ conditions as described in Materials and Methods. Expression (*n*-fold) was calculated as CAT activity (low Mg²⁺)/CAT activity (high Mg²⁺) and expressed relative to the CAT activity under low-Mg²⁺ conditions of plasmid-free SA113 in each experiment. Data are means of two to six (column N) experiments $\pm$ SEM.

FIG. 7. The indicated ORF is part of the *dlt* operon of *S. aureus* SA113. (A) Alignment of *dltABCD* with the upstream ORF, the transcription initiation site of polycistronic *dlt* mRNA (−250), and the probes used for Northern blot analysis. The numbering of nucleotides is relative to the first codon of *dltA*. (B) Northern blot analysis of total RNA isolated from *S. aureus* after growth in basal medium and probed with probes 1 (lane 1), 2 (lane 2), 3 (lane 3), and 4 (lane 4). (C) RT-PCR analysis using primers within *dltA* (+204) and either 217 (−217) or 599 (−599) bp upstream of *dltA*. Genomic DNA (gDNA) served as a positive control for the primers and RT− as a control for DNA contamination in cDNA samples. (D) Sequence upstream of *dlt* transcription start site. +1 corresponds to −250 bp from the *dltA* structural gene. The predicted −35 and −10 sites are underlined.

tween 325 and 171 bp upstream of the *dltA* translation initiation codon is needed both for activation of *dlt* transcription and for Mg^{2+} -induced inactivation/repression of transcription of the *dlt* operon.

An ORF upstream of *dltA* is part of the *dlt* operon. Between this regulatory region (−325 to −171) and the *dltA* initiation codon is an additional open reading frame located between −168 and −15 bp from the *dltA* initiation codon (Fig. 7A). To determine whether this ORF is part of the *dlt* operon, we generated several DNA probes (Fig. 7A) to regions of genomic DNA upstream of the ORF (probe 1), or within the ORF, *dltA*, or *dltD* (probes 2, 3, and 4, respectively). If the ORF is part of the *dlt* operon, there should be a single transcript reactive with probes 2, 3, and 4 but not with probe 1. A Northern blot revealed an mRNA species ~4.7 kb in length reactive with probes 2, 3, and 4 but not probe 1 (Fig. 7B). This finding suggests the presence of a polycistronic mRNA that includes the ORF as well as *dltABCD*, consistent with the ORF being part of the *dlt* operon. RT-PCR also revealed a transcript containing both the ORF and *dltA* but not a transcript also containing the next gene upstream (Fig. 7C), further supporting the hypothesis that the 5' end of the *dlt* operon includes the ORF.

Inclusion of the ORF in the *dlt* operon predicts a transcription start site that is upstream of the predicted ORF translation initiation codon (i.e., ≥168 bp upstream of the *dltA* translation initiation codon). To test this hypothesis we used the recently described SMART-RACE technique (39) to identify the start site of transcripts including the region containing *dltA*. Of 16 cloned products (see Materials and Methods), 9 corre-

sponded to a predicted transcription start site that is upstream of the predicted ORF, 7 of which mapped to the same nucleotide that is 250 bp upstream of the *dltA* ATG (Fig. 7A and data not shown). No other product was recovered from more than one clone. These results strengthen the view that the 5' region of the *dlt* operon contains the ORF present between 168 and 15 bp upstream of the *dltA* translation initiation codon.

DISCUSSION

The results presented here show that *S. aureus* responds to the addition of salt by decreasing the amount of *dlt* mRNA (Fig. 1 to 5). Changes in *dlt* mRNA levels parallel changes in *cat* mRNA and CAT activity of transcriptional reporter constructs (Fig. 2), indicating that the decline of *dlt* mRNA is due to decreased transcription.

In addition to NaCl, $CaCl_2$ and $MgCl_2$ inhibit *dlt* transcription, but at concentrations 10- to 30-fold lower than that required for NaCl-induced repression (Fig. 1). These results suggest that the concentration of cations, but not the concentration of Cl^- or osmolarity, must be most important in signaling transcriptional changes of the *dlt* operon. The much greater potency of divalent cations over monovalent cations in repressing *dlt* expression may suggest a more direct role of divalent cations in this process.

The role of extracellular divalent cations in bacterial signaling has been previously demonstrated. Probably the best-studied is the PhoPQ two-component system of *Salmonella enterica* serovar Typhimurium that regulates genes for adaptation to changing extracellular Mg^{2+} and Ca^{2+} concentration (11, 40).

FIG. 8. Model of transcriptional regulation of *dlt* operon. See the text for additional details.

Serovar Typhimurium has the ability to sense extracellular concentrations of Mg²⁺ and Ca²⁺ through distinct periplasmic binding sites of the transmembrane sensor histidine kinase PhoQ, which regulates the response regulator PhoP (40). A homolog of PhoQP, CsrSR, in the gram-positive organism *Streptococcus pyogenes* regulates expression of genes for hyaluronic acid capsule synthesis (*has*), also depending on extracellular concentrations of Mg²⁺ (14). However, unlike PhoQ, the sensing ability of CsrS appears to be limited to Mg²⁺, which, when in high concentrations (~10 mM), activates a repressor CsrR. Our findings suggest a mechanism for repression of the *dlt* operon that is activated by both ambient Mg²⁺ and Ca²⁺, but with greater sensitivity to Mg²⁺ (Fig. 1). We have identified a two-component system, ArlSR, initially on the basis of homology to CsrSR, that plays a role in cation-induced repression of the *dlt* operon. We cannot yet judge, however, whether the effects of ArlSR reflect direct interactions of ArlS with added (or displaced; see below) divalent cations or of ArlR with *cis* elements within the *dlt* operon or both. Other transcriptional regulatory effects of ArlSR have been reported, including those mediated by changes in DNA supercoiling induced by environmental changes such as temperature, osmolarity, and oxygen availability (9). However, as indicated above, the conditions favoring repression of *dlt* expression seem much more driven by (divalent) cation effects and not osmolarity, suggesting that the ArlSR system might contribute to cation-induced repression of *dlt* by a mechanism distinct from DNA supercoiling.

The strains used in our study were derived from *S. aureus* NCTC 8325. *S. aureus* NCTC 8325 and, therefore, RN 6390

and SA113 as well, have a deletion in *rsbU* making them σ^B defective (24). σ^B affects the expression of *arlRS* (2), raising the possibility that in staphylococcal strains with an intact σ^B operon, *arlRS*-mediated effects on *dlt* expression might be even more prominent. This possibility has not yet been directly tested. However, no differences in Mg²⁺-induced repression of *dlt* between σ^B -defective and σ^B -repaired *S. aureus* were observed (Fig. 1B and 5), suggesting that Mg²⁺-induced repression of *dlt* is independent of this alternative σ^B factor.

In addition to *trans* regulatory elements, we identified *cis* elements required for expression of the *dlt* operon. These elements are positioned between -325 and -171 bp from *dltA* and include both elements needed for expression and for Mg²⁺-induced repression of the *dlt* operon (Fig. 6). We cannot as yet judge from these results whether this region contains binding sites for an Mg²⁺-inactivated activator, Mg²⁺-activated repressor, and/or the RNA polymerase core promoter sequence. The attempt to define *dlt* transcription initiation sites by SMART-RACE revealed that *dlt* transcription initiates at 250 bp upstream from the 5' end of the *dltA* structural gene. Inspection of the region immediately upstream of this site reveals -10 (TATAAT) box and -35 (TTGAAT) consensus sequences (Fig. 7D). The consensus of the predicted -35 and -10 sites suggests a σ^A promoter (6) with high basal expression, favoring the Mg²⁺-activated repressor model of *dlt* regulation (Fig. 8). Our findings resemble those in *Listeria monocytogenes*, where primer extension analysis revealed a transcription start site 224 bp upstream of *dltA* and the σ^A promoter (1).

The apparent initiation of the *dlt* mRNA transcript at -250

bp suggests that a previously unappreciated ORF just upstream of *dltA* is part of the *dlt* operon. Northern blotting and RT-PCR analyses support this hypothesis (Fig. 7). Our findings provide experimental support for the predictions of Wang et al. (41), who used a mathematical algorithm to suggest that the ORF upstream of *dltA* is in the same operon as *dltABCD*. It has yet to be determined whether this ORF encodes a novel protein, with a role in D-alanylation of TA.

However cation-induced transcriptional repression is mediated, a key unanswered question remains whether such transcriptional changes in the *dlt* operon are important in the regulation of the chemical composition of the cell envelope. Studies that have been conducted on Mg^{2+} (Ca^{2+})-regulated lipid A palmitoylation in gram-negative bacteria may be instructive (20). In these organisms, deprivation of divalent cations by EDTA treatment up-regulates *pagP* expression and induces envelope alterations that increase the activity of PagP that is already present (20). Although it is the change in activity of PagP that accounts for the rapid remodeling of lipid A palmitoylation, later effects of transcriptional changes likely complement changed PagP activity as the bacteria are adapting to new environmental conditions. In a similar way, the salt sensitivity of D-alanine transfer between Dcp and LTA may provide a posttranslational mechanism for acute regulation of D-alanylation of LTA and WTA, whereas accompanying changes in *dlt* expression may be important for most efficiently maintaining modified steady-state levels of TA D-alanylation (Fig. 8). The continued presence and activity of products of the *dlt* operon in the absence of ongoing D-alanyl transfer to LTA (e.g., high salt) would lead to wasteful ATP consumption as follows (21): (i) $ATP + D\text{-alanine} + Dcl \rightarrow Dcl\text{-D-alanine} + PP_i$; (ii) $Dcl\text{-D-alanine} + Dcp \leftrightarrow Dcp\text{-D-alanine} + Dcl$; (iii) $Dcp\text{-D-alanine} \leftrightarrow Dcp + D\text{-alanine}$. Therefore, transcriptional repression of the *dlt* operon may be an important means to preserve cellular energy stores when there is reduced D-alanyl transfer to LTA.

Because of the polyanionic characteristics of TA, TA have significant cation, especially divalent cation, binding properties (16). D-Alanylation of TA reduces divalent cation binding and thus may be helpful when environmental levels of divalent cations are limiting by decreasing the scavenging of divalent cations by TA (Fig. 8) and allowing Mg^{2+} to be more readily available for essential intracellular metabolic pathways. However, when environmental levels of divalent cations are not limiting, these cations could provide needed counter-ions for TA in the cell envelope, thereby reducing the need for D-alanylation under such conditions. The similar hierarchies in the cation effects on *dlt* mRNA levels ($Mg^{2+} > Ca^{2+} \gg Na^+$) and cation binding to TA (26, 27, 35) are consistent with a direct role of cation binding and/or cation (Mg^{2+}) availability in the regulation of D-alanylation of TA and *dlt* expression. Identification of the molecular cue(s) signaling posttranslational and transcriptional regulation of D-alanylation of TA is an important target for future research.

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