Regulation of D-alanylation of lipoteichoic acid in *Streptococcus gordonii*

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D-Alanyl esters on lipoteichoic acid (LTA) are involved in adhesion, biofilm formation, resistance to cationic antimicrobial peptides, and immune stimulation. There is evidence that bacteria can modulate the level of D-alanyl esters on LTA in response to challenge, but the mechanism of regulation appears to be different among bacteria. In this study, expression of the *dlt* operon responsible for D-alanylation of LTA was examined in the commensal bacterium *Streptococcus gordonii*. *dlt* expression was assessed using the *dlt* promoter–*lacZ* reporter gene assay, LTA D-alanine content measurements and *dlt* mRNA quantification. The results showed that *dlt* expression was growth phase-dependent, with the greatest expression at the mid-exponential phase of growth. In contrast to *Staphylococcus aureus*, *dlt* expression in *Strep. gordonii* was not affected by the exogenous addition of Mg²⁺ or K⁺. Interestingly, *dlt* expression was upregulated under acidic conditions or when cells were stressed with polymyxin B, indicating that cell envelope stress may be a signal for *dlt* expression. In view of these results, mutants defective in the cell envelope stress LiaSR two-component regulatory system were constructed. The *liaS* and *liaR* mutants showed an increase in *dlt* expression over the parent strain at neutral pH. The mutants failed to respond to low pH and polymyxin B stress; *dlt* expression remained the same in the presence or absence of these stresses. These results suggest that *dlt* expression in *Strep. gordonii* is regulated by the LiaSR regulatory system in response to environmental signals such as pH and polymyxin B. The regulation appears to be complex, involving both repression and activation mechanisms.

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INTRODUCTION

Lipoteichoic acid (LTA) is an amphiphilic polymer of polyphosphoglycerol or polyphosphoribitol anchored to the cytoplasmic membrane by a glycolipid. Each phosphoglycerol or phosphoribitol on this molecule may be modified by glycosylation or, more often, substituted with D-alanyl esters (Fischer, 1988; Fischer *et al.*, 1990). The *dlt* operon is responsible for the D-alanylation of LTA (Neuhaus & Baddiley, 2003). The operon consists of four genes, *dltA*, *dltB*, *dltC* and *dltD*. The *dltA* gene encodes the D-alanyl carrier protein ligase (Dcl), which activates the D-alanine and ligates it to the D-alanyl carrier protein (Dcp). The function of the protein encoded by *dltB* has not been

experimentally established, but it is a transport protein that is thought to serve as a channel to secrete the unfolded D-alanyl Dcp. The *dltD* gene encodes a membrane-associated protein, which facilitates the binding of Dcp and Dcl for ligation of Dcp with D-alanine. Disruption of any one of the *dlt* genes will eliminate D-alanylation of LTA.

D-Alanylation confers a positive charge upon LTA and has been demonstrated to have a role in the regulation of autolytic activity (Fischer *et al.*, 1981), the binding of Mg²⁺ (Archibald *et al.*, 1973), intragenic coaggregation (Clemans *et al.*, 1999), biofilm formation (Fabretti *et al.*, 2006; Gross *et al.*, 2001), adherence to mammalian cells (Abachin *et al.*, 2002; Weidenmaier *et al.*, 2004), susceptibility to cationic antimicrobial peptides and phagocytic cells (Collins *et al.*, 2002; Henneke *et al.*, 2005; Kristian *et al.*, 2005; Poyart *et al.*, 2003), sensitivity to low pH (Boyd

Abbreviations: LTA, lipoteichoic acid; qRT-PCR, quantitative real-time PCR.

et al., 2000), and modulation of immune stimulation (Chan *et al.*, 2007; Morath *et al.*, 2001). In several bacteria, *dlt* mutants display an attenuated virulence phenotype in animal models (Abachin *et al.*, 2002; Kristian *et al.*, 2005; Poyart *et al.*, 2003).

There is evidence that bacteria can modulate the level of D-alanyl esters on LTA. An increase in the level of D-alanyl ester on LTA has been reported under low external pH conditions (Ellwood & Tempest, 1972; MacArthur & Archibald, 1984), and a decrease in D-alanyl ester contents has been reported when metal cations, such as Mg^{2+} , Ca^{2+} , Na^+ and K^+ , are present in the culture medium (Fischer & Rösel, 1980; Heptinstall *et al.*, 1970; Kiriukhin & Neuhaus, 2001; Koch *et al.*, 1985; Koprivnjak *et al.*, 2006; Reusch & Neuhaus, 1971). An alteration in the enzymic activity of esterification and hydrolysis by these environmental conditions has been suggested as a mechanism for the modulation (Haas *et al.*, 1984; Kiriukhin & Neuhaus, 2001), but transcriptional control of *dlt* expression has also been reported in three bacteria. In *Bacillus subtilis*, the *dlt* operon is transcribed from a σ^D -dependent promoter, with greatest transcription during exponential growth (Perego *et al.*, 1995). As cells enter stationary phase and sporulation, *dlt* expression is downregulated by the Spo0A and AbrB sporulation proteins. *Staphylococcus aureus* is thought to utilize the ArlSR two-component regulatory system, which responds to the presence of divalent cations, such as Mg^{2+} and Ca^{2+} , by transcriptionally repressing the *dlt* operon (Koprivnjak *et al.*, 2006). Regulation of the *dlt* operon in *Streptococcus agalactiae* is controlled by the unique *dltRS* two-component regulatory system located upstream of the *dlt* operon (Poyart *et al.*, 2001). The signal sensed by *dltRS* is not known. Thus, regulation of *dlt* expression is relatively unknown and is different among the three bacteria studied so far.

The cell envelope stress LiaSR two-component regulatory system is widespread among the Firmicutes (Jordan *et al.*, 2006, 2008). LiaS is an intramembrane-sensing histidine kinase and believed to sense signals that perturb membrane damage. LiaR is the cognate response regulator. A putative membrane protein (LiaF) has been shown to function as an inhibitor of LiaRS-mediated signal transduction in *B. subtilis*. The genetic context of *liaFSR* is conserved in the Firmicutes, with *liaF* located directly upstream of *liaSR* orthologues. It has been demonstrated in several bacteria that LiaSR regulates responses to cell envelope stress caused by cationic antibiotics, oxidative agents and low pH (Gardete *et al.*, 2006; Kuroda *et al.*, 2003; Li *et al.*, 2002; Martínez *et al.*, 2007; Mascher *et al.*, 2004; Suntharalingam *et al.*, 2009).

We previously reported that a *dltA* mutant of *Streptococcus gordonii* is highly sensitive to cationic antimicrobial peptides (Chan *et al.*, 2007), and our recent unpublished results showed that the mutant was also sensitive to acidic pH. Thus, it appears that D-alanylation and LiaSR confer some common phenotypes. In this study, we examined the regulation of *dlt* expression in *Strep. gordonii* and found

that acidic pH and polymyxin B induced *dlt* expression. Mutants defective in LiaS or LiaR were not able to respond to these stresses.

METHODS

Bacteria and growth conditions. *Strep. gordonii* and *Streptococcus mutans* were cultured in brain heart infusion (BHI), TVG [per millilitre: 5 mg glucose, 35 mg tryptone, 0.04 µg *p*-aminobenzoic acid, 0.2 µg thiamine-HCl, 1 µg nicotinamide and 0.2 µg riboflavin (Burne *et al.*, 1999)], HTVG (TVG containing 100 mM HEPES) or the chemically defined medium FMC (Terleckyj *et al.*, 1975) at 37 °C and 5% CO₂ without shaking. TVG and HTVG were adjusted to pH 7.8 with NaOH prior to autoclaving. When needed, 250 µg kanamycin ml⁻¹, 250 µg spectinomycin ml⁻¹ or 10 µg erythromycin ml⁻¹ was added to the media for cultivating *Strep. gordonii*. *Escherichia coli* was cultured in Luria-Bertani medium (1% tryptone, 0.5% yeast extract and 1% NaCl, w/v) containing 50 µg kanamycin ml⁻¹, 50 µg spectinomycin ml⁻¹ or 10 µg tetracycline ml⁻¹.

Construction of the *P_{dlt}-lacZ* fusion. The promoter region of the *dlt* operon (*P_{dlt}*; *dltA* GenBank ID: 157075452) was amplified by PCR using *Taq* DNA polymerase (New England Biolabs) and the primer pair SL624/SL625 (Table 1). The 408 bp PCR product was digested with *Xba*I and *Kpn*I, and ligated into the same sites upstream of the promoterless *lacZ* gene carried on pSL (Syvitski *et al.*, 2007), resulting in the creation of the plasmid pP_{dlt}-LacZ. The plasmid confirmed by restriction analysis and PCR was maintained in *E. coli* XL1-blue. *Strep. gordonii* strain DL1 was transformed with pP_{dlt}-LacZ or pSL using the methods described by Homonylo-McGavin & Lee (1996). The resulting transformants were named NM1 and SL, respectively.

LacZ assay. The LacZ assay was performed as described previously (Tremblay *et al.*, 2009). Preliminary experiments showed that *Strep. gordonii* SL produced no detectable LacZ activity, consistent with previous reports that pSL carrying the promoterless *lacZ* is incapable of producing LacZ (Syvitski *et al.*, 2007; Tremblay *et al.*, 2009). Thus, *Strep. gordonii* SL was omitted in subsequent experiments. The protein concentrations of samples were measured by the Bradford method, as modified by Spector (1978). LacZ activity was calculated as [(A₄₀₅ × 1000)/(mg protein × incubation time)] and is reported in modified Miller units.

In experiments to test the effect of metal cations on *dlt* expression, the chemically defined FMC medium was used to avoid the potential addition of metal cations from ingredients of a complex medium. Stock solutions of MgCl₂ or KCl were added to the medium at the indicated concentrations prior to inoculation. Cells were harvested at mid-exponential phase (OD₆₀₀ ~0.6) and LacZ activity was determined as described above.

LTA isolation and D-alanine content measurements. LTA was isolated from *Strep. gordonii* cell wall fractions by n-butanol extractions and hydrophobic interaction chromatography, as previously described (Chan *et al.*, 2007; Mayer *et al.*, 2009). The purified LTA gave a single band on a 15% SDS-PAGE gel stained with the cationic dye Stains-All (data not shown).

The purified LTA was lyophilized and the mass was determined. The D-alanine content of LTA samples was measured using the method of Peschel *et al.* (1999), with modifications as described previously (Chan *et al.*, 2007). Briefly, D-alanyl esters were released from the LTA backbone by an alkaline treatment and detected by reacting with D-amino acid oxidase and 2,4-dinitrophenyl hydrazine. The amount of D-alanine in the sample was determined from a D-alanine standard curve and expressed with respect to the amount of LTA.

Table 1. Primers used in this study

Primer	Sequence (5'–3'), restriction site*	Function
SL525	GAATTAACACATGCTCCACCGC	Reverse primer for 16S rRNA
SL550	CTGGGCCCCAGGCGGCCGGGCCAAAATTTGTTTGAT, <i>SfiI</i>	Forward primer for <i>ermAM</i>
SL551	CCTGGCCGGCCTGGCCGGCAGCGACTCATAGAAT, <i>SfiI</i>	Reverse primer for <i>ermAM</i>
SL624	GGGGTACCACTCAATTAGGGCTGATGA, <i>KpnI</i>	Forward primer for <i>dlt</i> promoter
SL625	GCTCTAGAATTCGTACGAAAGGATAC, <i>XbaI</i>	Reverse primer for <i>dlt</i> promoter
SL658	TGTCTTCAGCCTTTTCGGTT	Forward primer for <i>liaS</i> upstream fragment
SL659	CCTGGCCGCCTGGGCCCTTTTAATTGCTGCTCTACCT, <i>SfiI</i>	Reverse primer for <i>liaS</i> upstream fragment
SL660	CCTGGCCGGCCTGGCCAGTATCCTCTATTTTGGATA, <i>SfiI</i>	Forward primer for <i>liaS</i> downstream fragment
SL661	ATTGCAACTCCTTGCTTAGG	Reverse primer for <i>liaS</i> downstream fragment
SL692	ACTACATCTTCGTCCTACAG	Forward primer for <i>liaR</i> upstream fragment
SL693	CCTGGCCGCCTGGGCCCGAACCATTTTCGTGATCATC, <i>SfiI</i>	Reverse primer for <i>liaR</i> upstream fragment
SL694	CCTGGCCGGCCTGGCCCTTCCTCAGGAAGACTTCTGA, <i>SfiI</i>	Forward primer for <i>liaR</i> downstream fragment
SL695	ACAGGTAGCTTCTGGTAAAC	Reverse primer for <i>liaR</i> downstream fragment
SL697	ATTTATTGGGCGTAAAGCGAGCGC	Forward primer for 16S rRNA
SL726	GAGCACTATGCTCAGACACA	Forward primer for <i>dltA</i> used in qRT-PCR
SL727	CCCTTGACAGAATGAGTGAT	Reverse primer for <i>dltA</i> used in qRT-PCR

*Restriction site is underlined.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from cells using the hot acid phenol method of Peterson *et al.* (2000), as described previously (Tremblay *et al.*, 2009). Contaminating DNA was removed by digestion with amplification-grade DNase I (Invitrogen Life Technologies), and the resulting RNA was verified to be free of DNA by performing PCRs for 16S rRNA using primers SL525/SL697 (Table 1). cDNA was synthesized using random primers and SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's instructions.

qRT-PCR of *dlt* and 16S rRNA transcripts from the cDNA was performed using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratory) following the manufacturer's instructions. 16S rRNA was chosen as the endogenous reference gene, as its expression is relatively stable, irrespective of growth conditions (Stipp *et al.*, 2008). Duplicate reactions were performed for each sample. The primer pairs used for amplification of the *dlt* and 16S rRNA cDNA were SL726/SL727 (90% PCR efficiency) and SL525/SL697 (92%), respectively. qRT-PCRs were performed using a 7900 HT Fast Real-Time PCR system (Applied Biosystems) at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. Fluorescence was measured during each annealing and elongation phase and the cycle threshold (C_t) value was calculated at a threshold of 0.2 using SDS 2.2.2 software (Applied Biosystems). A dissociation curve was also constructed following the qRT-PCR. The relative expression level of *dlt* was calculated using the $\Delta\Delta C_t$ method (as described by Applied Biosystems (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf)) using the 16S rRNA C_t values to normalize each sample for total RNA content.

RT-PCR. cDNA was synthesized as described above, and levels of 16S rRNA and *dltA* transcripts were estimated by PCR with *Taq* DNA polymerase and primer pairs SL697/SL525 and SL726/SL727, respectively. PCRs were incubated at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final 5 min extension at 72 °C. The PCR products were analysed by gel electrophoresis.

Construction of the *liaS* and *liaR* mutants. To construct the *liaS* deletion cassette, the upstream (457 bp) and downstream (436 bp)

fragments of *liaS* were amplified by PCR from *Strep. gordonii* DL1 chromosomal DNA using the primer pairs SL658/SL659 and SL660/SL661, respectively (*liaS* GenBank ID: 157075096). The 0.8 kb *ermAM* gene was amplified from *Strep. mutans* strain YDNT005 chromosomal DNA (Tremblay *et al.*, 2009) using the primers SL550/SL551. The *liaS* PCR products were restricted with *SfiI* and ligated to the *ermAM* DNA separately. The ligated DNA was PCR-amplified to produce the 1.26 kb 5' *liaS-ermAM* and 1.24 kb *ermAM-3' liaS* DNA using the SL658/SL551 and SL550/SL661 primer pairs, respectively. The PCR products were mixed in a 1 : 1 ratio and further amplified by overlapping PCR using the primer pair SL658/SL661 to obtain the final 1.69 kb *liaS::ermAM* construct, which was introduced into *Strep. gordonii* NM1 via natural transformation (Homonylo-McGavin & Lee, 1996). Transformants were selected on erythromycin–kanamycin BHI agar. The mutant was verified to carry a 1.69 kb *liaS::ermAM* construct by PCR (data not shown).

The *liaR* mutant was constructed similarly to the *liaS* mutant. The regions upstream (466 bp) and downstream (532 bp) of *liaR* were amplified from DL1 chromosomal DNA by PCR with primer pairs SL692/SL693 and SL694/SL695, respectively (*liaR* GenBank ID: 157075406). The PCR products were ligated to *ermAM*, and the 1.6 kb ligated product was PCR-amplified using SL692 and SL695. The resulting *liaR::ermAM* construct was transformed into NM1 to obtain the *liaR* mutant. The mutant was verified by PCR with primer pair SL692/695 to carry the 1.6 kb *liaR::ermAM*, while the parent NM1 had the intact *liaR* (data not shown).

MIC assays. The MIC of polymyxin B for the parent strain and *lia* mutants was determined in 96-well polypropylene microplates by a microdilution method. Bacteria in BHI (50 μ l, 10^7 c.f.u.; final bacterial concentration 1×10^8 c.f.u. ml⁻¹) were added to the wells, which contained 50 μ l twofold-diluted polymyxin B. The starting concentration of polymyxin B was 800 μ g ml⁻¹. Plates were incubated at 37 °C for 24 h and examined visually for bacterial growth. The lowest concentration that inhibited growth was considered to be the MIC.

Polymyxin killing assays. BHI broth containing 400 μ g polymyxin B ml⁻¹ was inoculated with the parent strain NM1 or the *lia* mutants at a concentration of 1×10^8 cells ml⁻¹ in triplicate. Cultures were

incubated at 37 °C for 0, 2 and 4 h. Following incubation, survival was determined by plating in triplicate and counting the resulting colonies after incubation for 48 h.

Statistical analysis. Results were analysed by the two-tailed Student's *t* test. A *P* value of ≤ 0.05 was considered to be significant.

RESULTS

dlt expression is growth phase-dependent

Expression of the *dlt* operon by *Strep. gordonii* through growth was initially assessed using the P_{dlt} -*lacZ* reporter gene. LacZ activity increased with growth, with the greatest activity at mid-exponential phase (Fig. 1a). To confirm these results, D-alanine contents on LTA and *dlt* transcript levels were measured. Cells from the mid-exponential phase of growth contained the greatest amount of D-alanine on their LTA (Fig. 1b). In agreement with these results, cells from exponential phase produced higher levels of *dlt* mRNA than cells at the stationary growth stage (Fig. 1c). These results collectively indicate that *dlt* expression is growth phase-dependent, with higher expression during the exponential phase of growth.

dlt expression is not affected by Mg^{2+} and K^+

Earlier work in *Staph. aureus* has shown an increase in extracellular Mg^{2+} and Ca^{2+} correlated with repression of the *dlt* operon (Koprivnjak *et al.*, 2006). Thus, the effect of cations on *dlt* expression in *Strep. gordonii* was investigated. There was no change in the LacZ activity of cells grown in the presence or absence of $MgCl_2$ or KCl (Fig. 2), indicating that $MgCl_2$ and KCl do not alter expression of *lacZ* from P_{dlt} and suggesting that the regulation of *dlt* expression in *Strep. gordonii* is different from that in *Staph. aureus*.

dlt expression is upregulated by acidic pH and polymyxin B

As a change in external pH is commonly encountered by *Strep. gordonii*, the effect of culture pH on *dlt* expression was examined in buffered (HTVG) and unbuffered (TVG) media. *Strep. gordonii* exhibited the same growth curve and attained the same extent of growth in the two media (data not shown). The pH of the HTVG culture showed an initial decrease from 7.8 to 6.9 and maintained this pH to the end of growth, whereas that of the TVG culture decreased rapidly to pH 4.5 (Fig. 3a). *dlt* expression was assessed by LTA D-alanine content and *dlt* transcript measurements of cells at mid-exponential growth (i.e. at 6 h of growth, OD_{600} of 0.6). Cells from the TVG culture contained a higher amount of D-alanine in their LTA than those from the HTVG culture (Fig. 3b). In agreement with the D-alanine results, cells from the TVG culture produced a greater level of *dlt* mRNA than those from the HTVG culture (Fig. 3b). These results suggest that *dlt* expression is upregulated in acid conditions.

Fig. 1. *dlt* expression by *Strep. gordonii* during growth. (a) Growth curve of *Strep. gordonii* NM1 in TVG (□) and LacZ activity (◇) expressed by the P_{dlt} -*lacZ* construct carried by NM1. (b) D-Alanine content of LTA isolated from NM1 grown to early (OD_{600} ~0.3), mid- (OD_{600} ~0.6), late-exponential (OD_{600} ~1.8) and stationary phases (OD_{600} ~2.0) of growth; * $P < 0.05$. (c) RT-PCR of *dltA* and 16S rRNA from cells grown to the same growth phases as in (b). Results reported in (a) and (b) are means $\pm$ SD of triplicate cultures and are representative of two to three independent experiments.

Fig. 2. Effects of Mg²⁺ and K⁺ on *dlt* expression. LacZ activity produced by *Strep. gordonii* NM1 grown to mid-exponential phase in the chemically defined FMC medium in the presence of the indicated concentrations of MgCl₂ (closed bars) or KCl (open bars). Results are means $\pm$ SD of triplicate cultures.

We previously reported that the *Strep. gordonii* *dlt* mutant is more susceptible to cationic antimicrobial peptides (Chan *et al.*, 2007); thus, it is conceivable that *dlt* expression may respond to polymyxin B stress. To test this, mid-exponential *Strep. gordonii* cells were exposed to a subinhibitory concentration of polymyxin B for 1 h and the level of *dlt* transcript was determined. Cells produced a higher level of *dlt* transcript when challenged with polymyxin B, suggesting that polymyxin B can upregulate the *dlt* operon (Fig. 3c).

dlt expression in the *liaS* and *liaR* mutants

The above results suggest that *dlt* expression is responsive to stress to the cell envelope; therefore, mutants defective in the cell envelope stress LiaSR two-component regulatory system were constructed. The mutants were verified to have the *liaS* or *liaR* gene inactivated by the *ermAM* cassette via PCR (data not shown). LTA D-alanine contents were measured and compared between the mutants and the parent strain. LTA from both the *liaS* mutant and the *liaR* mutant contained more D-alanine than that from the parent when grown in HTVG (Fig. 4a). This result was confirmed by qRT-PCR measurement of the *dlt* transcript (Fig. 4b).

Interestingly, the *lia* mutants produced the same level of D-alanylated LTA when grown in HTVG (neutral) and TVG (acidic) media, whereas the parent strain clearly produced more D-alanylated LTA in TVG medium than in HTVG medium (Fig. 4a). qRT-PCR experiments gave similar results, showing that the *dlt* mRNA levels were identical between media for the *lia* mutants, whereas that for the parent strain was substantially higher in TVG than in HTVG (Fig. 4c).

Our results showed that *dlt* expression is upregulated by polymyxin B; hence, it was logical to examine expression in

Fig. 3. *dlt* expression is upregulated by acidic pH and polymyxin B. (a) Culture pH of *Strep. gordonii* NM1 grown in HTVG (○) and TVG (●). (b) D-Alanine content of LTA and *dlt* mRNA levels from cells harvested at 6 h of growth (OD₆₀₀ 0.6, mid-exponential). Open and closed bars are HTVG and TVG cultures, respectively. (c) *dlt* expression by mid-exponential cells following 1 h incubation with (closed bars) or without (open bars) 50 μg polymyxin B ml⁻¹. Results are means $\pm$ SD of triplicate cultures and are representative of two or three independent experiments. ****P* < 0.001.

the *lia* mutants. Both *lia* mutants produced a similar level of *dlt* transcript in the presence or absence of polymyxin B, while the parent strain produced more *dlt* transcript when challenged by the antibiotic (Fig. 4d).

***liaS* and *liaR* mutants are more sensitive to polymyxin B than the parent strain**

Given the results for the effect of LiaSR mutation on *dlt* expression, the susceptibility of the *lia* mutants to polymyxin B was tested. Both the *liaS* mutant and the *liaR* mutant were significantly more susceptible to killing by polymyxin B than the parent strain (Fig. 5). The MICs of polymyxin B for the parent, *liaS* and *liaR* strains were 400, 100 and 100 $\mu\text{g ml}^{-1}$, respectively.

DISCUSSION

In the present study, the expression and regulation of D-alanylation of LTA in *Strep. gordonii* was investigated.

Expression of *dlt* was growth phase-dependent, with higher expression during mid-exponential growth. This trend in *dlt* expression is also seen in *B. subtilis*, where the operon is negatively regulated by AbrB during the transition from vegetative growth to sporulation (Perego *et al.*, 1995). *Strep. gordonii* does not undergo sporulation; however, the peak in *dlt* expression of both *B. subtilis* and *Strep. gordonii* coincides with the peak in metabolic activity and cell proliferation of mid-exponential phase, perhaps to ensure adequate D-alanylation when cell envelope, and thus LTA, biosynthesis is at its greatest.

The correlation between decreasing external pH and increasing LTA D-alanine content has been reported in *Staph. aureus* and *B. subtilis* (Ellwood & Tempest, 1972; MacArthur & Archibald, 1984). Our results indicate that this is also the case in *Strep. gordonii* (Fig. 3b). Our qRT-PCR results further indicate that the pH effect is at the transcriptional level, providing direct evidence of transcriptional upregulation of *dlt* at low pH. The increase in *dlt* expression at low pH may be a part of the acid tolerance

Fig. 4. *dlt* expression in *liaS* and *liaR* mutants. (a) D-Alanine content of LTA produced by *Strep. gordonii* parent strain NM1 and the mutants *LiaS*⁻ and *LiaR*⁻ in HTVG or TVG. (b) *dltA* mRNA levels from cells grown in HTVG. (c) *dltA* mRNA levels of cells grown in HTVG relative to those grown in TVG. (d) *dltA* mRNA levels from cells grown in HTVG following 1 h incubation with or without 50 μg polymyxin B ml^{-1} . In all four panels, cells were obtained from the mid-exponential phase of growth. Results are means $\pm$ SD of triplicate cultures and are representative of two independent experiments. *** $P < 0.001$.

Fig. 5. Susceptibility of *liaS* and *liaR* mutants to polymyxin B. The parent strain (NM1) and the *liaS* and *liaR* mutants were incubated with polymyxin for 0, 2 or 4 h, and viable cell counts were performed following incubation. Values are means $\pm$ SD of triplicate cultures and are representative of two independent experiments. ** $P < 0.01$.

response of *Strep. gordonii*. Our unpublished results show that the *dlt* mutant of *Strep. gordonii* is unable to grow below pH 6.0. Similarly, the *dlt* mutant of *Strep. mutans* loses the ability to grow at low pH (Boyd *et al.*, 2000) and that of *Streptococcus pyogenes* is susceptible to vacuole acidification in neutrophils (Ganz, 2001). These findings collectively suggest that *dlt* upregulation may be a strategy employed by these bacteria for survival under conditions of low pH.

It has been documented that *dltA* mutants of several bacteria, including *Strep. gordonii*, show increased susceptibility to killing by cationic antimicrobial peptides (Chan *et al.*, 2007; Kristian *et al.*, 2005; Poyart *et al.*, 2003). Our results advanced these findings by demonstrating that *dlt* transcription is upregulated by polymyxin B. This upregulation is likely a means to counter polymyxin B, or other cationic antimicrobial peptides, by adding more D-alanine to LTA. It is interesting to note that polymyxin B induced a smaller fold-increase in *dlt* mRNA levels under acidic conditions compared with neutral pH (Fig. 3c). The reason for this is likely due to the already high *dlt* expression at low pH. Nonetheless, the results do indicate that upregulation of *dlt* expression is responsive to the presence of both low pH and polymyxin B.

Our results further showed that the LiaSR two-component regulatory system plays a regulatory role in *dlt* expression. This statement is supported by the findings that inactivation of LiaS or LiaR resulted in an increase in *dlt* expression and the abolition of *dlt* upregulation by low pH and polymyxin B. The regulation of *dlt* expression by LiaSR appears to be complex. The increase in *dlt* expression by the *lia* mutants suggests that LiaSR represses *dlt*. The loss in

response to low pH and polymyxin B suggests that LiaSR plays an activation role when these stimuli are present. Analysis of the *dlt* promoter region revealed an inverted repeat but not the putative 'LiaR-binding motif' (Jordan *et al.*, 2006). Gel shift assay results showed that LiaR purified from *E. coli* did not cause a mobility shift for the *dltA* promoter (data not shown). We have attempted to obtain a viable *liaSR* clone in *E. coli* but failed; hence, complementation experiments cannot be performed.

The *lia* mutants were more sensitive to polymyxin B than the parent strain. We previously showed that a *dlt* mutant of *Strep. gordonii* is more susceptible to polymyxin B, nisin, magainin II and human β -defensins than the parent strain (Chan *et al.*, 2007). The inability to upregulate *dlt* expression likely contributed to the increased susceptibility to polymyxin B of the *lia* mutants. In the oral cavity, *Strep. gordonii* faces the challenge of other cationic antimicrobial peptides, such as human β -defensins. The ability to regulate D-alanylation may present a mechanism to counter the deleterious effects of these naturally occurring cationic antimicrobial peptides.

In conclusion, D-alanylation of LTA in *Strep. gordonii* is growth phase-dependent and is upregulated by low pH and polymyxin B. The cell envelope stress LiaSR two-component regulatory system appears to play a role in regulating *dlt* expression.

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