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The *Streptococcus mutans* Cid and Lrg systems modulate virulence traits in response to multiple environmental signals

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The tight control of autolysis by *Streptococcus mutans* is critical for proper virulence gene expression and biofilm formation. A pair of dicistronic operons, SMU.575/574 (*lrgAB*) and SMU.1701/1700 (designated *cidAB*), encode putative membrane proteins that share structural features with the bacteriophage-encoded holin family of proteins, which modulate host cell lysis during lytic infection. Analysis of *S. mutans lrg* and *cid* mutants revealed a role for these operons in autolysis, biofilm formation, glucosyltransferase expression and oxidative stress tolerance. Expression of *lrgAB* was repressed during early exponential phase and was induced over 1000-fold as cells entered late exponential phase, whereas *cidAB* expression declined from early to late exponential phase. A two-component system encoded immediately upstream of *lrgAB* (LytST) was required for activation of *lrgAB* expression, but not for *cid* expression. In addition to availability of oxygen, glucose levels were revealed to affect *lrg* and *cid* transcription differentially and significantly, probably through CcpA (carbon catabolite protein A). Collectively, these findings demonstrate that the Cid/Lrg system can affect several virulence traits of *S. mutans*, and its expression is controlled by two major environmental signals, oxygen and glucose. Moreover, *cid*/*lrg* expression is tightly regulated by LytST and CcpA.

INTRODUCTION

The regulatory network for virulence factor expression by *Streptococcus mutans*, the principal aetiological agent of human dental caries (Loesche, 1986), is more complex than previously appreciated. Recent studies suggest that autolysins (Sturges & Rettger, 1922) may be central to modulation of virulence in many bacteria, including *S. mutans* (Ahn & Burne, 2006, 2007; Bayles, 2007; Rice *et al.*, 2007; Rice & Bayles, 2008; Shibata *et al.*, 2005). The autolytic process directly affects the biogenesis, stability and composition of the cell envelope, which is a dynamic and complex structure that expands as cells grow and is reshaped when cells divide or differentiate (Ghuysen *et al.*, 1966; Höltje, 1995; Perkins, 1980; Shockman & Holfje, 1994; Ward & Williamson, 1984). Autolysins thus impact many cellular processes, including cell-wall turnover, cell growth, antibiotic resistance, cell-to-surface adhesion, genetic competence, protein secretion and pathogenicity (Berry *et al.*, 1989; Blackman *et al.*, 1998; Groicher *et al.*, 2000; Heilmann *et al.*, 1997; Mercier *et al.*, 2002; Smith *et al.*, 2000; Wuenscher *et al.*, 1993).

Recently, autolysis has been proposed to be a form of programmed cell death (PCD) utilized by certain bacteria in an altruistic behaviour that enhances the survival of populations of organisms (Bayles, 2003, 2007; Engelberg-Kulka *et al.*, 2006; Lewis, 2000; Rice & Bayles, 2003; Yarmolinsky, 1995). These studies are based on the observation that single-celled organisms can display complex social behaviours when organized in multicellular communities, such as biofilms. PCD by cell lysis would be beneficial to the persistence of multicellular biofilm structures by eliminating damaged cells from the population, while concurrently enhancing the survival of the remaining population via liberation of nutrients from dying cells. Programmed altruistic behaviours

Abbreviations: BHI, brain heart infusion; eDNA, extracellular genomic DNA; Gtf, glycosyltransferase; PCD, programmed cell death; TCA, tricarboxylic acid; TCS, two-component system.

Supplementary figures showing structural similarities among LrgA, CidA and the prototypical bacteriophage lambda S holin, and aerobic growth of *Streptococcus mutans* wild-type and its derivatives are available with the online version of this paper.

may also be connected to the ability of bacteria to cope with changes in the environment, previous studies having revealed that autolysis is strongly influenced by multiple environmental factors, including salt concentration, pH, glucose and oxygen (Ahn & Burne, 2007; Bowman & Redmond, 1956; Gilpin *et al.*, 1972; Ochiai, 1999; Qoronfleh *et al.*, 1998; Tobin *et al.*, 1994; Wells & Russell, 1996; Yabu & Kaneda, 1995). Oxygen was recently demonstrated to have a profound impact on the regulatory pathways of AtlA, a major autolysin of *S. mutans* that modulates cell-surface biogenesis and composition in response to oxygen (Ahn & Burne, 2007; Ahn *et al.*, 2007). Thus, the autolytic process of *S. mutans* is modulated by its genetic and physiological responses to environmental stimuli, particularly oxygen.

In our efforts to identify the regulatory mechanisms and pathways that control autolysis in *S. mutans*, we previously identified a pair of dicistronic operons, *lrgAB* (SMU.575/574) and *cidAB* (SMU.1701/1700), that were regulated in response to aerobic growth (Ahn *et al.*, 2007). Here, we report that the *lrg* and *cid* operons are regulated in a complex manner and that their products have a significant impact on autolysis and virulence factor expression by *S. mutans*.

METHODS

Bacterial strains, plasmids, media and growth conditions. *Escherichia coli* DH10B was grown in Luria broth and *S. mutans* UA159 and its derivatives were grown in brain heart infusion (BHI) broth (Difco). For selection of antibiotic-resistant colonies after genetic transformation, ampicillin (100 μg ml^{-1} for *E. coli*), erythromycin (300 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} for *S. mutans*), kanamycin (50 μg ml^{-1} for *E. coli* and 1 mg ml^{-1} for *S. mutans*) and spectinomycin (50 μg ml^{-1} for *E. coli* and 1 mg ml^{-1} for *S. mutans*) were added to media, as required. For biofilm formation assays, *S. mutans* strains were grown in microtitre plates in the semi-defined medium BM (Loo *et al.*, 2000) supplemented with glucose or sucrose at a final concentration of 20 mM.

Construction of mutant strains. Standard DNA manipulation techniques were used to engineer plasmids and strains (Ahn *et al.*, 2006; Sambrook *et al.*, 1989). All mutants were created by using a PCR ligation mutagenesis approach (Lau *et al.*, 2002), whereby genes were disrupted by replacing nearly all of the ORF with a non-polar resistance marker, such as a spectinomycin (Sp^{R}), erythromycin (Em^{r}) or kanamycin (NPKm) marker, or a polar marker (ΩKm). Transformants were selected on BHI agar containing appropriate antibiotics, and double-crossover recombination into each gene was confirmed by PCR and sequencing to ensure that no mutations were introduced into flanking genes. The mutant strains of *S. mutans* constructed in this study are listed in Table 1.

Growth, biofilm and autolysis assays. For growth rate comparisons, fresh medium was inoculated with 1 : 100 dilutions of overnight cultures of *S. mutans*. The optical density at 600 nm (OD_{600}) was measured at 37 °C at fixed time intervals in a spectrophotometer or was monitored by using a Bioscreen C lab system (Ahn & Burne, 2007). To achieve anaerobic conditions, sterile mineral oil (50 μl per well) was placed on top of the cultures (Ahn & Burne, 2007; Ahn *et al.*, 2007). To measure the growth rate under oxidative stresses, the strains were grown for 48 h in BHI containing hydrogen peroxide (0.001 %, v/v) or paraquat (10 mM). Cells were also observed by phase-contrast microscopy to record chain length. For growth on agar plates, the strains were streaked from mid-exponential phase cultures onto the surface of BHI agar. The plates were then incubated for 72 h in an aerobic chamber, and growth was compared from three independent experiments. The ability to form stable biofilms in microtitre plates and the autolysis assay were carried out as described by Ahn & Burne (2006, 2007) and Shibata *et al.* (2005).

Table 1. *S. mutans* strains used in this study

Strain	Description	Relevant genotype	Source or reference
UA159	UA159	Wild-type	
SAB113	Δ*lrgA*::NPSp^{r}	*lrgA*$^{-}$	This study
SAB119	Δ*lrgB*::NPEm^{r}	*lrgB*$^{-}$	This study
SAB115	Δ*lrgAB*::ΩKm^{r}	*lrgAB*$^{-}$	This study
SAB111	Δ*lytS*::NPKm^{r}	*lytS*$^{-}$	This study
SAB112	Δ*lytS*::ΩKm^{r}	*lytST*$^{-}$	This study
SAB117	Δ*cidA*::NPKm^{r}	*cidA*$^{-}$	This study
SAB118	Δ*cidA*::ΩKm^{r}	*cidAB*$^{-}$	This study
SAB121	Δ*cidB*::NPKm^{r}	*cidB*$^{-}$	This study
TW1	Δ*ccpA*::ΩKm^{r}	*ccpA*$^{-}$	Wen & Burne (2002)

Protein electrophoresis and Western blotting. Protein extracts from *S. mutans* were prepared from cell pellets harvested from BHI cultures in mid-exponential phase (OD_{600}=0.5), as previously described (Ahn & Burne, 2006; Chen *et al.*, 1998). Proteins (10 μg) were separated by SDS-PAGE in a 10 % polyacrylamide gel with a 4.5 % stacking gel (Laemmli, 1970), or in a 3–8 % Tris/acetate gradient gel (Invitrogen). Proteins were transferred to Immobilon P membranes (Millipore) and were subjected to Western blot analysis according to standard protocols (Sambrook *et al.*, 1989). Membranes were incubated with anti-GtfB (Ahn *et al.*, 2007) polyclonal antisera. Peroxidase-labelled goat anti-rabbit IgG (KPL) and Sigma FAST (3,3′-diaminobenzidine tablets) were used to determine antibody reactivity. The protein concentration of samples was determined by a bicinchoninic acid assay (Sigma).

Transcriptional analysis. The potential for co-transcription of two genes was examined by RT-PCR. Levels of mRNA were quantified by Northern blotting and real-time RT-PCR analyses. To measure the expression of genes using real-time RT-PCR, *S. mutans* wild-type (UA159) and its derivatives were grown in 7 ml BHI, and cells were harvested in early (OD_{600}=0.2), mid- (OD_{600}=0.5), late (OD_{600}=0.9) exponential or stationary phase. Extraction of RNA, RT-PCR, real-time RT-PCR and data analysis were performed as described previously (Ahn *et al.*, 2005). The primers used for reverse transcription reactions and real-time PCR are shown in Table 2. For Northern blotting, total RNA was isolated from *S. mutans* UA159 cultures grown for 6 h, corresponding to late exponential phase, in Todd–Hewitt broth supplemented with 11, 15, 20, 25, 30, 35, 40 or 45 mM glucose. *S. mutans* UA159 and TW1 (Table 1), the latter being an otherwise-isogenic *ccpA* mutant, were also grown in the same broth containing glucose at a final concentration of either 11 or 45 mM. Cells were removed for RNA isolation at 2, 6 and 12 h growth, corresponding to early, late exponential and late stationary phases, respectively. Total RNA was isolated from each sample by using an RNeasy Mini kit (Qiagen) and FASTPREP (MP Biomedicals) as described previously (Patton *et al.*, 2005). Northern blotting was performed as described previously (Patton *et al.*, 2005). DIG-labelled DNA probes specific for the *cidB* and *lrgA* genes were synthesized by using a DIG PCR probe synthesis kit (Roche Applied Science) and the primer pairs listed in Table 2. Hybridization and processing of the blots were performed by using the DIG system (Roche Applied

Table 2. Primers used in this study for construction of deletion mutants and real-time PCR

Italic type indicates the restriction site integrated into the primer.

Primer purpose	5′-end amplicon		Deleted gene/ amplicon/probe*	3′-end amplicon	
	Primer	Nucleotide sequence (5′→3′)		Primer	Nucleotide sequence (5′→3′)
General					
	lrgA-A	GCAATTGGCACATCCTCCAC	*lrgA*	lrgA-*Sph*I-C	CTATCAAAA*GCATGC*ATGTGGCAAG
	lrgA-*Sph*I-B	CTGAAATAA*GCATGC*AAACGAGCAG		lrgA-D	CCATGGCAGTGATGGCAGTA
	lrgB-A	GCAATCGGGGACAGTTTTGA	*lrgB*	lrgB-*Eco*RI-C	GCAGCCTTT*GAATTC*GAATTAGGAG
	lrgB-*Sal*I-B	GCAAAGAAA*GTCGAC*TGTAAGAGAA		lrgB-D	ACAGACCGCTTTGAGGTTGC
	lytS-A	ACTGAACAGCCAGTGCACCA	*lytS*	lytS-*Bam*HI-C	GCAGTGCTA*GGATCC*TACACTTTGA
	lytS-*Bam*HI	GCCAGAATC*GGATCC*ATACCAAGTC		lytS-D	TCAAAACTGTCCCCGATTGC
	cidA-A	TGCGGTCAGTTTTGCTGTGG	*cidA*	cidA-*Bam*HI-C	GAGACATTA*GGATCC*AGACTTTCCA
	cidA-*Bam*HI-B	CAAATTCGC*GGATCC*AAGAAAAGAG		cidA-D	TGAGACAAAAGTGTTCCCAACC
	cidB-A	GCGCTTTTCAGGCAAGCAGA	*cidB*	cidB-*Bam*HI-C	ACGGGTTTG*GGATCC*GTCTTTGTAT
	cidB-*Bam*HI-B	TAGGCAAAT*GGATCC*AGCCAAAGAC		cidB-D	TGGCGCCAAATCTCTTACGC
RT-PCR† or real-time RT-PCR					
	lrgA-sense	TTGCCTAAAGCCTTACCGATTCC	*lrgA*	lrgA-antisense	GCCTGATGGGACAAACATAAAGC
	lrgB-sense	GGCAAAAGGATTGGGAACTGATG	*lrgB*	lrgB-antisense	TGGAACGGCAAAGGCAATGG
	lytS-sense	TTGTCAGTTCTGCTTTGGTAGG	*lytS*	lytS-antisense	CAATGACCTGCGAAGTAGATGG
	lytT-sense	CATCCTCCACTTGTCGTCTTTGC	*lytT*	lytS-antisense	CACACGCCCCTGCTCAAAAG
	cidA-sense	ATCCGTTTGCGTCATATCAATGC	*cidA*	cidA-antisense	CCATAATCCCCACTGCTGCTG
DIG-probe synthesis					
	lrgA-sense	CACAATCAAAATCAGCACCT	*lrgA*	lrgA-antisense	TCACCTTTTTGATAGACAGAA
	cidB-sense	TTTTTCGAATCCTCTTTTTG	*cidB*	cidB-antisense	CAACAACAACCAGTGTTACG

*Additional primers used for deletion of *lrgAB* were as follows: lrgA-*Bam*HI-B, CTGAAATAA*GGATCC*AAACGAGCAG; lrgB-*Bam*HI-C, GCAGCCTTT*GGATCC*GAATTAGGAG.

†Primer sets for RT-PCR were employed as follows: lrgA-sense/lrgB-antisense primers for *lrgA–lrgB*, and lrgB-sense/lytS-antisense primers for *lrgB–lytS*.

Science) following the supplier's recommendations for Northern blot analysis. Transcript sizes were estimated by comparison with an RNA molecular mass ladder (Invitrogen).

RESULTS

The *cid/lrg* operons of *S. mutans*

Previous studies revealed that oxygen modulates autolysis by *S. mutans* (Ahn & Burne, 2007). In a subsequent microarray analysis (Ahn *et al.*, 2007) designed to identify oxygen-responsive genes, two paralogously related operons, SMU.575/574 and SMU.1701/1700, were strongly upregulated or downregulated in cells grown aerobically, respectively. SMU.575 shares 20 % amino acid sequence similarity with that of SMU.1701, whereas SMU.574 shares 27 % amino acid sequence similarity with that of SMU.1700, by using a local BLAST search. The SMU.575/574 and SMU.1701/1700 operons share substantial sequence similarity with the bacterial *lrg* and *cid* operons, respectively, a family of genes widely conserved across a range of bacterial species (Bayles, 2007) that are known to control murein hydrolase activity in *Staphylococcus aureus* (Groicher *et al.*, 2000; Rice *et al.*, 2003). Thus, we designated SMU.575/574 as *lrgAB* and SMU.1701/1700 as *cidAB*.

The genetic organization of the *lrg* and *cid* loci of *S. mutans* is shown in Fig. 1. Notably, a two-component signal transduction system (TCS), annotated as *lytS* (sensor kinse) and *lytT* (response regulator), is encoded immediately upstream of *lrgA*. The *lytST* operon is conserved in a subset of Gram-positive bacterial genera, including *Bacillus* and *Staphylococcus* (Bayles, 2007). However, apparent homologues of *lyt–lrg* are absent in some oral streptococci, including *Streptococcus gordonii*, *Streptococcus mitis* and *Streptococcus sanguinis*, but are present in *Streptococcus agalactiae* (http://www.oralgen.lanl.gov/). The *S. mutans cidA* and *cidB* genes overlap by 4 nt, but do not appear to be linked to regulatory genes. Instead, these genes lie between genes encoding conserved hypothetical proteins, including a putative membrane-associated phosphatase and an rRNA *S*-adenosylmethionine (SAM)-dependent methyltransferase (Fig. 1). It is also noteworthy that LrgA and, to a lesser extent, CidA each share structural features with the bacteriophage-encoded holins and antiholins that control bacteriophage-mediated cell death and lysis (Wang *et al.*, 2000; Young & Blasi, 1995; Young, 2002). Similarities between LrgA and the prototypical holin, bacteriophage lamda S protein, include a relatively small size, two or more putative membrane-spanning domains, a polar N-terminal sequence and a charge-rich C-terminal domain (see

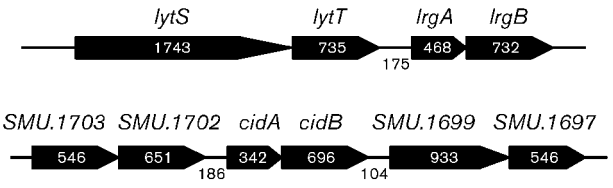

Fig. 1. Schematic diagram of the *lrg* and *cid* loci in the *S. mutans* UA159 genome. Gene assignments and gene numbers above the schematic diagram are based on the *S. mutans* UA159 genome (GenBank accession no. AE014133). Numbers inside the schematic diagram and between ORFs indicate the nucleotides (bp) in the ORFs and intergenic regions, respectively. Arrows indicate the direction of transcription. A dicistronic operon, *lytST*, is located immediately upstream of *lrgAB*. The *lytS* operon encodes the putative sensor kinase and *lytT* encodes the putative response regulator of a TCS. The *lrgA* and *lrgB* operons are annotated as a regulator and effector of murein hydroase, respectively. The *SMU.1699* and *SMU.1703* genes are annotated as conserved hypothetical proteins, and *SMU.1697* and *SMU.1702* are predicted to encode a possible rRNA methylase and uncharacterized phosphatase, respectively.

Supplementary Fig. S1, available with the online version of this paper). Unlike LrgA, the CidA protein of *S. mutans* does not possess a polar N-terminal sequence and shows a shorter charged C-terminal sequence (Supplementary Fig. S1). LrgB and CidB, the presumed hydrophobic partners of LrgA and CidA, respectively, lack sequence similarity to holin proteins or known murein hydrolases.

Role of the *cid* or *lrg* gene products in autolysis and biofilm formation of *S. mutans* cells

To begin to evaluate the role of LrgAB and CidAB in *S. mutans*, we constructed a series of mutant derivatives in the *lrg* and *cid* genes by replacing all or most of the structural genes with antibiotic resistance markers by using either a non-polar (NP) or polar (P) marker to inactivate a single gene or the entire operon (Table 1). A hallmark phenotype caused by the deficiency of *lrg* and *cid* in *S. aureus* is altered murein hydrolase activity (Bayles, 2000, 2003, 2007; Groicher *et al.*, 2000; Rice *et al.*, 2003; Rice & Bayles, 2008). To determine whether CidAB or LrgAB could impact lysis of *S. mutans*, autolysis assays were performed. Compared with the wild-type strain, the *lrgA* single mutant displayed enhanced autolysis and the *lrgB* single mutant displayed decreased autolysis (Fig. 2a). Within the first 6 h of the assay, the optical density (600 nm) of the cell suspension was reduced by 83 % in the *lrgA* mutant and 59 % in the *lrgB* mutant, whereas the wild-type cells showed a 70 % reduction. Inactivation of *lrgAB* (Fig. 2a) as well as *cidAB* genes (data not shown) did not have a discernible effect on autolysis.

Given that autolysis has been implicated in the ability of bacteria to develop biofilms, we evaluated whether loss of the *lrg* or *cid* genes in *S. mutans* affected its capacity to form biofilms. Interestingly, the ability of both *lrgA* and *lrgB* mutants to form biofilms was dramatically reduced in BM–glucose medium, compared with that of the parental strain (Fig. 2b). In BM–sucrose medium, the *lrgA* mutant displayed a dramatic reduction in biofilm formation ($P<0.05$, Student's *t*-test), whereas the *lrgB* mutant formed biofilms as efficiently as the wild-type strains (Fig. 2b). Sucrose-dependent biofilm accumulation in *S. mutans* is mediated primarily by the production of glucan polymers from sucrose via specialized glycosyltransferase (Gtf) enzymes. Binding to these glucans is mediated by the Gtfs themselves, as well as by multiple glucan binding proteins (Yamashita *et al.*, 1993). To examine whether the differences in the *lrgA* and *lrgB* mutants to form biofilms were due to altered levels of Gtfs, GtfB and GtfC production in these strains was monitored in mid-exponential phase BHI cultures via Western blot analysis by using an anti-GtfB serum (Ahn *et al.*, 2007; Wunder & Bowen, 2000). The amount of GtfB and GtfC production under these growth conditions was decreased in the *lrgA* mutant, whereas the *lrgB* mutant displayed no apparent difference in the amount of these enzymes, compared with the wild-type strain (Fig. 2c). These results correlate with the impaired ability of the *lrgA* mutant (but not the *lrgB* mutant) to form biofilm in the presence of sucrose (Fig. 2b). In addition to a lack of effect on autolysis, no obvious differences in biofilm formation and GtfB or GtfC levels were noted in the *cid* mutants (data not shown). It is unclear from these results why glucose-dependent biofilm formation is more greatly affected compared with sucrose-dependent biofilm formation in the *lrgA* and *lrgB* mutants. The release of extracellular genomic DNA (eDNA) via cell lysis is important for biofilm attachment and development (Mann *et al.*, 2009; Perry *et al.*, 2009), and this process is influenced by the Cid/Lrg system. Therefore, it is possible that the *lrgA* and *lrgB* gene products are affecting cell lysis and/or eDNA release during glucose-dependent biofilm development. Although there is only limited evidence to support a role for eDNA release in *S. mutans* biofilm formation (Perry *et al.*, 2009), we are currently investigating how *lrg* and *cid* might influence eDNA production and whether this could contribute to the observed phenotypes.

The Cid/Lrg system is required for efficient aerobic growth of *S. mutans*

Given that previously published microarray data showed that oxygenation profoundly regulates expression of *lrg* and *cid* in an opposing manner (Ahn *et al.*, 2007), we evaluated whether oxygen affects the growth of the *lrg*- or *cid*-deficient mutants in this study. Surprisingly, when the strains were cultured in an aerobic incubator on BHI agar plates, growth of the Δ*lrgAB*, Δ*cidAB* or Δ*cidB* mutants was almost completely inhibited (Supplementary Fig. S2). These growth defects were also observed in liquid media (data not shown). However, when the strains were cultured on solid BHI medium in an anaerobic jar, growth of all mutants was as robust as that of the wild-type strain (data

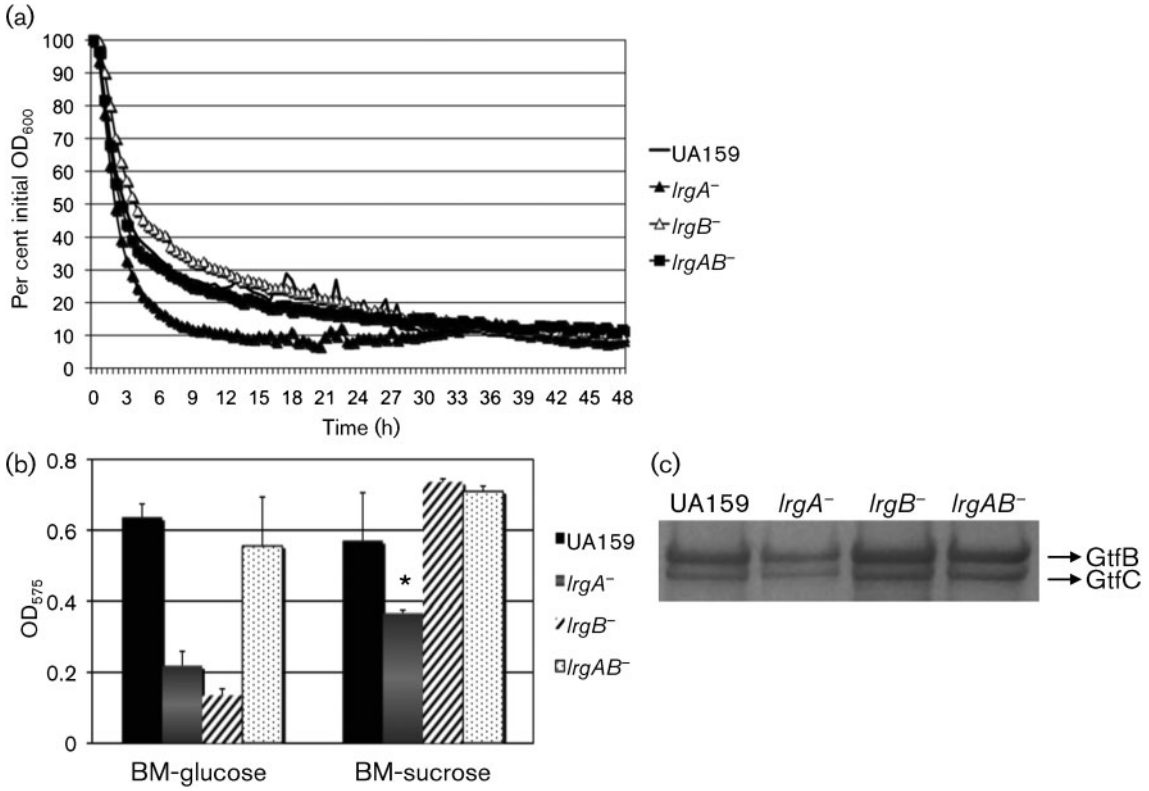

Fig. 2. Autolytic potential and biofilm-forming activity of *S. mutans* wild-type and its derivatives. (a) Autolysis assay. The autolytic activities of strains were monitored at 44 °C in a Bioscreen C system that was set to shake for 15 s before measurement every 30 min. Data are representative of three independent experiments and are presented as an average of triplicate samples. (b) Biofilm formation. Cultures were grown in BM medium supplemented with glucose or sucrose for 24 h. Data are representative of at least two separate experiments performed at least in triplicate. Error bars represent SD. *$P<0.05$; Student's *t*-test. (c) Production of GtfB and GtfC. Western blot analysis was performed by using bead-beaten, SDS-boiled extracts from *S. mutans* wild-type and its derivatives. Following SDS-PAGE, proteins were blotted onto an Immobilon P membrane and subjected to Western blotting with an anti-GtfB antiserum at a dilution of 1 : 500. Data are representative of at least three independent experiments. See text for details.

not shown). Notably, a deficiency of either *lrgA* or *lrgB* alone had no apparent effect on aerobic growth, compared with the wild-type strain (Supplementary Fig. S2). In contrast, deficiency of CidAB, or of CidB alone, critically impaired the growth of *S. mutans* in an aerobic environment, whereas the effect of a *cidA* mutation on aerobic growth of the organism was negligible (Supplementary Fig. S2). Therefore, the *cid* and *lrg* gene products, which are differentially regulated by aeration, appear to function in a way that augments the survival and growth of *S. mutans* in an oxidizing or oxygen-rich environment.

Deficiency of *cid* or *lrg* impacts oxidative stress tolerance by *S. mutans*

To determine whether the aerobic growth defects observed in the mutant strains were related to the capacity of cells to tolerate oxidative stress, the growth of each mutant was monitored under relatively anaerobic conditions (oil overlay) by using BHI medium containing hydrogen peroxide or paraquat. When the strains were grown in the presence of a low concentration of hydrogen peroxide (0.001 %, v/v), no obvious differences were observed in the growth of wild-type and all mutant strains (data not shown). In contrast, growth of the Δ*lrgAB*, Δ*lrgB*, Δ*cidAB* and Δ*cidB* mutants was profoundly affected by the presence of paraquat (10 mM), a superoxide anion-generating agent (Fig. 3). Although the *lrgA* mutant also displayed a growth defect (Fig. 3a), this was apparently not due to the presence of paraquat, as this mutant displayed similarly poor growth in the absence of paraquat (data not shown). These results suggest that the *cid*/*lrg* operons are required for growth of *S. mutans* during oxidative stress, and particularly that the *lrgB* and *cidB* gene products may be more important for stress tolerance compared with the *lrgA* and *cidA* gene products.

Acid tolerance is a major virulence attribute of *S. mutans* and we recently determined that growth under aerobic

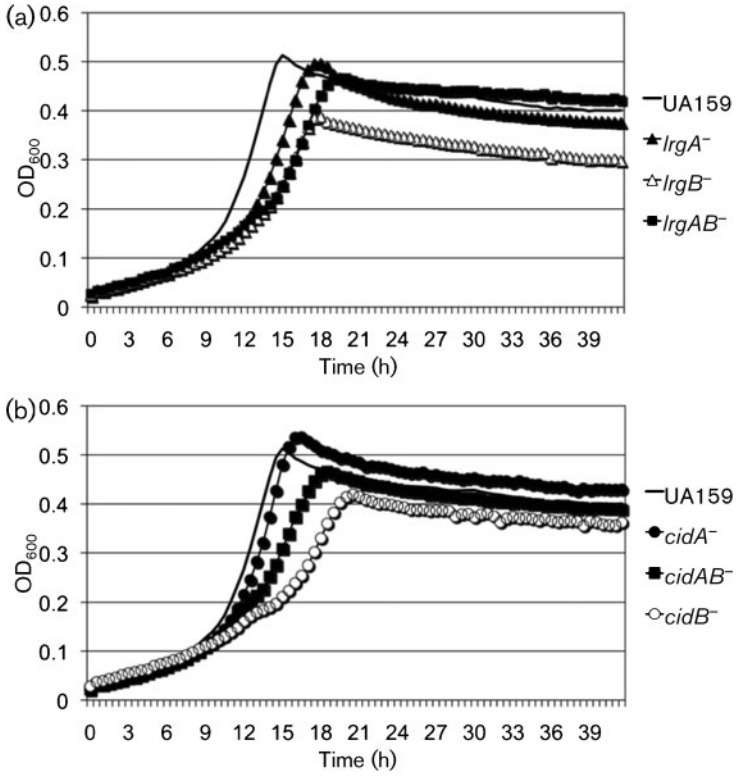

Fig. 3. Growth curves of *S. mutans* wild-type and its *lrg* (a) and *cid* (b) derivatives under oxidative stress. Strains were grown in BHI medium containing 10 mM paraquat under anaerobic conditions. Growth was monitored in a Bioscreen C system that was set to shake for 15 s every 30 min. For anaerobic growth, sterile mineral oil (50 μl) was placed on top of the broth cultures. The results are representative of two independent experiments.

conditions significantly increased the susceptibility of *S. mutans* to acid stress (Ahn *et al.*, 2009). Given the importance of *cid* and *lrg* for growth in the presence of oxidative stress, we investigated the impact of inactivation of these genes on acid tolerance. No differences were observed in the abilities of the strains to grow at pH 6.0, and all strains, including *S. mutans* UA159, did not grow well at pH 5.5 under the conditions tested (data not shown). Thus, the Cid/Lrg system, and in particular LrgB and CidB, may be important for tolerance of oxidative stress during aerobic growth of *S. mutans*, but these gene products do not appear to be required for growth at low pH under these experimental conditions (data not shown).

Expression of *lrg* and *cid* is growth-phase dependent

To begin to elucidate the factors influencing *lrg* and *cid* expression, we monitored *lrg* and *cid* mRNA levels during the growth cycle of planktonic cultures of UA159 by real-time PCR. Expression of *cid* and *lrg* was also evaluated in a *lytS*-deficient strain, as the LytSR TCS is known to regulate expression of *lrgAB* in *Staphylococcus aureus* (Groicher *et al.*, 2000). Strikingly, the levels of *lrgA* mRNA increased over 1000-fold during growth of the wild-type strain, with maximal expression occurring in late exponential and stationary phases (Fig. 4). In contrast, *cidA* mRNA levels were most abundant in early exponential phase, and then decreased more than 10-fold as cells entered the late exponential phase of growth. Fig. 4 also shows that lack of LytST resulted in lower basal levels of *lrgAB* expression and a complete lack of induction of the *lrg* genes later in the growth cycle. In contrast, no significant effect on the expression of the *cid* genes was observed in the *lytST* mutant. Given that the *lrgAB* operon is located immediately downstream of the *lytST* genes (Fig. 1), the possibility that the *lytST* mutation may have had a polar effect on *lrgAB* expression was ruled out by using RT-PCR to show that the *lytST* and *lrgAB* operons were not co-transcribed (data not shown). Together, these results suggest that *lrg* and *cid* expression is growth-phase dependent and that *lrgAB* expression is tightly controlled by LytST.

Expression of *lrg* and *cid* is regulated by glucose

Based on the observation that the *lrg* and *cid* genes are differentially expressed throughout the growth cycle in BHI medium, which contains 0.3 % glucose (w/v), we hypothesized that depletion of glucose could stimulate changes in *cid* and *lrg* gene expression during the transition from early exponential phase to stationary phase. In this respect, it is noteworthy that the *Bacillus subtilis ywbH* and *ywbG* genes, predicted homologues of *cidA* and *cidB*, were induced when the organism was grown in the presence of 1 % (55.5 mM) glucose (Moreno *et al.*, 2001), and that the *cidABC* operon of *Staphylococcus aureus* was induced in 35 mM glucose (Rice *et al.*, 2005). We examined whether glucose concentration influences the expression of *lrg* and *cid* by Northern blot analysis of RNA samples isolated from UA159 cultures grown for 6 h, corresponding to late exponential phase, in the presence of increasing concentrations of glucose (Fig. 5a). Interestingly, the *cidB* probe hybridized to three distinct

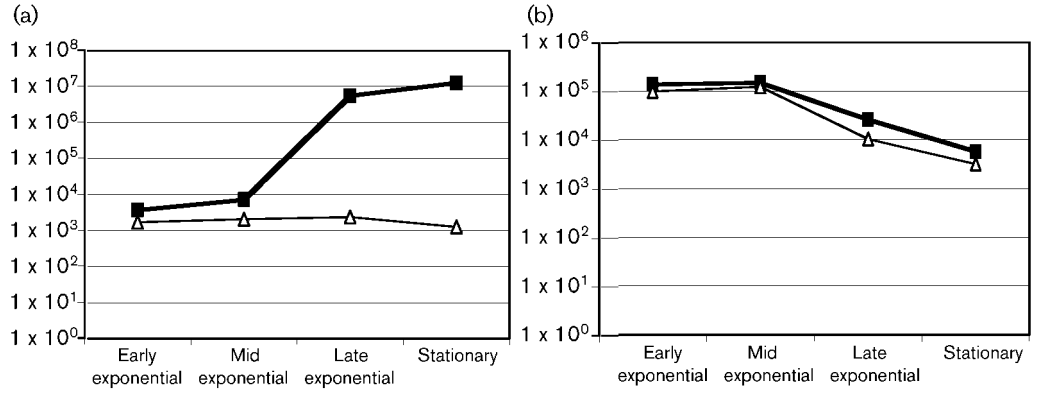

Fig. 4. Expression of *lrgA* and *cidA* with growth phase. The expression of *lrgA* (a) and *cidA* (b) genes was measured in UA159 (■) and its isogenic *lytST* mutant (△) in the early (OD_{600}=0.2), mid (OD_{600}=0.5), late (OD_{600}=0.9) exponential and stationary phases of growth, using real-time RT-PCR. Results are the average of triplicate samples from three independent experiments.

transcripts (1.1, 2.9 and 5.1 kb), of which the 1.1 kb transcript closely matched the predicted length of *cidAB* (1.045 kb). Subsequent Northern blot analysis with probes specific for *cidA* and the genes located immediately upstream and downstream of *cidAB* revealed that this locus comprises multiple overlapping transcripts: a 2.9 kb transcript contains *cidA*, *cidB* and two predicted ORFs (SMU.1697 and SMU.1699) located immediately downstream of *cidAB*, whereas the 5.1 kb transcript comprises these four genes and two predicted ORFs (SMU.1702 and SMU.1703) located immediately upstream of *cidAB* (data not shown). The amount of the transcript arising from the two dicistronic operons, *cidAB* and SMU.1697/1699, is similar to that of the *cidAB* transcript alone, indicating that transcription from the *cidAB* promoter contributes significantly to the expression of SMU.1697/1699.

Expression of *lrg* and *cid* was regulated in an opposite manner in response to glucose levels (Fig. 5a). The *lrgAB* genes were highly induced in cultures containing lower levels of glucose (11 and 15 mM) and were dramatically repressed in cultures containing glucose at concentrations of 20 mM and higher, although detectable expression persisted at concentrations of up to 45 mM. In contrast, all three overlapping transcripts of the *cid* locus were nearly undetectable when cells were cultured in the presence of lower glucose concentrations ($<$20 mM), but increased dramatically at higher glucose concentrations ($\geqslant$20 mM). Thus, expression of the *lrg* and *cid* genes is glucose-dependent in an opposite manner, and two dicistronic operons located immediately upstream and downstream of the *cid* locus are coordinately regulated and co-transcribed with the *cid* operon.

Expression of *lrg* and *cid* is under the control of CcpA

Given that *cid* and *lrg* expression is highly responsive to glucose levels in *S. mutans*, we investigated the involvement of CcpA (carbon catabolite protein A) in the regulation of *lrg* and *cid* genes. CcpA in *S. mutans* has been shown to serve as a major regulator of the expression of glycolytic and tricarboxylic acid (TCA) cycle enzymes, the pyruvate dehydrogenase complex, carbohydrate transporters and various catabolic pathways (Abranches *et al.*, 2008). It was also noted in a previous microarray study from our group (Abranches *et al.*, 2008) that expression of *lrg* and *cid* could be influenced by loss of CcpA and affected by growth under conditions that alleviate catabolite repression in *S. mutans*. Subsequently, we measured the expression of *lrg* and *cid* in the presence of the lower (11 mM) and higher (45 mM) levels of glucose by using Northern blot analysis (Fig. 5b). In medium containing the lower level of glucose, *cid* transcription was maximal in early exponential phase (2 h growth), whereas *lrgAB* was highly expressed in late exponential (6 h growth) and late stationary (12 h growth) phases, consistent with the results in Fig. 5, which were determined under similar conditions. Furthermore, the *cidAB* transcript (1.1 kb) as well as the other overlapping transcripts (2.9 and 5.1 kb) were differentially upregulated at all time points in media containing high levels (45 mM) of glucose (Fig. 5b, top).

In contrast with the observed effects of glucose on *cid* transcription, expression of the *lrgAB* operon was strongly repressed in stationary phase in the presence of 45 mM glucose (Fig. 5b, bottom). Interestingly, *cid* expression was upregulated in the *ccpA* mutant when grown in 11 mM glucose, relative to the parental strain UA159 (Fig. 5b, top), whereas *lrgAB* expression was similar between the two strains under these growth conditions. These results correlate well with recently published microarray data showing that *cidB* expression was upregulated threefold in the *ccpA* mutant of *S. mutans* (Abranches *et al.*, 2008). Also of note, the *cidAB* transcript (1.1 kb) was markedly increased as the *ccpA* mutant culture entered the late exponential phase of growth (6 h), and decreased to nearly undetectable levels in late stationary phase (12 h growth). However, when these strains were grown in 45 mM glucose, *lrgAB* expression was increased in the *ccpA* mutant

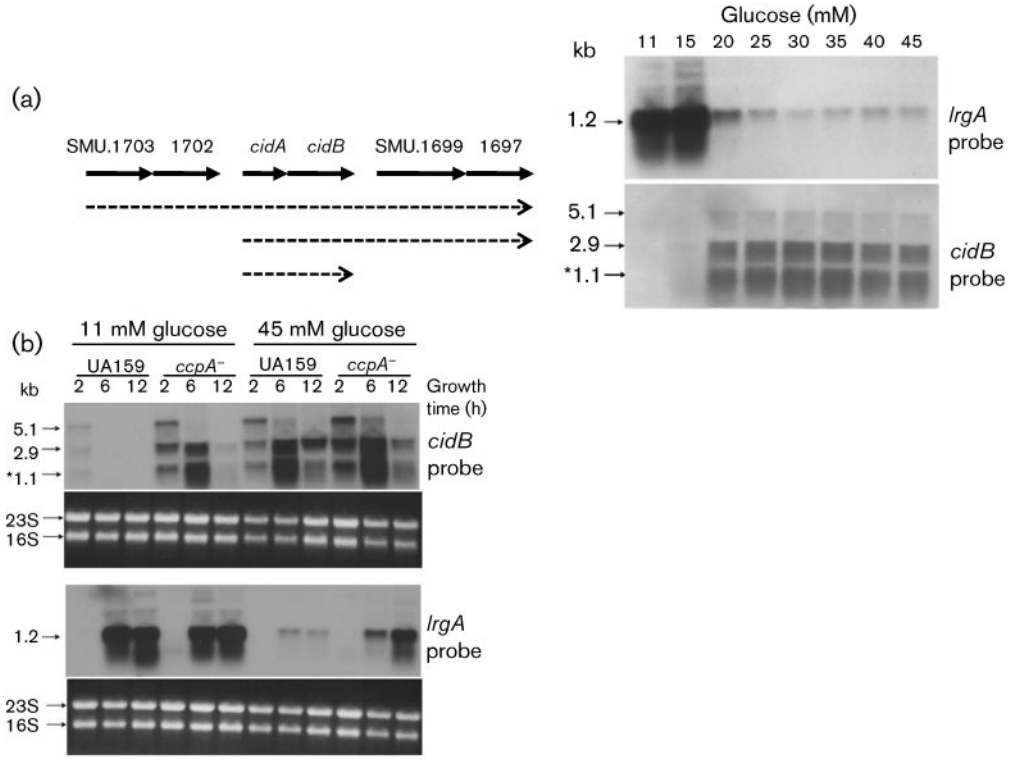

Fig. 5. Analysis of the effect of glucose concentration (a) and CcpA (b) on expression of the *S. mutans cid* and *lrg* operons by Northern blot. Total RNA was isolated from *S. mutans* UA159 cultures grown for 6 h in the presence of increasing concentrations of glucose (a), and UA159 and *ccpA*$^{-}$ (TW1) cultures grown in the presence of either 11 or 45 mM glucose, at 2 h (early exponential growth), 6 h (late exponential phase) or 12 h (late stationary phase) (b). RNA (5 μg) from each sample was subjected to Northern blotting with DIG-labelled DNA probes corresponding to either *lrgA* or *cidB*. The size of each transcript is indicated to the left of each blot, and is also shown in a schematic diagram of the *cid* locus by the dotted arrows. The 1.1 kb *cidAB* transcript is indicated by an asterisk. The corresponding ethidium-bromide-stained gel is presented beneath the blots with arrows highlighting the 23S and 16S RNA gene bands.

relative to UA159, whereas no apparent differences in *cid* expression were observed (Fig. 5b, bottom). It is not clear whether this CcpA-dependent regulation is due to direct interaction of CcpA with the *lrg* and *cid* promoters, or if this regulation is an indirect result of altered expression of other regulatory genes or due to an effect on glucose metabolism. In this respect, it is interesting to note that there appear to be two *cre*-like consensus elements, which are binding sites for CcpA (Miwa *et al.*, 2000), in the DNA sequence immediately upstream of *lrgAB* (Fig. 6), suggesting that CcpA may directly regulate expression of *lrgAB*. However, identifiable *cre* elements were not apparent in the sequences upstream of *cidAB* and *lytSR* (data not shown). Collectively, these results demonstrate that growth phase, glucose metabolism and CcpA are important factors that regulate expression of the *S. mutans cid* and *lrg* genes.

DISCUSSION

As part of an ongoing effort to identify the regulatory pathways controlling autolysis of *S. mutans*, we identified two dicistronic operons, *lrgAB* (SMU.575/574) and *cidAB* (SMU.1701/1700) (Ahn *et al.*, 2007), encoding products with similarities to bacteriophage holin : antiholin proteins that modulate cell lysis (Bayles, 2000, 2003; Rice & Bayles, 2003; Young, 1992; Young & Blasi, 1995). The present study shows that the *S. mutans* CidAB and LrgAB proteins play important roles in biofilm formation, oxidative stress tolerance and regulation of autolysis. Also of note, expression of these two operons is tightly regulated by multiple systems, including the LytST signal-transduction complex and CcpA. Furthermore, *cid*/*lrg* transcription is highly responsive to environmental stimuli, particularly oxygen and glucose. Given that oxygen levels have a profound effect on the phenotypic properties of *S. mutans* through metabolic changes (Ahn *et al.*, 2009), the exact mechanism by which *cid* and *lrg* gene products impact the virulence of this organism in the presence of oxygen and glucose remains unclear. However, the results presented herein show that the *lrgAB* and *cidAB* gene products probably interact to promote survival in a non-preferred oxidative environment (Fig. 3 and Supplementary Fig. S2). Additionally, the *lrgAB* gene products appear to play an

cre consensus: WTGNAANCGNWNNCW

lrgAB promoter region:
AGTTGGATATTGCAATTCAGCTTCTTTTTT**TTG**C**AA**T**CG**GGGA**CA**GTT**TTG**A**A**
AACGTTTTA**CA**CAAAATGAGCTACAATAACATTGTCAAGAAAGGAAGGAGTCG
TTCGTATT***ATG***

Fig. 6. Identification of *cre* sequences upstream of *lrgAB*. The *B. subtilis cre* consensus sequence derived by Miwa *et al.* (2000) is shown at the top, where W=A or T, and N=any nucleotide. The nucleotide sequence corresponding to 114 bp upstream of the ATG start codon (identified in bold italics) of the *S. mutans lrgA* gene is shown at the bottom. The putative *cre* elements are underlined and conserved nucleotides are shown in bold.

important role in modulating virulence behaviour, including biofilm formation and autolysis, of *S. mutans* (Fig. 2). The results presented in this study highlight some fundamental differences between the Lrg/Cid systems of *S. mutans* and *Staphylococcus aureus*, the latter having been more intensively studied to date (Groicher *et al.*, 2000; Rice *et al.*, 2003). Namely, a role for the *cid* gene products in regulating cell autolysis and biofilm formation was not demonstrated for *S. mutans* in the present study. However, recent analysis of *cid* and *lrg* homologues in *Bacillus anthracis* found that inactivation of either *lrgAB* or *clhAB* did not appear to affect murein hydrolase activity or autolysis, but instead altered sporulation efficiency (Chandramohan *et al.*, 2009). Collectively, these studies suggest that the Cid/Lrg systems may have evolved to respond to environmental, metabolic and developmental signals depending on the lifestyle of a particular organism.

Based on the results of the current study, we provide a working model for the role and regulation of the *S. mutans cid/lrg* systems (Fig. 7). Primarily, Lrg and Cid levels are regulated at the transcriptional level in a growth-phase-dependent fashion that is integrated with availability of oxygen and glucose. In early exponential phase, where levels of oxygen and glucose are elevated, *cid* expression is dominant and *lrg* is repressed. As cells enter stationary phase, or in oxygen/glucose-limited cells, *lrg* seems to be de-repressed and activated through the LytST complex, whereas *cid* expression diminishes under these conditions. In this way, the expression levels of *lrg* and *cid* are counter-balanced throughout the growth cycle. Given that *Staphylococcus aureus cid* and *lrg* expression is regulated by acetic acid production during metabolism of excess glucose (Rice *et al.*, 2005), it is possible that metabolic acids and/or low pH produced by *S. mutans* glucose fermentation act as the actual signals that regulate *cid*/*lrg* expression. We are currently investigating the effect and role of these acidic metabolites (i.e. lactate, acetate and formate) on *S. mutans cid*/*lrg* expression. However, it should be noted that the *S. mutans lrg* and *cid* mutant strains did not differ in their ability to grow at low pH, suggesting that the function of these gene products does not appear to be influenced by low pH.

Although certain aspects of this working model have yet to be tested directly, we propose that this regulatory pathway integrates environmental signals to control whether *S. mutans* is destined for active growth or for cell death/lysis in response to multiple environmental inputs, a process that is critical for the development of pathogenic biofilms. Although the *cid* and *lrg* genes were found to be regulated by CcpA in this study, the basis for this observation is still under investigation. Given that putative *cre* sites were identified in the *lrg* promoter region but not in the *cid* promoter region, it is likely that CcpA directly regulates *lrgAB* expression and indirectly regulates *cid* expression. As

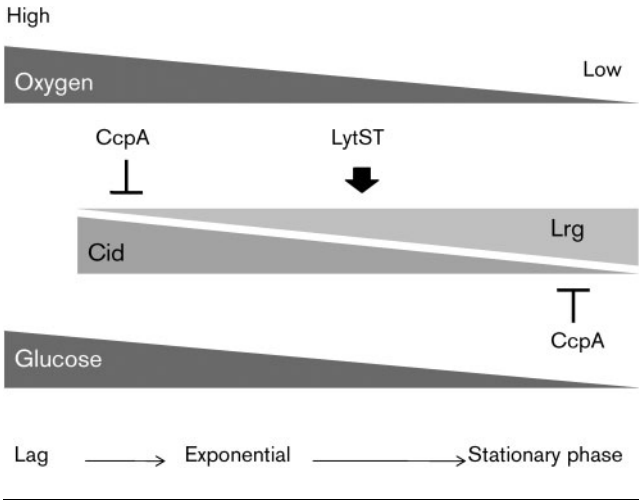

Fig. 7. Working model for glucose and oxygen-dependent regulation of the *S. mutans* Cid/Lrg system. Lrg and Cid activities are regulated in a growth-phase-dependent fashion by oxygen and glucose levels, which would change dramatically as cells progress from early exponential to stationary phase. As cells actively grow (high levels of oxygen and glucose available), *cid* expression is dominant but *lrg* is minimal and probably under the control of CcpA. As cells enter stationary phase (oxygen/glucose-limited cells), *lrg* expression is dramatically increased by the LytST TCS. In contrast, *cid* expression is gradually diminished, in part due to CcpA-dependent regulation. The expression level of *lrg* and *cid* seems to be tightly balanced throughout growth, and direct interactions between Lrg and Cid proteins may be involved in achieving this balance. Therefore, by sensing multiple environmental inputs and modulating the balance between Lrg and Cid, *S. mutans* is able to modulate growth and biofilm formation, which are critical for virulence expression by this pathogen.

CcpA in *S. mutans* has been shown to serve as an important regulator of the expression of glycolytic and TCA cycle enzymes, carbohydrate transporters and catabolic pathways (Abranches *et al.*, 2008), there are a number of possible ways by which CcpA deficiency could indirectly affect *cid* gene expression. For example, the CcpA-deficient strain may display alterations in *lrgA* expression that would affect *cid* transcription, or cause changes in metabolic end products, for example acetate, that could affect expression of *cid* genes. Alternatively, loss of CcpA may also affect the expression of transcriptional regulators that influence *cid* expression.

Clearly, *lrg* expression, but not that of *cid*, is under the tight control of LytST TCS. The primary regulatory role of LytST appears to be to activate *lrg* transcription in late exponential or stationary phase (Fig. 4), most likely by binding the *lrg* promoter region. Recently, by using a generic phylogenic footprinting/shadowing approach, the LytTR-family of response regulators was found to be associated with a specific operator motif consisting of direct repeats separated by 10–11 nt (de Been *et al.*, 2008; Francke *et al.*, 2008). A conserved motif (TGCAATTCAG-N11-TGCAATCGGG) was identified in the promoter region of *lrgAB* in *S. mutans*. By analysing the operator sequence identified in low-G+C Gram-positive bacteria, LytSR was also found to be involved in affecting or regulating transport of carbohydrates, peptides and/or amino acids (de Been *et al.*, 2008), supporting our idea that activation of *lrg* genes by LytST is controlled by carbohydrate levels or metabolism. Given our previous microarray data that *lytST* is highly responsive to oxygen, the Cid/Lrg system appears to be hierarchically regulated in a signalling network involving carbohydrate availability and/or oxygen.

Additional interesting findings presented here are that Northern blot analysis with *cid* probes revealed three overlapping transcripts, and that the levels of these transcripts were coordinately regulated in response to the concentration of glucose in the growth medium. In particular, the amount of the 1.1 kb (*cidAB*) and 2.9 kb (*cidAB*–SMU.1699/1697) transcripts was very similar, which suggests that the *cid* operon may be regulated by, or functionally connected to, the dicistronic operon (SMU.1699/1697) located immediately downstream of *cid*. Interestingly, SMU.1697 is predicted to encode a putative rRNA SAM-dependent methyltransferase. These enzymes catalyse many diverse reactions, including methylation, isomerization, sulfur insertion, ring formation, anaerobic oxidation and protein radical formation (Sofia *et al.*, 2001). As *cid* expression appears to be regulated by growth phase, oxidative stress and glucose metabolism, it is possible that these downstream ORFs participate in redox reactions that provide some protection to the cells. Importantly, however, the expression of these genes was not altered in the *cidA* or *cidB* mutants examined in these studies (data not shown), so the phenotypes described for these mutants are attributable to loss of CidA or CidB and not to polar effects on other genes. Studies are ongoing to determine whether the genes in this larger operon contribute to the function of the *cid* gene products and stress tolerance.

Based on the results presented here, it is not clear whether the Cid and Lrg systems of *S. mutans* are true holin : antiholin pairs. Specifically, inactivation of the *cid* genes did not have an opposing effect on the phenotypes measured in this study (growth rate, autolysis and biofilm formation) relative to inactivation of the *lrg* genes. Nevertheless, the fact that these genes display opposite patterns of expression in response to growth phase, glucose concentration, oxygenation (Ahn *et al.* 2007) and regulation by CcpA suggests that the functions of these gene products are interrelated and potentially antagonistic in *S. mutans*. Interestingly, a computational analysis via STRING (http://string.embl.de/), a database of known and predicted protein interactions, presents possible interactions of LrgA with LrgB or CidB (data not shown). In fact, loss of *cidB* was shown to reverse the effects of a *cidA* mutation (Fig. 3b). Moreover, autolysis is enhanced in an *lrgA* mutant and diminished in an LrgB-deficient strain. Thus, various possibilities exist for how LrgAB and CidAB may interact to regulate their biochemical activities and expression of these genes. Nonetheless, the data suggest that Lrg/Cid interations are critical for homeostasis in *S. mutans*. Indeed, a *cidB* mutation does not seem to be complemented by simply producing CidB *in trans*, as the *cidB* mutant strain harbouring a shuttle plasmid pMSP3535–CidB$^+$ was still not able to grow on BHI agar plates under aerobic conditions (data not shown). It is also noteworthy that *lrg* expression was not induced in late exponential phase (when grown in BHI) when expression of *cidAB* genes was constitutively driven by the *ldh* promoter of *S. mutans* (unpublished data). Given that the *lrg* and *cid* mutants displayed very different phenotypes in the assays described above, and that they are regulated in an opposite manner in response to environmental conditions, the levels of *cid* and *lrg* gene products are critical and they may interact in as yet unknown ways. This may result in cross-regulation of these two operons, possibly explaining why complementation was not achieved in the experiment described above. Thus, the stoichiometry of these proteins in the cell appears to be critical. We are currently investigating whether there are any direct interactions between Lrg and Cid proteins and probing in more detail the potential for cross-regulation of expression of the *lrg* and *cid* operons.

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