molecular oral microbiology

molecular oral microbiology

Association of CiaRH with resistance of *Streptococcus mutans* to antimicrobial peptides in biofilms

Y. Mazda[1,2], M. Kawada-Matsuo[1], K. Kanbara[1], Y. Oogai[1], Y. Shibata[3], Y. Yamashita[3], S. Miyawaki[2] and H. Komatsuzawa[1]

1 Department of Oral Microbiology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan
2 Department of Orthodontics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan
3 Section of Preventive and Public Health Dentistry, Division of Oral Health, Growth and Development, Kyushu University Faculty of Dental Science, Kyushu, Japan

Correspondence: Hitoshi Komatsuzawa, Department of Oral Microbiology, Kagoshima University Graduate School of Medical and Dental Sciences, Sakuragaoka 8-35-1, Kagoshima City, Kagoshima 890-8544, Japan Tel.: +81 99 275 6150; fax: +81 99 275 6158; E-mail: hkomatsu@dent.kagoshima-u.ac.jp

Keywords: cathelicidin; defensin; innate immunity; two-component system
Accepted 17 December 2011
DOI: 10.1111/j.2041-1014.2012.00637.x

SUMMARY

Streptococcus mutans **is a cariogenic pathogen in humans. To persist in the oral cavity, *S. mutans* is resistant against several antibacterial factors derived from the host. In this study, we investigated the mechanism of resistance to cationic antimicrobial peptides (AMPs), which are innate immune factors in humans. Because *dltA-D* (teichoic acid biosynthesis) was reported to affect the susceptibility to AMPs in other bacterial species, we evaluated the susceptibility of a *dltC* knockout mutant of *S. mutans* to the AMPs human beta-defensin-1 (hBD1), hBD2, hBD3 and LL37. The *dltC* mutant exhibited significantly increased susceptibility to AMPs. Regulation of *dltC* expression involved CiaRH, a two-component system. Expression of *dltC* in the wild-type strain was significantly increased in biofilm cells compared with that in planktonic cells, whereas expression was not increased in a *ciaRH* knockout mutant. In biofilm cells, we found that susceptibility to LL37 was increased in the *ciaRH* mutant compared with that in the wild type. From these results, it is concluded that Dlt is involved in the susceptibility of *S. mutans* to AMPs and is regulated by CiaRH in biofilm cells.**

INTRODUCTION

Streptococcus mutans is one of the commensal bacteria in the human oral cavity and is known to be cariogenic (Hamada & Slade, 1980; Kuramitsu, 1993; van Houte, 1994). *Streptococcus mutans* can attach to the smooth surfaces of teeth and form biofilms, known as dental plaque, with other oral bacteria. Biofilms are considered to be barriers against physical and chemical factors (Roberts & Mullany, 2010). In the oral cavity, oral bacteria are exposed to various host immune factors, lysozyme, antimicrobial peptides, lactoferrin and complement, derived from saliva and serum (Hamada & Slade, 1980; Tenovuo *et al.*, 1991). To persist in the oral cavity, *S. mutans* needs to express factors to resist the effects of host immune factors.

Recently, several bacterial species have been shown to possess the ability to resist the effects of cationic antimicrobial peptides (AMPs) produced by

humans, such as defensins and LL37 (Peschel *et al.*, 2001; Abachin *et al.*, 2002; Poyart *et al.*, 2003; Jin *et al.*, 2004; Kristian *et al.*, 2005; Li *et al.*, 2007b). AMPs are one of the innate immune factors (Lehrer & Ganz, 1999; Zaiou & Gallo, 2002) and are found in saliva and periodontal tissues (Hosokawa *et al.*, 2006; Gorr & Abdolhosseini, 2011). These peptides are attracted to the weak negatively charged bacterial membrane and then form pores or disrupt the membrane, which then has a bactericidal effect (Komatsuzawa *et al.*, 2007). Among factors related to AMP resistance, bacterial surface charge is critical for susceptibility to AMPs. In *Staphylococcus aureus*, *dlt* and *mprF* have been demonstrated to be involved in determining surface charge (Peschel *et al.*, 1999, 2001). The *dlt* operon, encoding five gene products (including a putative DltX), is responsible for alanine incorporation into teichoic acid, whereas MprF is responsible for lysine addition to phosphatidylglycerol, a major component of cell membranes. These factors result in the addition of positive charge to the cell surface, which causes weakening of the negative charge of the cell surface. Additionally, a two-component system (TCS), designated ApsRS, was demonstrated to regulate these two factors in *Staph. aureus* (Li *et al.*, 2007a; Kraus *et al.*, 2008). The TCS is composed of a histidine kinase, for sensing stimuli such as pH, osmolarity and antibacterial agents, and a response regulator for regulating the expression of several genes for adaptation to the stimuli (Hoch, 2000).

In studying mechanisms of *S. mutans* persistence in the oral cavity, we focused on the interaction of *S. mutans* with antimicrobial peptides. Here, we demonstrated that the TCS named CiaRH was associated with the regulation of *dlt* in *S. mutans* biofilms. CiaRH is one of 15 sets of TCS, including orphan TCS (Ahn *et al.*, 2006). It is composed of three factors, including an additional factor CiaX, which has the ability to bind calcium (He *et al.*, 2008). Previously, CiaXRH was shown to be related to mutacin production, competence development, stress tolerance, biofilm formation and acid tolerance (Ahn *et al.*, 2006; Lévesque *et al.*, 2007; Liu & Burne, 2009). In this study, we show a new aspect of CiaXRH that is involved in the susceptibility to antimicrobial peptides in *S. mutans* biofilms.

METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Streptococcus mutans* and *Escherichia coli* were grown in trypticase soy broth (TSB; BDH Systems, Cockeysville, MD, USA) and Luria–Bertani broth, respectively. When *S. mutans* was grown in biofilms, sucrose was added to the medium at 2% final concentration. Erythromycin was added at a final concentration of 10 μg ml^{-1} for *S. mutans* or 300 μg ml^{-1} for *E. coli* when necessary. Spectinomycin was added at a final concentration of 500 μg ml^{-1} for *S. mutans* or 50 μg ml^{-1} for *E. coli* when necessary.

Construction of *ciaRH* and *ciaH* knockout mutants

Knockout mutants of *S. mutans* UA159 and NCTC10449 were constructed using a method described elsewhere (Kawada-Matsuo *et al.*, 2009). Briefly, the erythromycin-resistance (Em^r) gene amplified with two specific primers from plasmid ResEm-Not (Shiroza & Kuramitsu, 1993) was cloned into pBluescript SK II (+). Each flanking region of the target gene (*ciaRH*) at both ends (*ciaR* upstream: 946 bp, *ciaH* downstream: 861 bp) was then amplified with specific primers from the *S. mutans* genome, and each fragment was cloned into both ends of the Em^r gene to generate an Em^r gene with the flanking region of the target gene. After amplification of the whole gene by polymerase chain reaction (PCR), the PCR fragment was transformed into *S. mutans*. By selection for erythromycin resistance, the mutants were isolated. The *ciaH* mutant was constructed using the same method as described above. Primers used for the construction are listed in Table 1. The *dltC* mutant in the UA159 strain was constructed previously (Shibata *et al.*, 2011).

For genetic complementation, we failed to construct a *ciaRH* complemented mutant for unknown reasons. We also used a *ciaR*-knockout mutant that was constructed previously (Kawada-Matsuo *et al.*, 2009). In this mutant, the *ciaR* gene was replaced with the Em^r gene. We verified that *ciaX* and *ciaH* were expressed in the *ciaR* mutant. We then constructed the DNA fragment to insert the gene containing the

Table 1 Strains and primers used in this study

Strains	Character	Reference
Streptococcus mutans		
UA159	Clinical strain, wild type	Murchison *et al.*, 1986
Δ*ciaRH*	*ciaRH* (SMU. 1129-28[1]) deletion mutant in UA159, Em^{r2}	This study
Δ*dltC*	*dltC* (SMU. 1689) deletion mutant in UA159, Em^r	Shibata *et al.*, 2011
Δ*ciaR*	*ciaR* (SMU. 1129) deletion mutant in UA159, Em^r	Kawada-Matsuo *et al.*, 2009
Δ*ciaR+*	*ciaR* complementation in *ftf* gene, Em^r, Spc^{r3}	This study
Δ*ciaH*	*ciaH* (SMU. 1128) deletion mutant in UA159, Em^r	This study
NCTC10449	Laboratory strain (ATCC 25175), wild type	Clarke, 1924
Δ*ciaRH*	*ciaRH* (SMU. 1129-28) deletion mutant in NCTC10449, Em^r	This study
Escherichia coli		
XL-II Blue	*end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac*F′ [*pro*AB *lac*IqZΔM15 Tn10 (Tet^r)]	Stratagene
M15(pREP4)	Host strain for the expression of His-tagged protein	Qiagen

Primers

	Primer, forward	Primer, reverse
Knockout mutant construction DNA fragment		
Em^r For Δ*ciaRH* mutant	gaaggaaacttaagagtg	ttatttcctcccgttaaa
Upstream of *ciaR*	cggggtaccgtgagaattgggcttgga	cggctcgagctattatagcatgacttgg
Downstream of *ciaH* For Δ*ciaH* mutant	cggactagtctcgcctgcttgctaata	cgggcggccgcaaaatggccagtctgc
Upstream of *ciaH*	cggctcgagcatgcaggtttttgatggtg	cgggtcgacctcatcctgcttttgttt
Downstream of *ciaH*	cggactagtctcgcctgcttgctaata	cgggcggccgccgtgctgaacttaacgtt
Knock-in mutant construction DNA fragment		
spc^r	acaggctcttcgttcgtgaatacatg	tactttctgtttccaccatttttttca
ciaR	gtggaaacagaaagtaaaaaaaggggt	gtcccatcatattctcatcctgcttt
Ftf-N-terminal	aatgatatcgtgtttgtt	cgaacgaagagcctgttctgttagac
Ftf-C-terminal	gagaatatgatgggactacaaaagtc	aaccaatgcttacacaga
Quantitative PCR Gene name		
ciaR (SMU. 1129)	gctgtatcaacggttaactcac	aggacacggttttgaattagg
dltC (SMU. 1689)	ggctgtattccaatcatcac	tgatgcaggtgttttagatagc
htrA	gacccgctcttggtatttca	cgctaccttcttcccatcaa
EMSA DNA fragment		
dlt-1	attattttaaggtatatttaaata	ataaattctccctgaccttga
dlt-2	atggcaatctgaatgagg	ataaattctccctgaccttga
ciaX-1	gtctcttccatgacagca	ctggttaatacagcttttg
ciaX-2	catggttagttagactaa	agccatcatccattttga
ciaR(for His-tag protein)	gcggatccataaagttattattagtagaag	cgcaagcttatattctcatcctgcttt
Race		
dltA-RACE	acgttctaaggctg-5′-phosphate	

[1]The GenBank locus tag was from the *S. mutans* UA159 genome at the Oral Pathogen Sequence Database site.
[2]Erythromycin resistance.
[3]Spectinomycin resistance.

spectinomycin-resistance (spc^r) gene and *ciaR* into the *ftf* gene coding for fructosyltransferase. First, spc^r, *ciaR*, the *ftf* partial gene at the N-terminal region, and the *ftf* partial gene at the C-terminal region were amplified with the specific primers. The primers added an extra eight nucleotides to anneal for each PCR fragment. The mixture of the *ftf* partial gene at the N-terminal region and spc^r was then heated at 95°C for 5 min and left for 30 min at 37°C. DNA polymerase and dNTPs were added to the mixture and allowed to react at 68°C for 15 min; then, PCR was performed using both ends of the primers. Finally, all the fragments were joined by PCR. The fragment was then transformed into the *ciaR* mutant in *S. mutans*. By selection for erythromycin and spectinomycin resistance, the complemented strain was

isolated. Finally, in the strain obtained, *spc*^{r} and *ciaR* gene insertion into the *ftf* gene was verified by PCR.

Analysis of gene expression by quantitative PCR

A small sample (10^8 cells) of *S. mutans* cultured overnight was inoculated into 10 ml fresh TSB with or without 2% sucrose. The *S. mutans* cells were then grown at 37°C with 5% CO_2, and bacterial cells in the planktonic or biofilm condition were collected. Total RNA was extracted from bacterial cells using a FastRNA Pro Blue kit (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Roche, Tokyo, Japan). Using cDNA as template DNA, quantitative PCR was performed with the MyiQ system (Bio-Rad, Tokyo, Japan). Gene expression was calculated from three independent experiments and the significance (*P*-value) was calculated using Student's *t*-test or Tukey's honestly significant difference. We also performed quantitative PCR using total RNA without cDNA synthesis to confirm no DNA contamination. Results were normalized against *gyrA* expression that was used as a housekeeping gene reference (Wu *et al.*, 2010). Primers used in this study are shown in Table 1.

Rapid amplification of cDNA ends (RACE)

To determine the promoter region of *dltA* in *S. mutans* UA159, RACE was performed. A sample of *S. mutans* UA159 (10^8 cells) cultured overnight was inoculated into 10 ml fresh TSB with 2% sucrose. The *S. mutans* cells were then grown at 37°C with 5% CO_2 for 16 h. Next, the bacterial cells were collected, and total RNA was extracted using the method described above. RACE was performed using a 5′-Full RACE Core Set (Takara Bio Inc., Ohtsu, Japan) according to the manufacturer's protocol. Primers used for the RACE experiment are listed in Table 1.

Electophoresis mobility shift assay (EMSA)

To clarify the binding of CiaR to the *dltA* promoter region, EMSA was performed. For the positive control, we also investigated binding to the *ciaX* promoter region, which was previously demonstrated as CiaR binding (Wu *et al.*, 2010). The promoter regions in *dltA* and *ciaX* with or without the predicted CiaR-binding sites were amplified with the specific primers listed in Table 1. DNA fragments at the 3′ end were labeled with digoxigenin (DIG) using DIG Gel Shift kit, Second Generation (Roche, Mannheim, Germany). Recombinant CiaR protein was constructed using the pQE system (Qiagen, Tokyo, Japan). The PCR fragment of the *ciaR* gene, amplified with specific primers (Table 1), was cloned into pQE30 vector using *Bam*HI and *Hind*III sites. The plasmid was electroporated into *E. coli* M15 harboring pREP4 and expression of protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested, suspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole), and were disrupted with an Ultrasonic disruptor (Tomy Seiko, Tokyo, Japan). After centrifugation at 25,000 ***g*** for 30 min, a cell-free lysate was obtained. Then, recombinant protein was purified according to the manufacturer's protocol.

Purified CiaR was phosphorylated using a method described elsewhere (Gao *et al.*, 2005). CiaR protein was incubated for 2 h at room temperature in 50 mM Tris–HCl (pH 8.0) containing 10 mM $MgCl_2$, 3 mM dithiothreitol and 32 mM acetyl phosphate. Each DIG-labeled fragment (1 ng) was reacted with various amounts of CiaR protein (6, 12.5, 25, 50 and 100 ng) in binding buffer. After native polyacrylamide gel electrophoresis through 6% acrylamide, DNA fragments were transferred to a positively charged nylon membrane (Roche, Mannheim, Germany), and then DNA fragments were visualized according to the manufacturer's protocol. The experiment was performed three times independently.

Assay to determine activity of antimicrobial peptides

An antibacterial assay was performed following a protocol described elsewhere (Midorikawa *et al.*, 2003). Briefly, *S. mutans* strains grown to exponential phase [optical density at 660 nm (OD_{660}) 0.3] or stationary phase (OD_{660} > 1.0) were harvested, washed with phosphate-buffered saline, and suspended in 10 mM sodium phosphate buffer pH 6.8. The bacterial suspension was diluted to 10^7–10^8 cells ml^{-1} with phosphate buffer, and 10 μl of the bacterial suspension (10^5–10^6 cells) was inoculated into 0.5 ml phosphate

buffer with or without antimicrobial peptides and incubated aerobically for 2 h at 37°C. Human β-defensin-1 (hBD1), hBD2 and hBD3 were purchased from Peptide Institute, Inc., Osaka, Japan. LL37 was purified using a method described elsewhere (Midorikawa *et al.*, 2003). Dilutions of the reaction mixture (100 μl) were plated on agar media and incubated at 37°C overnight. The colony-forming units were determined as the total number of colonies identified on each plate. The antibacterial effect was calculated from three independent experiments as the ratio of the number of surviving cells (survival rate, %) to the total number of bacteria incubated in control phosphate buffer solution after exposure to antimicrobial peptides.

Analysis of biofilm by confocal laser scanning microscopy (CLSM)

A sample (10^8 cells) from an overnight culture of *S. mutans* was inoculated into 5 ml fresh TSB containing 2% sucrose in a glass-bottomed dish. After 18 h at 37°C with 5% CO_2, biofilm cells were washed with phosphate-buffered saline and then stained with the BD Cell Viability kit (BD Biosciences, San Jose, CA, USA), which enables visualization of dead or live cells. CLSM was performed using a Carl Zeiss LSM700 microscope (Carl Zeiss MicroImaging Co. Ltd., Tokyo, Japan). The microscope was equipped with detectors for monitoring red fluorescence (excitation wavelength 555 nm, dichroic mirror wavelength 585 nm) and green fluorescence (excitation wavelength 488 nm, dichroic mirror wavelength 590 nm). Confocal images were obtained using a 63 × 1.4 oil lens for an optical section thickness of approximately 0.7 μm. The experiment was performed three times independently.

After obtaining images of dead or live cells in each horizontal section, image processing was performed using ZEN 2009 (Carl Zeiss). All layers were stacked to construct an image of the three-dimensional architecture of the biofilm and an image of the vertical section. Additionally, image analysis was performed using ImageJ 1.44i (National Institutes of Health, Bethesda, MD, USA). We calculated the ratio of the area occupied by bacteria to the total area scanned in each layer and the ratio of live bacteria to the total bacteria including live and dead cells. Finally, the average of the rate of surviving cells to the total number of cells in all sections was calculated to compare the effect of LL37 among strains. The summary of this method is indicated in supplementary material, Fig. S1.

To identify the effect of antimicrobial peptide on biofilm cells, LL37 (500 μg ml^{-1} for UA159 and 750 μg ml^{-1} for NCTC10449) was reacted with biofilms in 10 mM sodium phosphate buffer (pH 6.8) for 30 min before staining the cells. CLSM analysis was then performed using the method described above.

RESULTS

AMP susceptibility in the *dltC* knockout mutant

We investigated the susceptibility of the *dltC* knockout mutant (*dltC* mutant) in UA159 to various AMPs (Fig. 1). The susceptibilities to hBD1 (1.25 μg ml^{-1}), hBD2 (0.5 μg ml^{-1}), hBD3 (0.25 μg ml^{-1}) and LL37 (0.25 μg ml^{-1}) in the *dltC* mutant were significantly increased compared with those of the wild type. The percentages of surviving cells of the wild type against hBD1, hBD2, hBD3 and LL37 were 73.9, 64.4, 82.6 and 100%, whereas those of the *dltC* mutant were 14.5, 23.8, 50.7 and 39.7%, respectively.

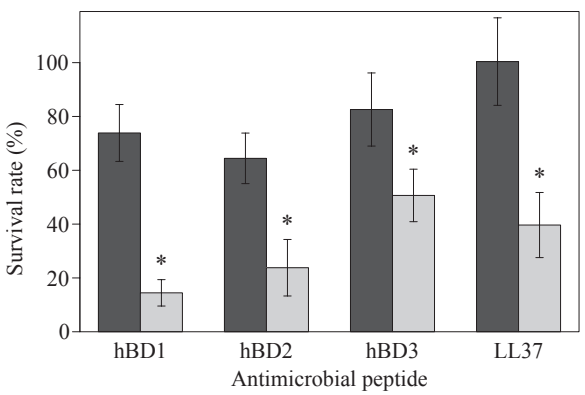

Figure 1 Antimicrobial peptide (AMP) susceptibility in the *dltC* mutant in planktonic cells. *Streptococcus mutans* strains in exponential phase were tested for AMP susceptibility as described in the Methods. The antibacterial effect was calculated as the ratio of the number of surviving cells (survival rate, %) to the total number of bacteria incubated in control phosphate buffer solution after exposure to antimicrobial peptides. Black and gray bars indicate the wild type and the *dltC* mutant, respectively. *indicates significant difference as determined by *t*-test ($P < 0.05$). The error bar represents SD.

dltC and *ciaR* expression in planktonic and biofilm cells

The expression of *dltC* in planktonic or biofilm cells was investigated using *S. mutans* UA159 and NCTC10449. In both strains, *dltC* expression in biofilm cells showed a two-fold to five-fold higher level than that in planktonic cells (Fig. 2A). Similar results were observed in two other clinically isolated strains (data not shown). We also checked *dltC* expression in planktonic and biofilm cells in the UA159 strain at 12 or 24 h after bacterial inoculation and found results similar to those at 18 h (data not shown).

Because *ciaRH* in *Streptococcus pneumoniae* was reported to be associated with *dlt* expression (Mascher *et al.*, 2003), we investigated whether *ciaRH* in *S. mutans* was associated with the *dlt* expression. In biofilm cells it was found that *ciaR* expression had a pattern similar to that of the *dltC* expression, showing a significant increase in biofilm cells compared with planktonic cells (Fig. 2B).

Characterization of the *ciaRH* knockout mutant

To determine the association of *ciaRH* with *dlt*, we investigated *dltC* expression of the *ciaRH* mutants of strains UA159 and NCTC10449 in planktonic or biofilm cells. Although the *ciaRH* and *ciaR* mutants grew more slowly compared with the wild type, the amounts of cells at stationary phase were almost the same between the wild type and mutants in planktonic or biofilm cells (data not shown). In biofilm cells, *dltC* expression in the *ciaRH* mutant of strain UA159 was significantly lower than that of the wild type (Fig. 2C). With the *ciaRH* mutant of strain NCTC10449, the result was almost the same, although expression of *dltC* in NCTC10449 grown in biofilm cells was higher than that of the UA159 strain. The *ciaR* mutant of UA159 showed a similar phenotype to the *ciaRH* mutant, showing low-level *dltC*

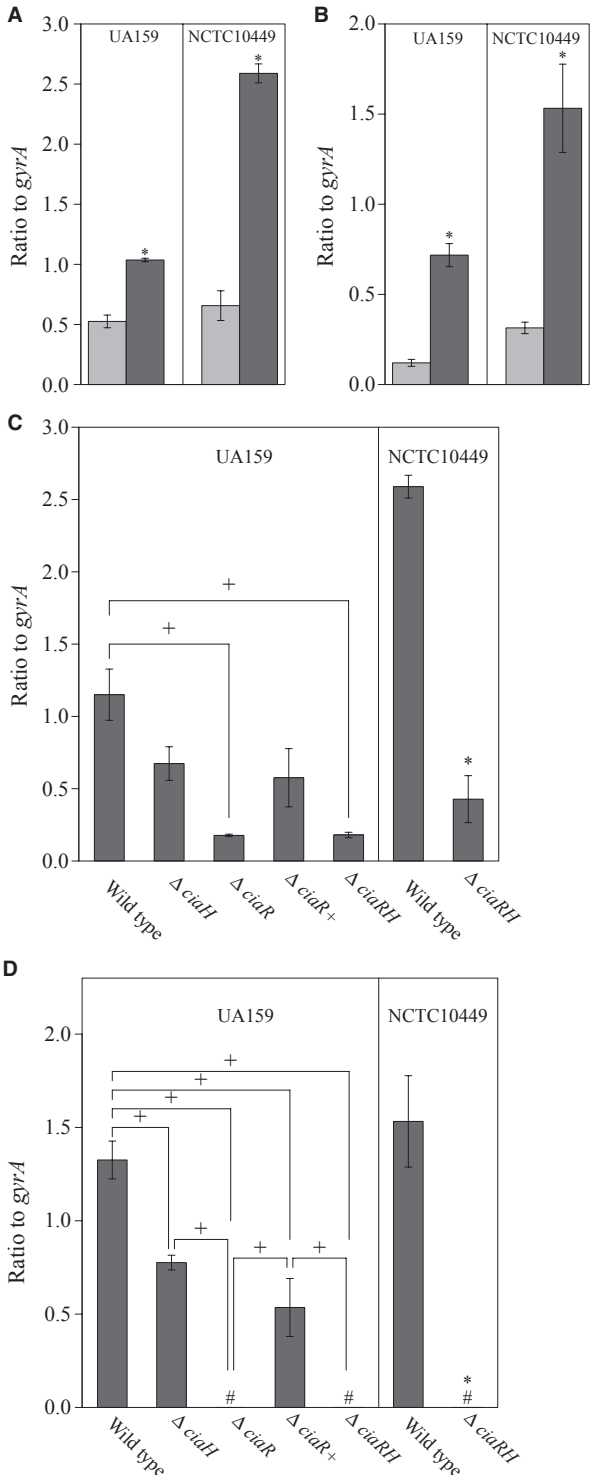

Figure 2 *ciaR* and *dltC* expression in planktonic or biofilm cells. Quantitative polymerase chain reaction was performed on cell extracts as described in the Methods. (A) *dltC* expression in strains UA159 and NCTC10449 grown as planktonic and biofilm cells; (B) *ciaR* expression in strains UA159 and NCTC10449 grown as planktonic and biofilm cells; (C) *dltC* expression in strains UA159 (wild type, Δ*ciaH*, Δ*ciaR*, Δ*ciaR+*, Δ*ciaRH*), and NCTC10449 (wild type and Δ*ciaRH*) grown in biofilms. (D) *ciaR* expression in UA159 (wild type, Δ*ciaH*, Δ*ciaR*, Δ*ciaR+*, Δ*ciaRH*), and NCTC10449 (wild type and Δ*ciaRH*) grown in biofilms. Black and gray bars indicate biofilm cells and planktonic cells, respectively. *indicates significant differences determined by *t*-test ($P < 0.05$); +indicates significant differences determined by Tukey's honestly Significant Difference ($P < 0.05$). #indicates not detected. The error bar represents SD.

expression in biofilm cells. In a *ciaR* complementation experiment, we found that *ciaR* expression was observed in the *ciaR* knock-in mutant (Fig. 2D). Complementation of the *ciaR* mutation led to increased *dltC* expression compared with that of the *ciaR* mutant, demonstrating that CiaR regulated *dltC* expression. In contrast, the *ciaRH* mutant in planktonic cells showed a similar amount of *dltC* expression to the wild type (see Fig. S2A). In planktonic cells of the *ciaH* mutant, we confirmed the previous result that *ciaR* expression was increased (Wu *et al.*, 2010) (see, Fig. S2B). However, in biofilm cells, *ciaR* expression in the *ciaH* mutant was slightly decreased compared with that of the wild type, simultaneous with decreased *dltC* expression (Fig. 2C,D). To confirm *ciaR* regulation in the *ciaH* mutant, we also investigated gene expression of *htrA*, which was reported to be regulated by CiaR (Ahn *et al.*, 2006). We found that *htrA* expression was decreased in the *ciaH* mutant compared with the wild type, in biofilm cells (data not shown).

CiaR binds to the *dltA* promoter region

First, we identified the transcriptional start site of *dltA* mRNA using RACE and found that this was 163 bp upstream of the *dltA* start codon (Fig. 3A). This transcriptional start site in strain UA159 was different from that reported previously in strain UA130 (4 bp upstream of the site in UA159). Additionally, the DNA sequence of this region in the UA159 strain was slightly different from that reported previously (Spatafora *et al.*, 1999). Based on the result of our RACE experiment, we predicted the promoter region of *dltA*, although the -10 region in the *dltA* promoter has two matches to the canonical -10 sequence. Because the consensus region (NTTAAG-n5-WTTAAG) of the CiaR binding site was reported in *S. mutans* and *S. pneumoniae* (Wu *et al.*, 2010; Halfmann *et al.*, 2011), we searched upstream of *dltA* and found one region upstream of the promoter (Fig. 3B).

Next, we performed EMSA to investigate whether the predicted CiaR binding site was actually bound by CiaR protein. We obtained four DNA fragments: the *dltA* promoter region containing the predicted binding site (dlt-1; 183 bp); the *dltA* promoter region with a deletion of the predicted binding site (dlt-2; 136 bp); the *ciaX* promoter region containing the predicted binding site (ciaX-1; 155 bp); and the *ciaX* promoter region with a deletion of the predicted binding site (ciaX-2; 199 bp). Figure 3C clearly shows that CiaR protein bound to dltA-1 and ciaX-1 in a dose-dependent manner, whereas the protein did not bind to dltA-2 and ciaX-2. These results indicate that CiaR

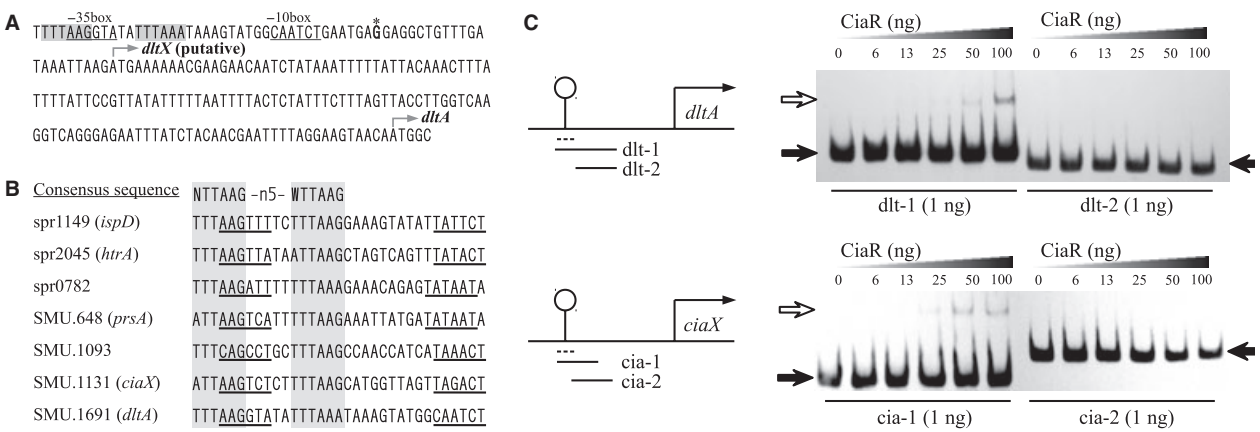

Figure 3 Analysis of CiaR binding upstream of the *dltA* promoter region. (A) Nucleotide sequence of the *dltA* promoter region in *Streptococcus mutans* UA159. The transcriptional start site and -10 and -35 promoter regions are indicated by an asterisk and underlining, respectively. The start codon of putative *dltX* is indicated upstream of *dltA*. The gray box represents the predicted CiaR binding site. (B) The consensus region of the CiaR binding site. The CiaR binding sites and the promoter regions (-10 and -35 region) are indicated by gray box and vertical lines, respectively. Spr and SMU indicate gene ID from *Streptococcus pneumoniae* R6 and *Streptococcus mutans* UA159, respectively. (C) Electromobility shift assay (EMSA) of the *dltA* and *ciaX* promoter regions. The DNA fragments were labeled with digoxigenin (DIG) and reacted with recombinant CiaR protein as described in the Methods. After electrophoresis, CiaR-bound DNA (open arrow) and unbound (solid arrow) were detected with the method described in the Methods. In the left figure, dashed line and line represent the CiaR binding site and the PCR fragment, respectively.

protein bound to the predicted CiaR binding sites upstream of *dltA* and *ciaX*.

AMP susceptibility of *dlt* and *ciaRH* mutants in biofilm cells

We first analysed the susceptibility to AMPs in planktonic cells of the wild type and the *ciaRH* mutant. The *ciaRH* mutant of NCTC10449 showed a slightly increased susceptibility to hBD3 and LL37, whereas the mutant of strain UA159 showed almost the same susceptibility as the wild type (data not shown). This result was in good accordance with *dlt* expression levels in planktonic cells of the wild type and the *ciaRH* mutants of strains UA159 or NCTC10449 (see, Fig. S2A).

Next, we analysed the susceptibility to LL37 of the *ciaRH* mutant and wild-type UA159 in biofilm cells by CLSM (Fig. 4A,B). The *dltC* mutant in biofilm cells without LL37 treatment showed a high proportion of dead cells compared with other strains. About 36.5% of the *dltC* mutant cells in biofilm without LL37 treatment were dead, whereas the proportion of dead cells in the wild type and *ciaRH* mutant were 22.0 and 11.8%, respectively (data not shown). When biofilm cells were treated with LL37, the proportion of dead cells in the wild type and two mutants increased. The *dltC* and *ciaRH* mutants of strain UA159 showed a higher proportion of dead cells, 70.5% for the *dltC* mutant and 41.2% for the *ciaRH* mutant, compared with the wild type (2.9%) (Fig. 4E). With the *ciaRH* mutant of strain NCTC10449, the result was almost the same as that for the mutant in UA159 (Fig. 4C,D). The amount of biofilm in *dltC* and *ciaRH* mutants of strain UA159 was less than that of the wild type, with about two-thirds the height of that of the wild type. However, biofilm morphology of the wild-type strain NCTC10449 was similar to that of the *ciaRH* mutant.

Furthermore, we investigated the susceptibility of the *ciaR* mutant and complemented strain to LL37. The *ciaR* mutant showed an increased susceptibility to LL37, a similar phenotype to the *ciaRH* mutant, whereas the complemented strain showed decreased susceptibility compared with the *ciaR* mutant (see, Fig. S3).

DISCUSSION

In the present study, we showed that the *dlt* locus is associated with susceptibility to antimicrobial peptides in *S. mutans*. In other species, including *Staph. aureus*, *Streptococcus agalactiae*, *Streptococcus gordonii* and *Streptococcus pyogenes*, alanine incorporation into teichoic acids or lipoteichoic acids mediated by Dlt confers resistance to cationic antimicrobial peptides (Peschel *et al.*, 2001; Collins *et al.*, 2002; Poyart *et al.*, 2003; Jin *et al.*, 2004; Kristian *et al.*, 2005; Chan *et al.*, 2007). Although we did not analyse D-alanine content in lipoteichoic acids in the present study, the increased susceptibility to cationic peptides, together with previous reports (Boyd *et al.*, 2000; Chan *et al.*, 2007), suggest that D-alanine content was decreased in the *dltC* mutant in *S. mutans*. Hence, in gram-positive bacteria, *dlt* is considered to be a common factor responsible for resistance to cationic antimicrobial peptides. Additionally, our results showed that *S. mutans* in biofilms highly expressed *dlt*, resulting in lower susceptibility to AMPs. This suggests that *S. mutans* in dental plaque might resist these peptides in the oral cavity.

To evaluate the susceptibility of antimicrobial peptides in biofilm cells, we used CLSM. The analysis using a cell viability kit was useful to evaluate not only antibacterial activity in biofilm cells but also the structure of the biofilm itself. Without treatment of antimicrobial peptides, almost all biofilm cells of the wild-type strain were alive after 18 h of incubation, with the exception of a small proportion of dead internal cells, whereas the *dltC* mutant showed a high proportion (36.5%) of dead cells, suggesting that some proportion of the *dltC* mutant cells died during biofilm formation. Previously, *dlt* mutants in *S. mutans* and *S. gordonii* showed increased sensitivity to acidic pH (Clemans *et al.*, 1999; Boyd *et al.*, 2000). Under our biofilm-forming conditions, with 2% sucrose, the *dltC* mutant may produce acids resulting in a higher ratio of dead cells compared with that in the wild type. Hence, increased *dlt* expression in the biofilm is responsible for survival in acidic conditions, as in dental plaque, although the induction molecules for *ciaRH* and *dlt* expression in biofilm cells remain unknown. With LL37 treatment, dead cells in the wild type and the mutants were uniformly observed among biofilm cells, indicating that AMPs could react with internal cells in biofilm. Because we found increased AMP susceptibility in the *dltC* mutant, our results indicate that *dlt* expression may be responsible for reduced susceptibility to cationic antimicrobial peptides in biofilm cells.

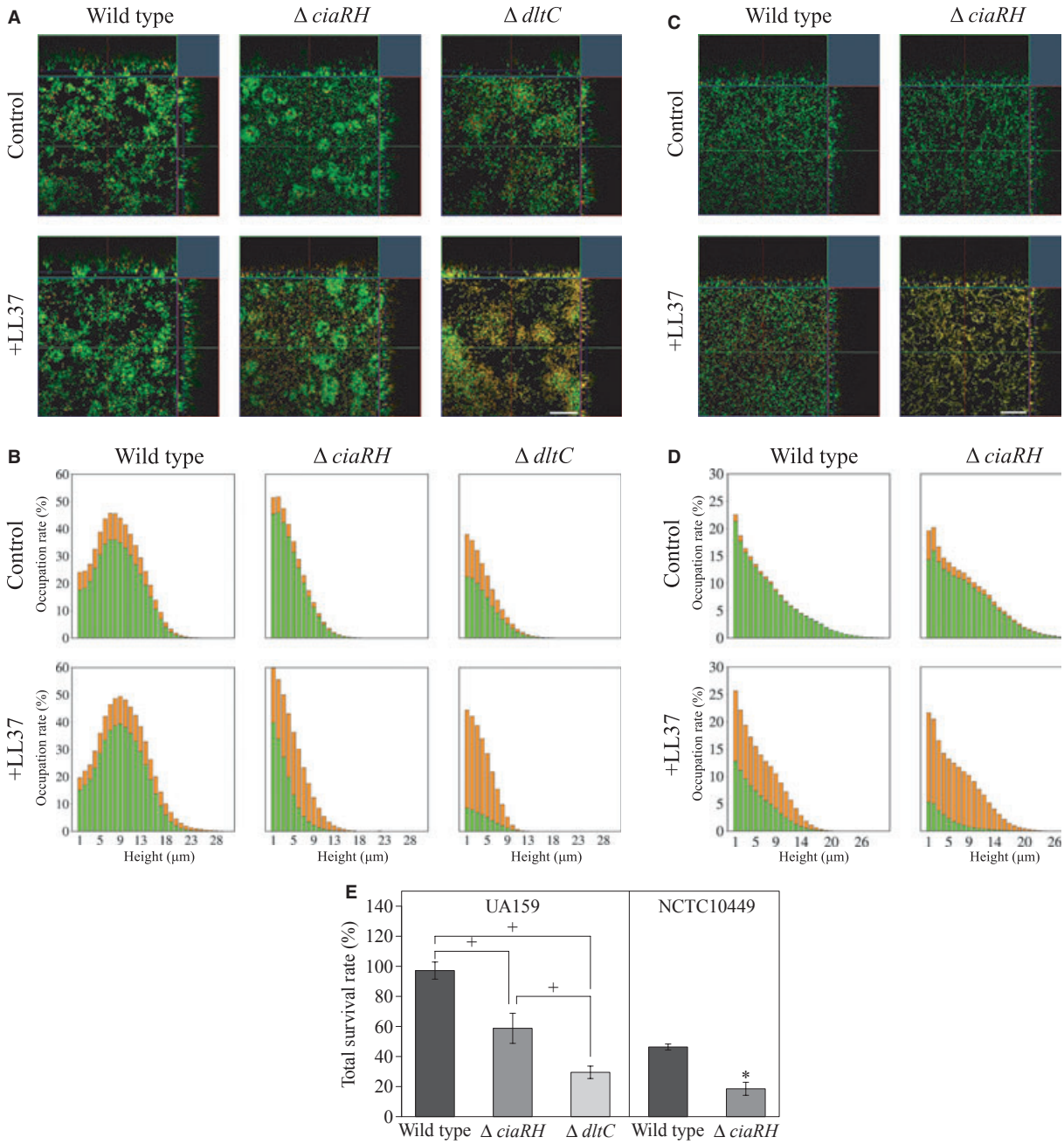

Figure 4 LL37 susceptibility of *dlt* and *ciaRH* mutants of strains UA159 and NCTC10449 in biofilms by confocal laser scanning microscopy (CLSM). Biofilm formation was performed as described in the Methods and LL37 (500 μg ml^{-1} for UA159 and 750 μg ml^{-1} for NCTC10449) was reacted with biofilms in 10 mM phosphate buffer (pH 6.8) for 30 min. BD Cell Viability stain was used to assess the viability of cells. CLSM analysis was then performed as described in the Methods. Green and orange cells indicate viable and dead cells, respectively. CLSM images of wild type (left), the *ciaRH* mutant (middle), and the *dltC* mutant (right) in strain UA159 (A), and those of wild type (left) and the *ciaRH* mutant (right) in strain NCTC10449 (C). Upper figures represent normal biofilm cells (control), and lower figures represent biofilm cells treated with LL37. Bar represents 20 μm. The occupation rate of wild type (left), Δ*ciaRH* (middle) and Δ*dltC* (right) in UA159 (B), and those of wild type (left) and Δ*ciaRH* (right) in NCTC10449 (D). In each bar, the occupation rate of dead and live cells to each area in each section is represented. Susceptibility to LL37 in wild type (UA159 and NCTC10449), Δ*ciaRH* (UA159 and NCTC10449) and Δ*dltC* (UA159) (E). The average of the rate of surviving cells to the total number of cells in all sections was calculated. +represents significant differences determined by Tukey's honestly significant difference ($P < 0.05$); *indicates statistically significant difference as determined by *t*-test ($P < 0.05$). The error bar represents SD.

In this study, we showed that *dltC* expression was regulated by *ciaRH* in biofilm cells. Wu *et al.* (2010) undertook a comprehensive analysis of the genes affected by *ciaXRH*. However, they did not find a relationship between *dlt* and *ciaXRH.* In the current study, we demonstrated linkage between *dlt* and *ciaRH* expression in biofilm cells but not in planktonic cells. Expression of *dltC* and *ciaR* was increased in biofilm cells, whereas *ciaH* inactivation was reported to enhance expression of *ciaR*, having an influence on expression of many genes (Wu *et al.*, 2010). We constructed the *ciaH* mutant of UA159 and found that *ciaR* expression in planktonic cells was increased compared with the wild type. Unexpectedly, *ciaR* expression was decreased in the *ciaH* mutant compared with the wild type under biofilm conditions. As a result, *dltC* expression was also decreased in the *ciaH* mutant compared with the wild type. These results indicate that CiaR activation in biofilm conditions is mediated by CiaH and results in increased the *dlt* expression. This is similar to the findings in *S. pneumoniae*, where the CiaR target genes included *dlt* (Mascher *et al.*, 2003). Together with our EMSA analysis of the *dlt* promoter, we conclude that *S. mutans* CiaR binds to the *dlt* promoter region, resulting in direct regulation of *dlt* expression.

In conclusion, we have shown that *dlt* is responsible for resistance to antimicrobial peptides and that *dlt* expression is regulated by *ciaXRH*. Our results indicate that *S. mutans* in dental plaque is resistant to antimicrobial peptides as a result of high expression of *dlt* mediated by *ciaXRH*.

ACKNOWLEDGMENTS

We wish to thank the Joint Research Laboratory, Kagoshima University Graduate School of Medical and Dental Sciences for the use of their facilities. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

REFERENCES

Abachin, E., Poyart, C., Pellegrini, E. *et al.* (2002) Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. *Mol Microbiol* **43**: 1–14.

Ahn, S.J., Wen, Z.T. and Burne, R.A. (2006) Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. *Infect Immun* **74**: 1631–1642.

Boyd, D.A., Cvitkovitch, D.G., Bleiweis, A.S. *et al.* (2000) Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. *J Bacteriol* **182**: 6055–6065.

Chan, K.G., Mayer, M., Davis, E.M. *et al.* (2007) Role of D-alanylation of *Streptococcus gordonii* lipoteichoic acid in innate and adaptive immunity. *Infect Immun* **75**: 3033–3042.

Clarke, J.K. (1924) On the bacterial factor in the aetiology of dental caries. *Br J Exp Pathol* **5**: 141–147.

Clemans, D.L., Kolenbrander, P.E., Debabov, D.V. *et al.* (1999) Insertional inactivation of genes responsible for the D-alanylation of lipoteichoic acid in *Streptococcus gordonii* DL1 (Challis) affects intrageneric coaggregations. *Infect Immun* **67**: 2464–2474.

Collins, L.V., Kristian, S.A., Weidenmaier, C. *et al.* (2002) *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J Infect Dis* **186**: 214–219.

Gao, J., Gusa, A.A., Scott, J.R. *et al.* (2005) Binding of the global response regulator protein CovR to the *sag* promoter of *Streptococcus pyogenes* reveals a new mode of CovR-DNA interaction. *J Biol Chem* **280**: 38948–38956.

Gorr, S.U. and Abdolhosseini, M. (2011) Antimicrobial peptides and periodontal disease. *J Clin Periodontol* **38**(Suppl 11): 126–141.

Halfmann, A., Schnorpfeil, A., Müller, M. *et al.* (2011) Activity of the two-component regulatory system CiaRH in *Streptococcus pneumoniae* R6. *J Mol Microbiol Biotechnol* **20**: 96–104.

Hamada, S. and Slade, H.D. (1980) Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* **44**: 331–384.

He, X., Wu, C., Yarbrough, D. *et al.* (2008) The *cia* operon of *Streptococcus mutans* encodes a unique component required for calcium-mediated autoregulation. *Mol Microbiol* **70**: 112–126.

Hoch, J.A. (2000) Two-component and phosphorelay signal transduction. *Curr Opin Microbiol* **3**: 165–170.

Hosokawa, I., Hosokawa, Y., Komatsuzawa, H. *et al.* (2006) Innate immune peptide LL-37 displays distinct expression pattern from beta-defensins in inflamed gingival tissue. *Clin Exp Immunol* **146**: 218–225.

van Houte, J. (1994) Role of micro-organisms in caries etiology. *J Dent Res* **73**: 672–681.

Jin, T., Bokarewa, M., Foster, T. *et al.* (2004) *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J Immunol* **172**: 1169–1176.

Kawada-Matsuo, M., Shibata, Y. and Yamashita, Y. (2009) Role of two component signaling response regulators in acid tolerance of *Streptococcus mutans*. *Oral Microbiol Immunol* **24**: 173–176.

Komatsuzawa, H., Ouhara, K., Kawai, T. *et al.* (2007) Susceptibility of periodontopathogenic and cariogenic bacteria to defensins and potential therapeutic use of defensins in oral diseases. *Curr Pharm Des* **13**: 3084–3095.

Kraus, D., Herbert, S., Kristian, S.A. *et al.* (2008) The GraRS regulatory system controls *Staphylococcus aureus* susceptibility to antimicrobial host defenses. *BMC Microbiol* **8**: 85.

Kristian, S.A., Datta, V., Weidenmaier, C. *et al.* (2005) D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. *J Bacteriol* **187**: 6719–6725.

Kuramitsu, H.K. (1993) Virulence factors of mutans streptococci: role of molecular genetics. *Crit Rev Oral Biol Med* **4**: 159–176.

Lehrer, R.I. and Ganz, T. (1999) Antimicrobial peptides in mammalian and insect host defence. *Curr Opin Immunol* **11**: 23–27.

Lévesque, C.M., Mair, R.W., Perry, J.A. *et al.* (2007) Systemic inactivation and phenotypic characterization of two-component systems in expression of *Streptococcus mutans* virulence properties. *Lett Appl Microbiol* **45**: 398–404.

Li, M., Cha, D.J., Lai, Y. *et al.* (2007a) The antimicrobial peptide-sensing system *aps* of *Staphylococcus aureus*. *Mol Microbiol* **66**: 1136–1147.

Li, M., Lai, Y., Villaruz, A.E. *et al.* (2007b) Gram-positive three-component antimicrobial peptide-sensing system. *Proc Natl Acad Sci U S A* **104**: 9469–9474.

Liu, Y. and Burne, R.A. (2009) Multiple two-component systems of *Streptococcus mutans* regulate agmatine deiminase gene expression and stress tolerance. *J Bacteriol* **191**: 7363–7366.

Mascher, T., Zähner, D., Merai, M. *et al.* (2003) The *Streptococcus pneumoniae cia* regulon: CiaR target sites and transcription profile analysis. *J Bacteriol* **185**: 60–70.

Midorikawa, K., Ouhara, K., Komatsuzawa, H. *et al.* (2003) *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. *Infect Immun* **71**: 3730–3739.

Murchison, H.H., Barrett, J.F., Cardineau, G.A. and Curtiss, R. (1986) Transformation of *Streptococcus mutans* with chromosomal and shuttle plasmid (pYA629) DNAs. *Infect Immun* **54**: 273–282.

Peschel, A., Otto, M., Jack, R.W. *et al.* (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem* **274**: 8405–8410.

Peschel, A., Jack, R.W., Otto, M. *et al.* (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *J Exp Med* **193**: 1067–1076.

Poyart, C., Pellegrini, E., Marceau, M. *et al.* (2003) Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. *Mol Microbiol* **49**: 1615–1625.

Roberts, A.P. and Mullany, P. (2010) Oral biofilms: a reservoir of transferable, bacterial, antimicrobial resistance. *Expert Rev Anti Infect Ther* **8**: 1441–1450.

Shibata, Y., Kawada-Matsuo, M., Shirai, Y. *et al.* (2011) *Streptococcus mutans* diacylglycerol kinase homologue: a potential target for anti-caries chemotherapy. *J Med Microbiol* **60**: 625–630.

Shiroza, T. and Kuramitsu, H.K. (1993) Construction of a model secretion system for oral streptococci. *Infect Immun* **61**: 3745–3755.

Spatafora, G.A., Sheets, M., June, R. *et al.* (1999) Regulated expression of the *Streptococcus mutans dlt* genes correlates with intracellular polysaccharide accumulation. *J Bacteriol* **181**: 2363–2372.

Tenovuo, J., Lumikari, M. and Soukka, T. (1991) Salivary lysozyme, lactoferrin and peroxidases: antibacterial effects on cariogenic bacteria and clinical applications in preventive dentistry. *Proc Finn Dent Soc* **87**: 197–208.

Wu, C., Ayala, E.A., Downey, J.S. *et al.* (2010) Regulation of *cia*XRH operon expression and identification of the CiaR regulon in *Streptococcus mutans*. *J Bacteriol* **192**: 4669–4679.

Zaiou, M. and Gallo, R.L. (2002) Cathelicidins, essential gene-encoded mammalian antibiotics. *J Mol Med* **80**: 549–561.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Analysis of biofilm by confocal laser scanning microscopy (CLSM).

Figure S2. *ciaR* and *dltC* expression in *Streptococcus mutans* wild type (WT) and the mutants grown in planktonic cells.

Figure S3. LL37 susceptibility of the *ciaR* mutant and the *ciaR* mutant complemented with *ciaR* of UA159 in biofilm cells by confocal laser microscopy.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.