Received: 26 March 2021 | Revised: 7 May 2021 | Accepted: 14 May 2021

DOI: 10.1002/mbo3.1203

ORIGINAL ARTICLE

MicrobiologyOpen Open Access WILEY

The c-di-AMP signaling system influences stress tolerance and biofilm formation of *Streptococcus mitis*

Gro Herredsvela Rørvik | Ali-Oddin Naemi | Per Kristian Thorén Edvardsen | Roger Simm

Institute of Oral Biology, University of Oslo, Oslo, Norway

Correspondence
Roger Simm, Institute of Oral Biology, University of Oslo, Oslo, Norway.
Email: roger.simm@odont.uio.no

Present address
Per Kristian Thorén Edvardsen, Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

Abstract

Streptococcus mitis is a commensal bacterial species of the oral cavity, with the potential for opportunistic pathogenesis. For successful colonization, *S. mitis* must be able to adhere to surfaces of the oral cavity and survive and adapt to frequently changing environmental conditions. Cyclic-di-AMP (c-di-AMP) is a nucleotide second messenger, involved in the regulation of stress responses and biofilm formation in several bacterial species. Cyclic-di-AMP is produced by diadenylate cyclases and degraded by phosphodiesterases. We have previously shown that in *S. mitis*, one diadenylate cyclase (CdaA) and at least two phosphodiesterases (Pde1 and Pde2) regulate the intracellular concentration of c-di-AMP. In this study, we utilized *S. mitis* deletion mutants of *cdaA*, *pde1*, and *pde2* to analyze the role of c-di-AMP signaling in various stress responses, biofilm formation, and adhesion to eukaryotic cells. Here, we demonstrate that the Δ*pde1* mutant displayed a tendency toward increased susceptibility to acetic acid at pH 4.0. Deletion of *cdaA* increases auto-aggregation of *S. mitis* but reduces biofilm formation on an abiotic surface. These phenotypes are more pronounced under acidic extracellular conditions. Inactivation of *pde1* or *pde2* reduced the tolerance to ciprofloxacin, and UV radiation and the Δ*pde1* mutant was more susceptible to Triton X-100, indicating a role for c-di-AMP signaling in responses to DNA damage and cell membrane perturbation. Finally, the Δ*pde2* mutant displayed a tendency toward a reduced ability to adhere to oral keratinocytes. Taken together, our results indicate an important role for c-di-AMP signaling in cellular processes important for colonization of the mouth.

KEYWORDS
acid stress, biofilm, c-di-AMP, DNA damage, *Streptococcus mitis*, β-lactam antibiotics

1 | INTRODUCTION

The commensal bacterial species *Streptococcus mitis* (*S. mitis*) is a prominent member of the oral microbiota, colonizing most of the surfaces in the oral cavity of healthy individuals (Aas et al., 2005). The association between *S. mitis* and the host is established early in life (Sulyanto et al., 2019), and it is usually a harmonic and lifelong partnership where disease rarely occurs. The hallmark of this commensal relationship is the intricate and balanced interaction between the bacteria and the host's immune system. Altered conditions in the

TABLE 1 Strains and mutants used in this study

Name	Description	Origin	Reference
S. mitis CCUG 31611	Type strain *Streptococcus mitis*, corresponding to NCTC 12261 and ATCC 49456		CCUG
S. mitis Δ*cdaA*	Markerless in-frame deletion of SM12261_1351	*S. mitis* CCUG 31611	Rørvik et al., (2020)
S. mitis Δ*pde1*	Markerless in-frame deletion of SM12261_1779	*S. mitis* CCUG 31611	Rørvik et al., (2020)
S. mitis Δ*pde2*	Markerless in-frame deletion of SM12261_1122	*S. mitis* CCUG 31611	Rørvik et al., (2020)
S. mitis Δ*pde1pde2*	Markerless in-frame deletion of SM12261_1779 and SM12261_1122	*S. mitis* Δ*pde2*	Rørvik et al., (2020)
S. mitis cdaA-KB	SM12261_1351 re-introduced into the original locus	*S. mitis* Δ*cdaA*	Rørvik et al., (2020)
S. mitis pde1-KB	SM12261_1779 re-introduced into the original locus	*S. mitis* Δ*pde1*	Rørvik et al., (2020)
S. mitis pde2-KB	SM12261_1122 re-introduced into the original locus	*S. mitis* Δ*pde2*	Rørvik et al., (2020)

oral cavity can cause changes in the equilibrium of host–microbe interactions resulting in a transition from a commensal to a parasitic relationship where *S. mitis* behaves as an opportunistic pathogen.

Immunocompromised individuals such as neutropenic cancer patients are especially vulnerable to bacteremia caused by *S. mitis* (Basaranoglu et al., 2019; Marron et al., 2000; Shelburne et al., 2014). In addition, local infections where *S. mitis* has been identified as the disease-causing agent have been reported sporadically (Al-Farsi et al., 2018; Byrd & Nemeth, 2017; Lu et al., 2003; Nygren et al., 2018). Persistent colonization of the oral cavity requires that bacteria can adhere to epithelial cells, form biofilm on the teeth, and avoid inducing a strong host immune response. They must be able to survive changing pH, temperature, and availability of oxygen and nutrients and withstand exposure to antibacterial chemicals used in the treatment of infections and oral hygiene products.

Cyclic-di-adenosine monophosphate (c-di-AMP) is a nucleotide second messenger, relaying information from the environment into cellular responses, allowing the bacteria to sense and respond to its surroundings (Witte et al., 2008). This signaling system has been characterized to a different extent in various species, among them several streptococci including the oral colonizer and cariogenic species *Streptococcus mutans* (*S. mutans*) (Bai et al., 2013; Cheng et al., 2016; Du et al., 2014; Kamegaya et al., 2011; Peng et al., 2016; Teh et al., 2019). The c-di-AMP signaling system has been shown to regulate phenotypes relevant for growth and colonization of the human body, such as biofilm formation (Du et al., 2014; Teh et al., 2019), osmotic stress tolerance (Devaux, Sleiman, et al., 2018; Smith et al., 2012; Teh et al., 2019), acidic stress tolerance (Rao et al., 2010; Witte et al., 2013; Zarrella et al., 2018), the ability to adhere to eukaryotic cells (Du et al., 2014; Teh et al., 2019), and susceptibility to DNA damaging and cell wall-targeting antibiotics (Cho & Kang, 2013; Corrigan et al., 2011; Luo & Helmann, 2012; Witte et al., 2013). Changes in the intracellular concentration of c-di-AMP can alter the ability of many bacteria to cause disease in mouse models, and thereby participate in the regulation of virulence, *in vivo* (Bai et al., 2013; Cho & Kang, 2013; Du et al., 2014; Fahmi et al., 2019; Hu et al., 2020). In addition, c-di-AMP is recognized by several receptors of the innate immune system, leading to type I interferon production or NF-κB activation (Barker et al., 2013; McFarland et al., 2017; Parvatiyar et al., 2012; Woodward et al., 2010; Xia et al., 2018), and it is therefore directly involved in crosstalk between the microbe and the host (Andrade et al., 2016; Woodward et al., 2010). We have previously identified and characterized one c-di-AMP-producing diadenylate cyclase (CdaA) and two c-di-AMP-degrading phosphodiesterases (Pde1 and Pde2) in *S. mitis* CCUG 31611 (Rørvik et al., 2020) and showed that the c-di-AMP signaling system regulates growth, metabolism, colony morphology, and chain length in *S. mitis*.

In this study, we utilized our collection of *S. mitis* deletion mutants of genes encoding c-di-AMP turnover proteins to investigate the role of c-di-AMP signaling in biofilm formation and stress tolerance relevant for persistent colonization of the oral cavity. The ability of *S. mitis* to tolerate different types of stress, such as acidic conditions, DNA damage, and cell envelope stress, was influenced by the c-di-AMP signaling system. In addition, we found that interference with c-di-AMP homeostasis affected biofilm formation on abiotic surfaces and there was a tendency toward an effect on the capacity of *S. mitis* to adhere to eukaryotic cells under the conditions tested in this study.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Unless otherwise stated, all chemicals were purchased from MilliporeSigma.

2.2 | Bacterial strains and growth conditions

The bacterial strains used in this study are summarized in Table 1. The deletion mutants and knock-back (KB) strains had been generated by markerless gene editing in a previous study (Rørvik et al., 2020). Bacteria were, unless otherwise stated, grown in tryptone soya broth (TSB) (Oxoid) at 37°C in a humidified atmosphere containing 5% CO_2. Pre-cultures were prepared by growing bacteria from glycerol stocks (stored at −80°C) on blood agar plates [Blood agar base No.2 (Merck) + 5% defibrinated sheep blood (Oxoid)]

overnight, followed by inoculation and growth in TSB to an OD_{600} ≈ 0.5. Glycerol was added to a final concentration of 15%, before aliquotation into Eppendorf tubes and storage at −80°C until further use.

For experiments, bacteria were inoculated in TSB, either directly from blood agar plates or by diluting pre-cultures 1:10, and grown under standard conditions to mid-exponential phase (OD_{600} ≈ 0.5). The OD_{600} was then adjusted to 0.1, and these bacterial suspensions (referred to as "synchronized cultures") were used to start the respective experiments.

Viability assays were performed by making 10-fold dilutions of samples, spotting five µl of each dilution in duplicates on blood agar plates, and incubating the plates at 37°C, in an atmosphere supplemented with 5% CO_2 for 24 h. The number of colony-forming units (CFU) was determined.

2.3 | Stress tolerance

2.3.1 | Susceptibility to the acidic environment

This experiment was carried out according to three slightly different protocols: (1) The pH of synchronized cultures was adjusted to 4 by addition of acetic acid (VWR, Radnor, PA, USA) or 2) HCl (VWR, Radnor, PA, USA), and the acidified cultures were incubated at 37°C for 40 min. Samples for viability testing were collected before acidification (untreated control sample) and after 40 min incubation of the acidified cultures. (3) Exponentially growing bacteria (OD_{600} 0.5) were pelleted and resuspended in either PBS (pH 7.2) or acetic acid (pH 4) and incubated for 45 min. At this point, samples were collected for viability testing. The experiments were performed in at least three biological replicates.

2.3.2 | Susceptibility to DNA damaging radiation

This experiment was performed as described by Bai et al. (2013), with minor modifications. Synchronized cultures were used to make 10-fold serial dilutions in phosphate-buffered saline (PBS). Five microliters of each dilution were spotted in duplicate onto two blood agar plates. One of the plates was treated with UV radiation (2.5 mJ/cm^2) by using a UV crosslinker (UVC 500, Hoefer Pharmacia Biotech Inc.), and the other plate served as untreated control and was used to calculate the total number of CFU in the cultures. The experiment was repeated at least seven times.

2.3.3 | Susceptibility to heat

Synchronized cultures were incubated at 52°C for 30 min in a water bath. Samples for viability testing were collected before (untreated control) and after the 30-min incubation at 52°C. At least two individual experiments were performed.

2.3.4 | Survival in Triton X-100

Synchronized cultures were incubated in 0.1% Triton X-100 (Sigma) for 10 min at room temperature. Samples were collected before (untreated control) and after the 10-min incubation for viability assays. The experiment was repeated at least two individual times.

2.3.5 | Minimum inhibitory concentration of antibacterial chemicals

The minimum inhibitory concentration (MIC) was determined in broth microdilution assays. Synchronized cultures were diluted to an OD of 0.001 in Mueller–Hinton broth and distributed (100 µl per well) in U-shaped 96-well Nunc microtiter plates (Thermo Fisher Scientific). Twofold serial dilutions of ciprofloxacin (32 mg/L–0.25 mg/L), ampicillin (2 mg/L–0.016 mg/L), xylitol (200,000 mg/L–781 mg/L), chlorhexidine (50 mg/L–0.195 mg/L), or sodium fluoride (50,000 mg/L–195 mg/L) were added to the bacterial suspensions, and the plates were incubated at 37°C, in a humidified atmosphere of 5% CO_2. Plates were inspected for growth after 24 and 48 h. MIC was determined as the lowest concentration that inhibited visible growth after 24 h. If the results in some wells were inconclusive after 24 h, the plates were incubated for 48 h before the MIC was finalized. At least three biological replicates were performed.

2.4 | Bacterial growth curves

Synchronized cultures were diluted to OD_{600} of 0.01 in TSB with the supplementations indicated for each experiment. Cultures were distributed in flat-bottom 96-well Nunc microtiter plates (Thermo Fisher Scientific) and incubated at 37°C in the ambient atmosphere in a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek). The OD of static cultures was measured every 30 minutes for 20 h, and the OD of shaken cultures was measured every 15 min for 20 h. Growth in unmodified TSB (pH 7.2), and TSB buffered to pH 5.5, and 7.2, was measured in both static and shaken cultures. The buffered TSB adjusted to pH 5.5 and pH 7.2 was buffered with PBS. The addition of PBS to the TSB resulted in an increase in pH from 7.2 to 7.4. The pH in the PBS-buffered TSB was therefore adjusted to pH 7.2 or pH 5.5 by the addition of HCl. Growth in TSB supplemented with ampicillin (0.0625 mg/L), ciprofloxacin (1 mg/L), xylitol (100 g/L), chlorhexidine (0.781 mg/L), and sodium fluoride (391 mg/L) was measured in shaken cultures. The growth curves were repeated at least two individual times.

2.5 | Biofilm formation assay

Synchronized cultures were 10-fold diluted in TSB or TSB buffered to pH 5.5 or 7.2. One mL of the bacterial suspensions were distributed,

in duplicates, into wells of flat-bottom 24-well polystyrene plates (Thermo Fisher Scientific). Plates were incubated in an ambient atmosphere or a humidified atmosphere supplemented with 5% CO_2 for 20 h, or in an anaerobic atmosphere (5% H_2, 5% CO_2, and 90% N_2) for 72 h.

The biomass of the biofilms was determined by removing the growth medium and the cells not attached to the substratum. The biofilms were stained with 0.1% safranin (Merck) for 30 min at room temperature. The unabsorbed safranin was removed, and the biofilms were washed twice with PBS. Acetic acid (30%) was used to release the safranin from the biofilm, and the amount of safranin was quantified by measuring the absorbance at 530 nm in a Cytation™ 3 Cell Imaging Multi-Mode Reader. The experiments were repeated at least two individual times.

2.6 | Adhesion to oral keratinocytes

The oral squamous cell carcinoma cell line PE/CA-PJ49 (ECACC 00060606, Salisbury, UK) was grown to confluence in 12-well Nunc cell culturing plates (Nunc) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum albumin (FBS) and antibiotics (penicillin (100 kU/L), streptomycin (100 mg/L); Thermo Fisher Scientific) and pre-warmed to 37°C. Adhesion to oral keratinocytes was investigated by the method described by Rukke et al. (2016), with minor modifications. Briefly, on the day of the experiment, the cells were washed twice in PBS pre-warmed to 37°C to remove growth medium supplemented with antibiotics. One mL IMDM supplemented with 1% FBS, without supplementation of antibiotics, was pre-warmed to 37°C and added to each well. Synchronized bacterial cultures were pelleted and resuspended in IMDM with 1% FBS pre-warmed to 37°C. Bacterial suspensions were added to the cells at a final multiplicity of infection (MOI) of 20 or 50 bacteria per eukaryotic cell. Plates were centrifuged at 1000 × g for 1 min and incubated for 30 min at 37°C, in a humidified atmosphere supplemented with 5% CO_2. After incubation, the medium was removed, and the cells were washed twice with PBS pre-warmed to 37°C. Cells were either released from the substratum by incubation in the presence of trypsin (0.5%) for 20 min or the cells were lysed by incubation in Triton X-100 (0.1% for 10 min). Serial dilutions of the samples were prepared in PBS, and 5 µl of the dilutions were spotted in duplicate on blood agar plates and incubated at 37°C, in a humidified atmosphere supplemented with 5% CO_2, for determination of CFU. The experiments were performed at least two times.

2.7 | Statistical analyses

Statistical significance was determined as $p < 0.05$ and was calculated using the Kruskal–Wallis test followed by Dunn's multiple comparison test. Each mutant sample was compared to the WT sample. Statistical analyses were carried out using Excel or GraphPad Prism.

3 | RESULTS

3.1 | Disruption of c-di-AMP homeostasis affects the ability of *S. mitis* to survive certain stressful conditions

We used in-frame markerless deletion mutants of *cdaA*, *pde1*, and *pde2* to analyze the role of the c-di-AMP signaling system in stress tolerance of *S. mitis* CCUG 31611. First, we performed pilot experiments testing the ability of the WT to survive different pH, temperatures, and UV radiation doses. We estimated that a 100-fold difference in the number of CFU under the test conditions compared to the control conditions should allow for detection of both increased and decreased survival of the mutants compared to the WT. This criterion was fulfilled by incubating the WT in the presence of 175 mM acetic acid (pH 4.0) for 40 min, in PBS at 52°C for 30 min, or by exposing a newly inoculated blood agar plate with 2.5 mJ/cm^2 UV radiation. These conditions were used for further experiments.

The Δ*pde1* mutant displayed a tendency toward reduced survival compared to the WT, in an environment acidified by acetic acidic, as indicated by an almost 20-fold difference in mean values and non-overlapping confidence intervals. However, the result was not statistically significant ($p < 0.05$) according to the statistical analysis (Figure 1a). There was no difference in survival of the mutants compared to the WT during incubation for 40 min in an environment where the pH had been adjusted to 4.0 using the inorganic acid HCl.

The Δ*pde1* and Δ*pde2* mutants had a significantly reduced ability to survive exposure to 2.5 mJ/cm^2 of UV radiation compared with the WT (Figure 1b). The ability to survive UV stress was completely restored in the *pde1*-KB and *pde2*-KB strains. Deletion of *cdaA* did not result in the altered ability of *S. mitis* to survive UV stress (Figure 1b). The *cdaA*-KB showed a lower ability to survive UV radiation when compared to the WT (Figure 1b).

There was no statistically significant difference in survival between the Δ*cdaA*, Δ*pde1*, or Δ*pde2* mutants compared to the WT after exposure to 52°C for 30 min (Figure 1c).

Survival in the presence of Triton X-100 was tested at the concentration used for eukaryotic cell lysis (0.1%). The Δ*pde1* mutant displayed a significantly decreased survival in the presence of Triton X-100 (Figure 1d). The susceptibility to Triton X-100 was fully restored to WT levels in the *pde1*-KB strain.

3.2 | The c-di-AMP signaling system affects susceptibility to antibacterial compounds

Disruption of c-di-AMP homeostasis can affect the susceptibility of certain bacteria to cell wall-disrupting and/or DNA-damaging antibiotics (Corrigan et al., 2011; Dengler et al., 2013; Fahmi et al., 2019; Griffiths & O'Neill, 2012; Luo & Helmann, 2012; Massa et al., 2020; Witte et al., 2013). To establish whether this was the case in *S. mitis*, we determined the MIC of ampicillin and ciprofloxacin for the WT and Δ*cdaA*, Δ*pde1* and Δ*pde2* mutants. In addition, we determined

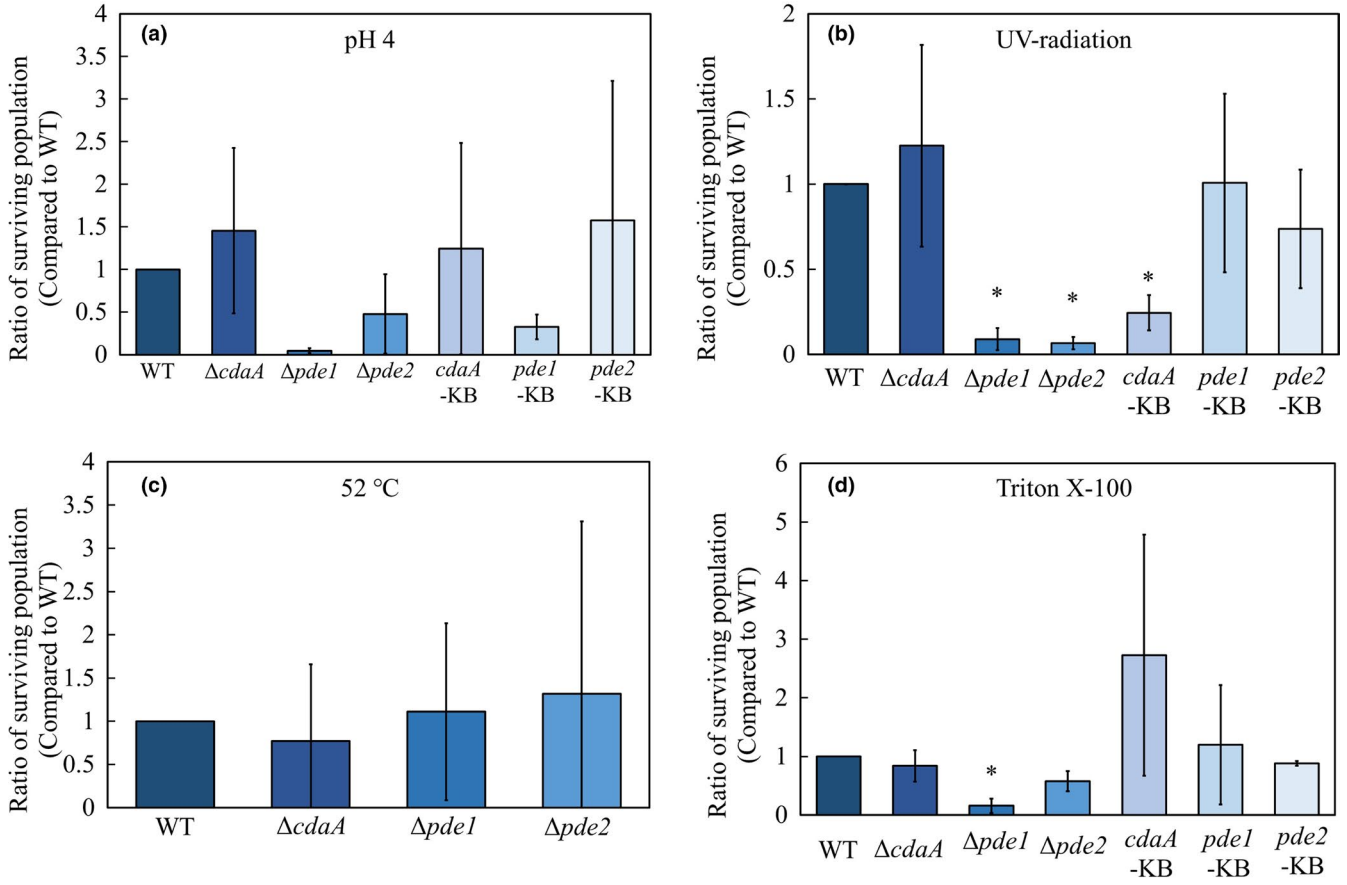


FIGURE 1 Stress tolerance of *Streptococcus mitis* is influenced by the c-di-AMP signaling system. The number of colony-forming units (CFU) before and after exposure to stress was determined, and the ratio was calculated and normalized against the WT. (a) Acid conditions (TSB adjusted with acetic acid to pH 4, 40 min), (b) UV radiation (2.5 mJ/cm^2), (c) high temperature (52°C, 30 min), and (d) detergent (0.1% Triton X-100, 10 min). For the WT, there was approximately 10^5 CFU per µl in the untreated sample and this number was reduced approximately 100-fold in the treated sample. This applied to all treatments. Shown are averages and confidence intervals of the mean of at least two independent experiments. * indicates a statistically significant difference when compared to the WT ($p < 0.05$) calculated by the Kruskal–Wallis test followed by Dunn's post hoc test

TABLE 2 Minimum inhibitory concentrations of antibacterial compounds

	Antibacterial compound (mg/L)				
	AMP	CIP	XYL	NaF	CHX
Strain	(0.016–2)	(0.25–32)	(781–200,000)	(195–50,000)	(0.195–50)
WT	0.125	4.0	n.d.[a]	1560	1.56
Δ*cdaA*	0.06125	8.0	n.d.[a]	780	1.56
Δ*pde1*	0.125	2.0	n.d.[a]	1560	1.56
Δ*pde2*	0.06125	2.0	200,000	780	1.56

Abbreviations: AMP, ampicillin; CHX, chlorhexidine; CIP, ciprofloxacin; NaF, sodium fluoride; XYL, xylitol.

[a]n.d. not determined; bacteria grew at the highest concentration of antibacterial compound used in the assay. Numbers in parentheses signify the concentration range used in the experiment.

the MIC of chlorhexidine, fluoride, and xylitol, compounds with antibacterial activity that *S. mitis* can be exposed to in the oral cavity (Kontiokari et al., 1995; Marquis et al., 2003; Russell & Day, 1993). Compared to the WT, the Δ*cdaA* mutant displayed higher tolerance to ciprofloxacin and higher susceptibility to ampicillin and fluoride and the Δ*pde2* mutant displayed higher susceptibility to ampicillin, ciprofloxacin, xylitol, and fluoride, whereas the Δ*pde1* mutant displayed higher susceptibility to ciprofloxacin (Table 2).

Although the MIC of the antibacterial compounds did not vary much between the WT and the Δ*cdaA*, Δ*pde1*, and Δ*pde2* mutants in general, our visual inspection indicated that the MICs were reached at different time points for the different strains. To analyze a potential effect

on growth rate in more detail, we performed growth experiments in the presence of sub-lethal concentrations of antibacterial compounds and compared it to the growth in the absence of antibacterial supplements. In TSB without any supplementation, the WT and Δ*cdaA* and Δ*pde1* mutants grew relatively similarly, but the Δ*cdaA* mutant generally reached a slightly lower OD in the stationary phase (Figure 2a, b and Rørvik et al., 2020). The Δ*pde2* mutant on the other hand displayed a reduced growth rate and reached a lower OD in the stationary phase than the other strains (Figure 2c and Rørvik et al., 2020). In TSB supplemented with ampicillin (0.0625 mg/L), the Δ*pde1* mutant grew slightly faster than the wild type in the exponential growth phase compared to the WT, while the Δ*pde2* and the Δ*cdaA* mutant grew slower compared to the WT (Figure 2d–f). The observed effect of ampicillin on the Δ*cdaA* and the Δ*pde2* mutants was over-compensated in their corresponding knock-back (KB) strains (Figure 2d, f). In TSB supplemented with ciprofloxacin (1 mg/L; Figure 2g–i), the Δ*cdaA* mutant displayed slightly increased growth in the exponential growth phase, while the end-OD was slightly reduced compared to the WT (Figure 2g). The Δ*pde1* mutant grew slower than the WT in the exponential growth phase, while the Δ*pde2* mutant reached a lower OD compared to the WT at all time points measured (Figure 2h,i). At lower concentrations of ciprofloxacin or ampicillin, all strains displayed growth kinetics similar to growth in TSB without antibacterial supplements.

In the presence of xylitol (100 g/L), the Δ*cdaA* and Δ*pde2* mutants grew slower than the WT and Δ*pde1* mutant (Figure 3a-c). Growth was restored to WT levels in the corresponding knock-back strains. In TSB supplemented with chlorhexidine (0.78 mg/L), the Δ*cdaA* mutant grew slightly faster in the exponential phase but reached a lower end-OD compared to WT (Figure 3d). The Δ*pde2* mutant grew slower than the WT, and growth was restored in the Δ*pde2*-KB (Figure 3f). In the presence of fluoride (390 mg/L) the Δ*cdaA* and Δ*pde2* mutants grew slower compared to the WT (Figure 3g,i). The *cdaA*-KB and *pde2*-KB strains grew with similar growth kinetics as the WT (Figure 3g,i). The Δ*pde1* mutant did not display an aberrant phenotype compared to the WT when challenged with xylitol, chlorhexidine, or fluoride (Figure 3b, e and h).

3.3 | Acidic growth conditions result in aggregation of the *ΔcdaA* mutant

The c-di-AMP signaling system is involved in regulating the growth of *S. mitis* in unmodified TSB at 37°C in shaking culture (Figure 2a-c and

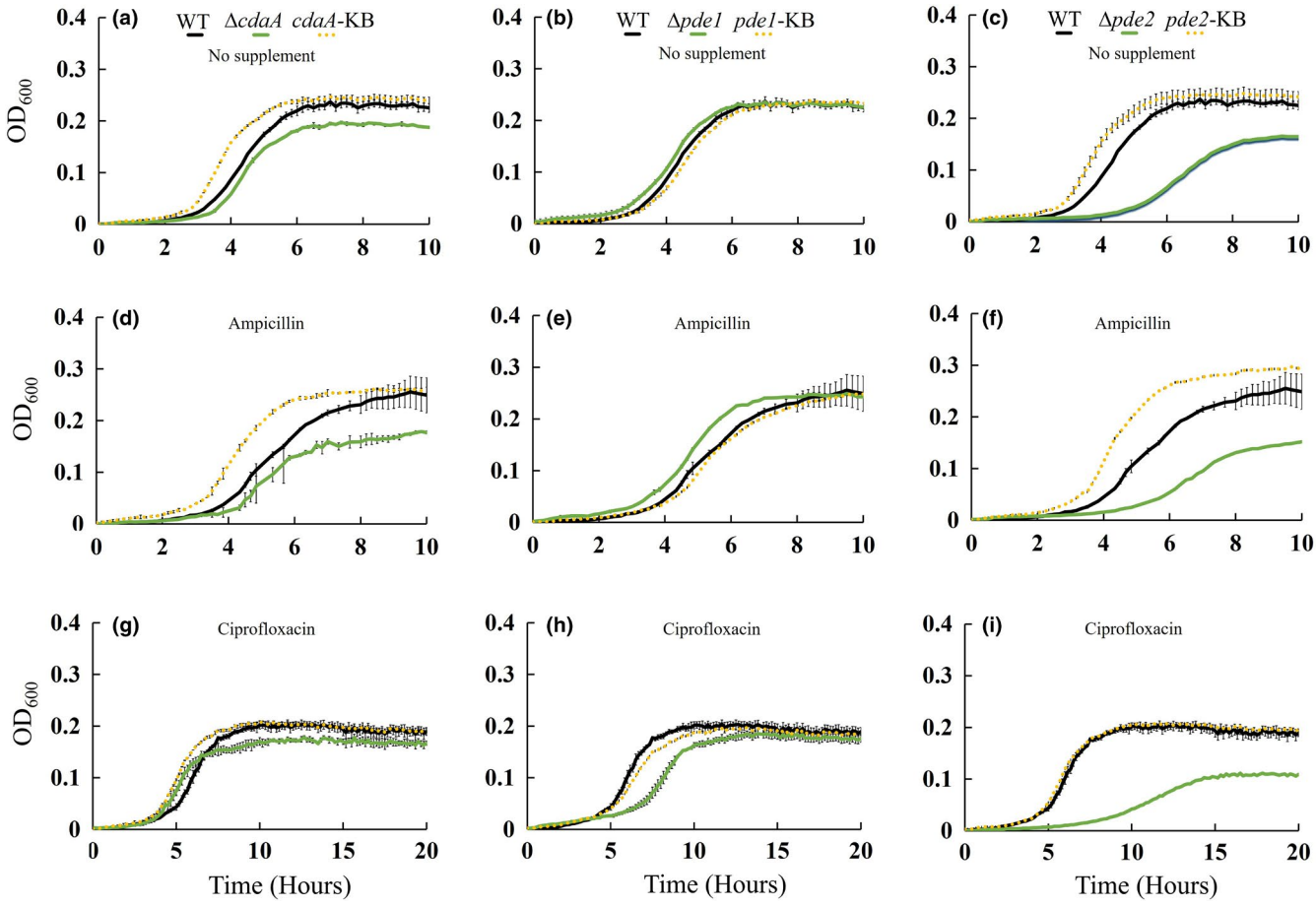


FIGURE 2 Antimicrobial compounds affect growth differently in WT and mutants. Growth in the presence of sub-lethal concentrations of antibacterial compounds was investigated by measuring OD_{600} every 10 min until the stationary phase was reached. Growth in TSB supplemented with (a–c) no supplement, (d–f) ampicillin (0.0625 mg/L), and (g–i) ciprofloxacin (1 mg/L). Shown are representative experiments performed in triplicate, and the error bars represent the standard deviation of three replicates

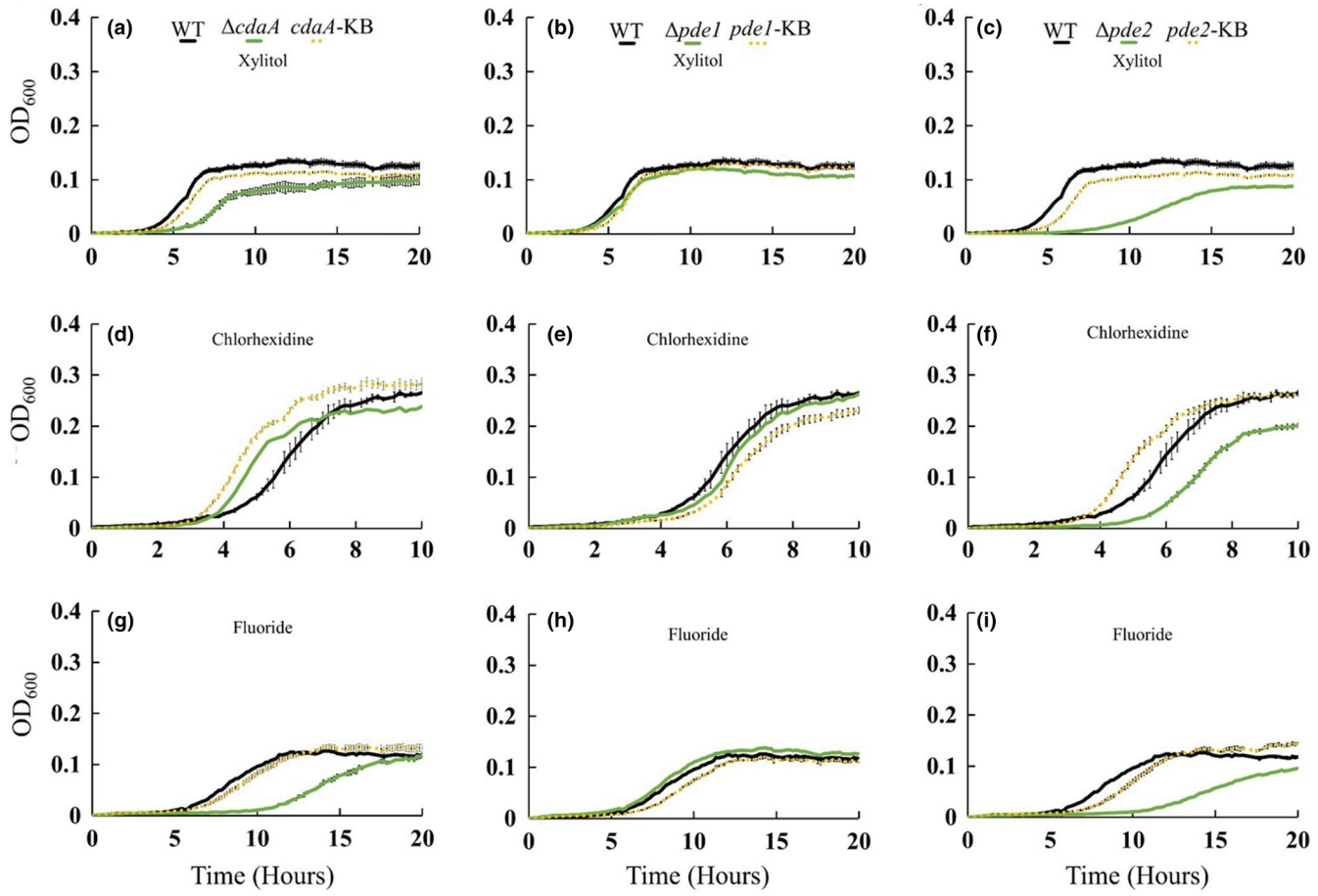


FIGURE 3 Chemicals found in oral hygiene products have different effects on the growth of the Δ*cdaA*, Δ*pde1*, and Δ*pde2* mutants, compared to WT. Growth in the presence of sub-lethal concentrations of antibacterial compounds was investigated by measuring OD_{600} every 10 min until the samples reached the stationary growth phase. (a–c) xylitol (100 g/L), (d–f) chlorhexidine (0.78 mg/L), and (g–i) fluoride (390 mg/L). Shown are representative experiments performed in triplicates, and error bars represent the standard deviation of three replicates

(Rørvik et al., 2020)). Growth was also analyzed under static growth conditions. The WT and Δ*pde1* mutants displayed similar growth kinetics in TSB under these conditions, whereas the Δ*cdaA* mutant displayed higher OD in the stationary phase and the Δ*pde2* mutant grew more slowly in the exponential phase but reached a comparable OD to the WT in the stationary phase (Figure 4a; Table 3). *S. mitis* extracts energy from the fermentation of carbohydrates and produces organic acids as a by-product. This results in the accumulation of organic acid by-products over time. Since the difference in OD of the Δ*cdaA* mutant compared to the WT differed between shaken and static conditions and this was most pronounced in the stationary phase, we hypothesized that it was due to acidification of the growth medium. To test this hypothesis, growth was analyzed in TSB buffered at pH 5.5 and pH 7.2 under both shaking and static conditions. At pH 5.5, the Δ*cdaA* mutant reached a higher OD in the stationary phase compared to the WT under static conditions (Figure 4b) and grew slower and reached a lower OD under shaking conditions (Figure 4d; Table 3). At pH 7.2, the Δ*cdaA* mutant grew similar to the WT under both shaking and static conditions (Figure 4c,e; Table 3). Growth of the Δ*pde1* mutant was similar to the WT under shaking and static conditions at both pH 5.5 and 7.2 (Figure 4b–e; Table 3). The Δ*pde2* mutant grew significantly worse than the WT under all four conditions (Figure 4b–e; Table 3).

3.4 | Biofilm formation of *S. mitis* is under influence by the c-di-AMP signaling system

The apparent pH-induced differences in growth of the Δ*cdaA* mutant in static and shaking cultures suggest that aggregation and/or biofilm formation of *S. mitis* are controlled by the c-di-AMP signaling system. We therefore investigated the biofilm-forming ability of the WT and Δ*cdaA*, Δ*pde1*, and Δ*pde2* mutants and their respective KB strains in unmodified TSB as well as in TSB buffered to pH 5.5 and pH 7.2 in both ambient air and an atmosphere supplemented with 5% CO_2. The amount of biofilm produced by the WT was higher under acidic conditions than under neutral conditions. In a humidified atmosphere supplemented with 5% CO_2, the Δ*cdaA* mutant produced significantly less biofilm than the WT (Figure 5a). Biofilm formation was restored to WT levels

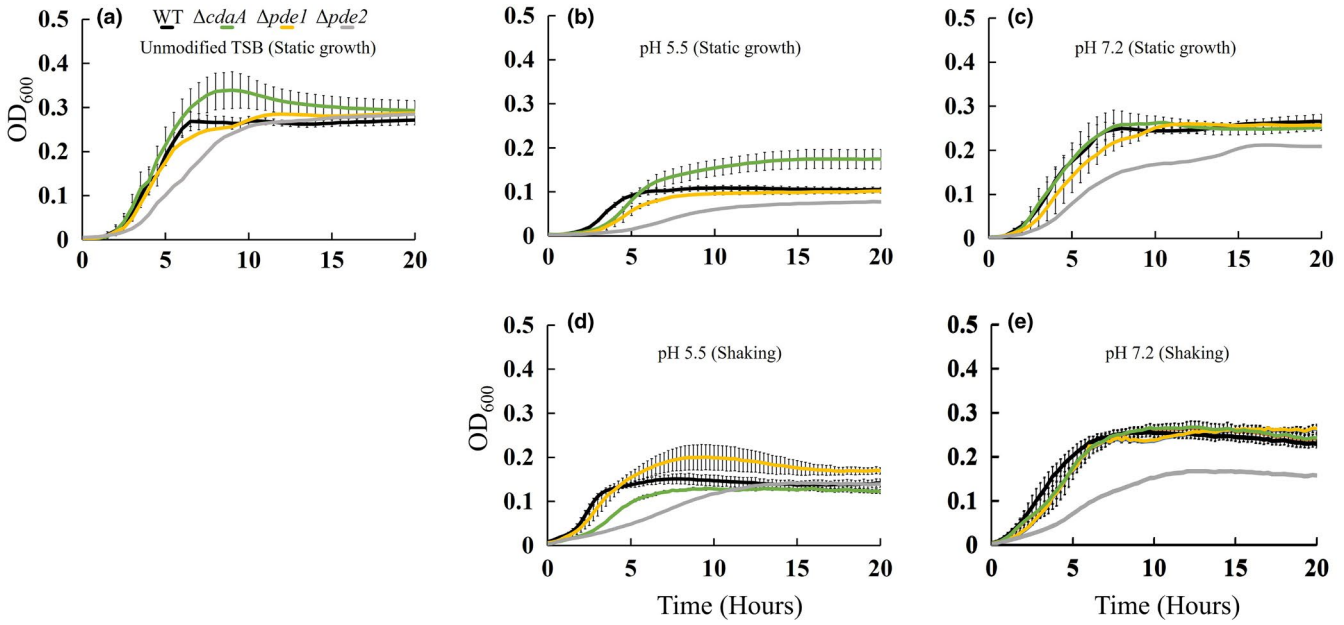


FIGURE 4 The c-di-AMP signaling system influences the growth of *Streptococcus mitis* under acidic conditions. Bacteria were grown at 37°C for 20 h in a plate reader with or without shaking as indicated below. The pH of the PBS-buffered TSB was adjusted from pH 7.4 to 5.5 or 7.2 using HCl. Cultures were incubated under static conditions in (a) unmodified TSB (b) PBS-buffered TSB adjusted to a starting pH of 5.5 (c) PBS-buffered TSB adjusted to a starting pH of 7.2. Cultures were incubated with recurrent shaking in (d) PBS-buffered TSB adjusted to a starting pH of 5.5 (e) PBS-buffered TSB adjusted to a starting pH of 7.2. Results represent the average of at least two independent experiments, performed in at least duplicates, and the error bars represent the standard error of the mean

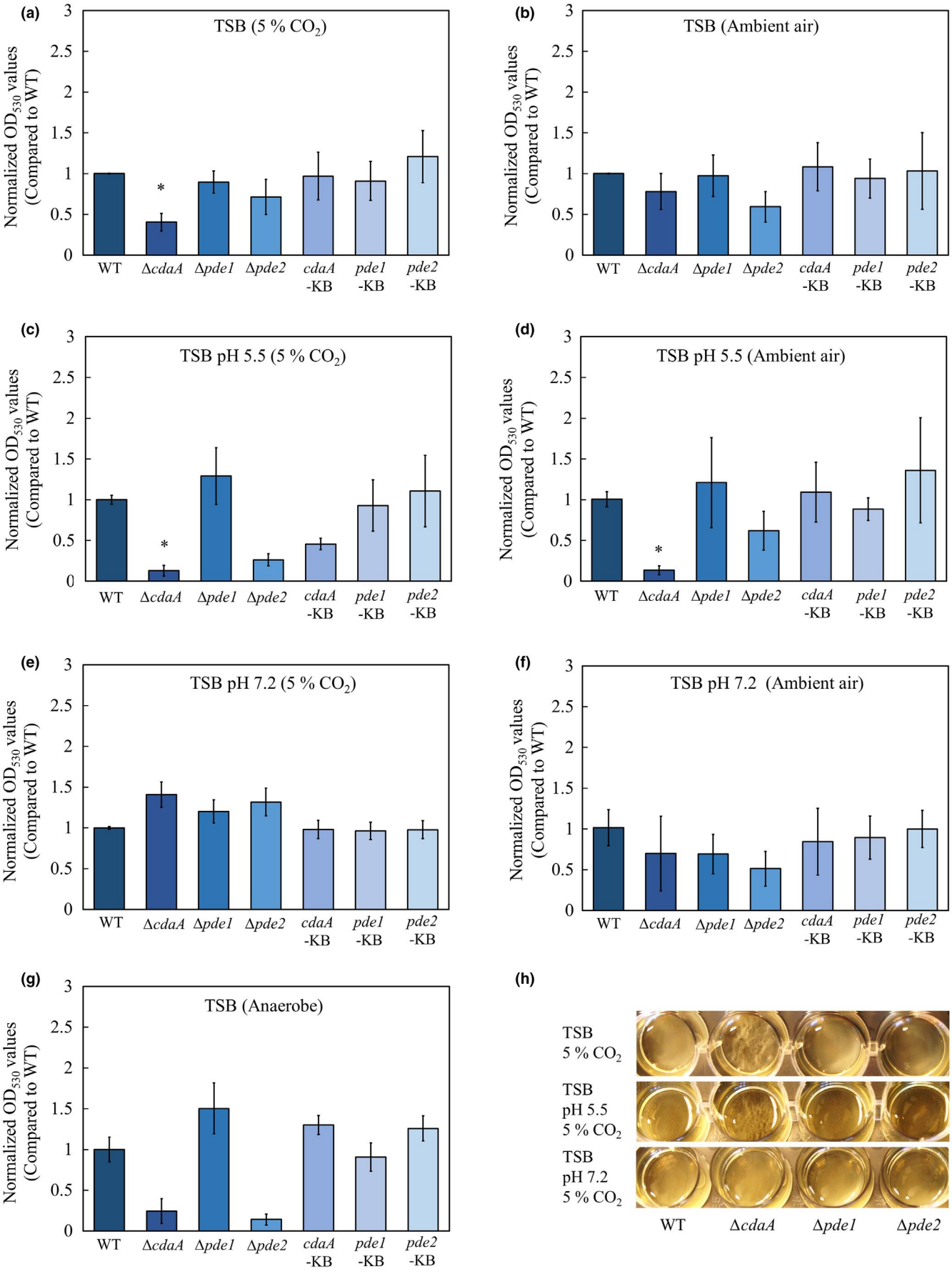
TABLE 3 Generation time

	TSB—shaking			TSB—static		
	Unmodified	pH 5.5	pH 7.2	Unmodified	pH 5.5	pH 7.2
WT	41.6 ± 3.7	45.0 ± 6.0	47.7 ± 8.9	25.6 ± 2.4	41.8 ± 3.7	40.1 ± 2.0
Δ*cdaA*	47.0 ± 1.7	69.8 ± 9.6	46.8 ± 13.9	26.5 ± 2.7	56.1 ± 4.5	35.8 ± 5.7
Δ*pde1*	50.3 ± 7.1	47.8 ± 7.8	46.3 ± 14.3	33.4 ± 6.2	46.9 ± 9.0	44.9 ± 5.5
Δ*pde2*	69.5 ± 9.1	145.1±18.6	98.0 ± 16.9	53.1 ± 11.2	100 ± 11.2	56.1 ± 1.6

Note: Generation time (min) calculated from at least two independent experiments. ± Indicate standard deviation.

following the reintroduction of *cdaA*, into the Δ*cdaA* mutant. In ambient air and unmodified TSB, there was a trend toward reduced biofilm formation by the Δ*pde2* mutant as suggested by the twofold difference in mean values and non-overlapping confidence intervals, but the difference was not statistically significant ($p < 0.05$) according to the statistical analysis (Figure 5b). Biofilm formation of the Δ*cdaA* mutant was reduced to a greater extent in TSB buffered to pH 5.5 compared to unmodified TSB in both a humidified atmosphere supplemented with 5% CO_2 and ambient air. (Figure 5c,d). There was no difference in biofilm formation between the WT and mutants in TSB buffered at pH 7.2 in a humidified atmosphere supplemented with 5% CO_2 or ambient air

FIGURE 5 Biofilm formation of *Streptococcus mitis* depends on environmental pH and atmospheric conditions and is influenced by the c-di-AMP signaling system. Biofilms were formed at 37°C in (a) unmodified TSB in a humidified atmosphere with 5% CO_2 (20 h), (b) unmodified TSB in ambient air (20 h), (c) PBS-buffered TSB, with pH 5.5 in a humidified atmosphere with 5% CO_2 (20 h), (d) PBS-buffered TSB with pH 5.5 in ambient air (20 h), (e) PBS-buffered TSB with pH 7.2 in a humidified atmosphere with 5% CO_2 (20 h), (f) PBS-buffered TSB with pH 7.2 in ambient air (20 h), (g) unmodified TSB in anaerobic atmosphere (5% H_2, 5% CO_2, and 90% N_2) for 72 h, and (h) appearance of cultures after 24 h incubation at 37°C in unmodified TSB, PBS-buffered TSB with pH 7.2, or PBS-buffered TSB with pH 5.5 in a humidified atmosphere supplemented with 5% CO_2. The brightness, sharpness, and contrast of the pictures have been adjusted for better visualization of the cultures. The pH of the PBS-buffered TSB was adjusted from pH 7.4 to pH 5.5 or pH 7.2 using HCl. Shown are averages and confidence intervals of the mean of at least two independent experiments. * indicates a statistically significant difference when compared to the WT ($p < 0.05$) calculated by the Kruskal–Wallis test followed by Dunn's post hoc test


(a)
TSB (5 % CO2)
(b)
TSB (Ambient air)
(c)
TSB pH 5.5 (5 % CO2)
(d)
TSB pH 5.5 (Ambient air)
(e)
TSB pH 7.2 (5 % CO2)
(f)
TSB pH 7.2 (Ambient air)
(g)
TSB (Anaerobe)
Normalized OD530 values (Compared to WT)
WT
ΔcdaA
Δpde1
Δpde2
cdaA -KB
pde1 -KB
pde2 -KB
(h)
TSB 5 % CO2
TSB pH 5.5 5 % CO2
TSB pH 7.2 5 % CO2
WT
ΔcdaA
Δpde1
Δpde2

(Figure 5e,f). Interestingly, in unmodified TSB and TSB buffered at pH 5.5 in the humidified atmosphere supplemented with 5% CO_2, the Δ*cdaA* mutant displayed an aggregating growth phenotype, where the cells appeared to clump together (Figure 5h). This phenotype was not visible in TSB buffered at pH 7.2. Under all conditions tested, cultures of the WT and Δ*pde1* and Δ*pde2* mutants appeared homogenous regarding bacterial dispersion during growth (Figure 5h).

Since the gas composition of the atmosphere appeared to affect the biofilm-forming ability of *S. mitis*, we also tested the phenotype in unmodified TSB under anaerobic conditions. The Δ*cdaA* and Δ*pde2* mutants displayed heavily reduced biofilm-forming capability compared to the WT as suggested by 5- to 10-fold differences in mean values and non-overlapping confidence intervals, but the effects were not statistically significant ($p < 0.05$) according to the statistical analysis (Figure 5g).

Under all conditions tested, the biofilm-forming ability of the mutants was, according to the statistical analysis, restored to WT levels in the respective KB strains (Figure 5).

3.5 | Adhesion to oral keratinocytes may be negatively affected in *Δpde2* mutant

Adherence to epithelial cells of the oral cavity is important for bacterial colonization. We therefore investigated the ability of the WT and mutants to adhere to a confluent layer of the human oral squamous cell carcinoma cell line PE/CA-PJ49. *pde2* mutant displayed a tendency toward reduced ability to adhere to the oral keratinocytes, but the effect was not significant ($p < 0.05$) in the statistical analysis (Figure 6a and b). Since 0.1% Triton X-100 also showed a tendency toward an effect on the number of viable *S. mitis* cells (Figure 1d), the experiment was repeated using 0.5% trypsin to disrupt the surface interactions between bacteria and keratinocytes. Under these conditions, the Δ*pde2* and Δ*pde1*Δ*pde2* mutants displayed an apparent reduction in the ability to adhere to keratinocytes at both MOI 20 and MOI 50 in the form of differences in mean values and non-overlapping confidence intervals (Figure 6c and d). However, the results were not significant in the statistical analysis ($p < 0.05$). Reintroduction of *pde2* into the Δ*pde2* mutant resulted in complete

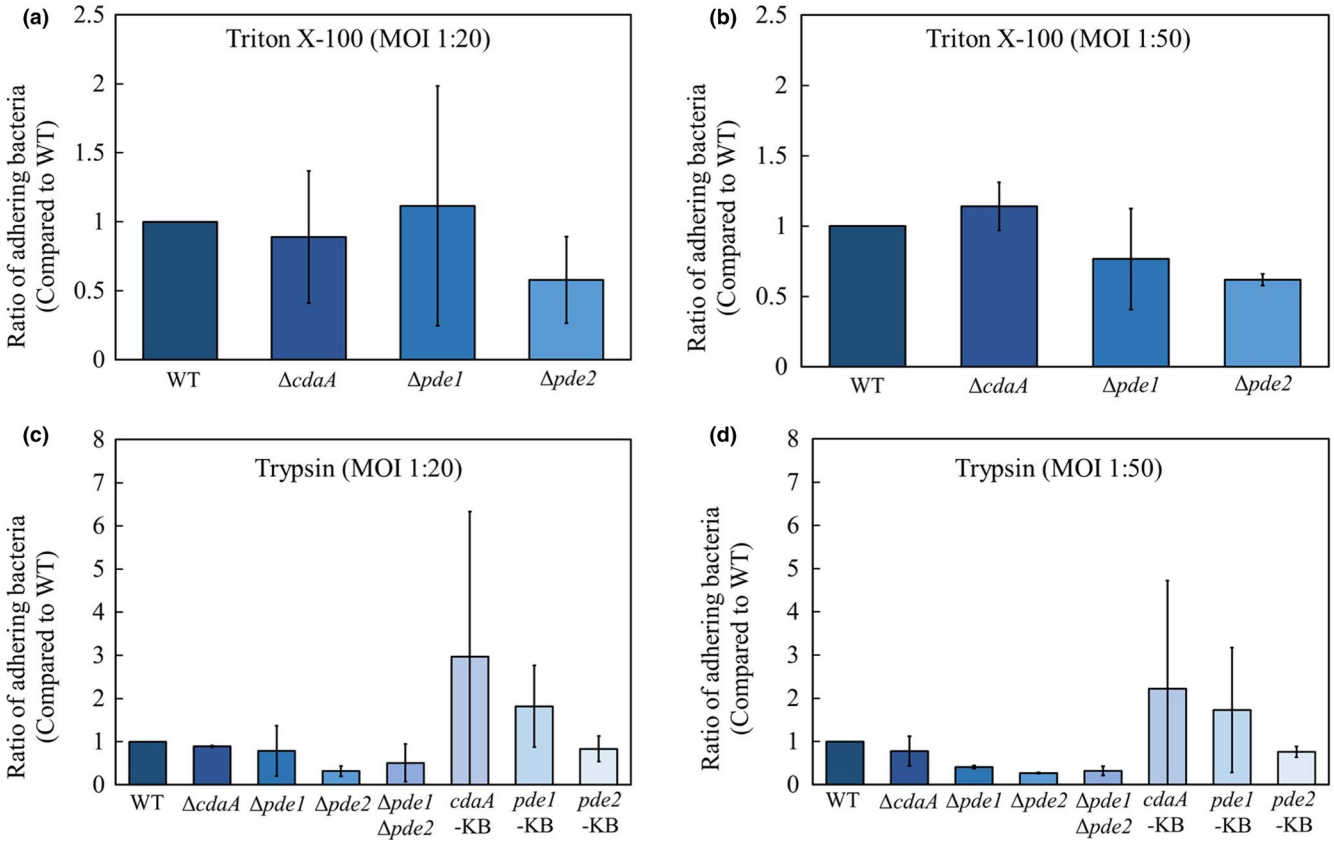


FIGURE 6 The c-di-AMP signaling system may influence adhesion to oral keratinocytes. The oral squamous cell carcinoma cell line PE/CA-PJ49 was grown to confluence and co-incubated with *Streptococcus mitis* WT and mutants with MOI 20 or MOI 50 for 30 min. The medium was removed, and cells were either lysed with 0.1% Triton X-100 for 10 min (a) MOI 20, and (b) MOI 50, or the cells were released from the surface using 0.5% trypsin for 20 min (c) MOI 20 and (d) MOI 50. The CFUs were determined, and the ratio of adhering bacteria to the cells was calculated and values of the mutants were normalized against the WT. For the WT, approximately 10 million and 25 million CFU were added to each well for MOI 20 and MOI 50, respectively. Approximately 1% of the original number of bacteria were attached to the keratinocytes after 30 min co-incubation. Shown are averages and confidence intervals of the mean of at least two independent experiments

restoration of the WT phenotype at MOI 20 and MOI 50, respectively (Figure 6c,d).

4 | DISCUSSION

The oral cavity constitutes a dynamic ecosystem that is continuously provoked by biotic and abiotic factors. Consequently, microorganisms that successfully colonize the oral cavity have to survive and quickly adapt to frequent changes in their environment. The c-di-AMP signaling system is involved in regulating bacterial responses to environmental changes and interference with cellular c-di-AMP homeostasis can influence survival and stress tolerance of bacteria, biofilm formation, and host–microbe interactions (Bremer & Krämer, 2019; Devaux et al., 2018; Xiong et al., 2020; Zarrella & Bai, 2020). In this study, we show that the c-di-AMP signaling system is influencing the ability of *S. mitis* to form a biofilm and cope with DNA damage, and there is also a tendency toward an effect on susceptibility to acid stress and membrane perturbation.

4.1 | c-di-AMP is involved in DNA damage repair

We have previously shown that in the exponential phase, the c-di-AMP concentration is abolished in the Δ*cdaA* mutant and significantly increased in the Δ*pde1* and Δ*pde2* mutants (Rørvik et al., 2020). The high intracellular concentration of c-di-AMP in the Δ*pde1* and Δ*pde2* mutants sensitizes *S. mitis* to UV radiation, suggesting that c-di-AMP is involved in regulating DNA repair mechanisms (Figure 1b). Similar results have been reported for *S. pneumonia* (Bai et al., 2013). The susceptibility to the fluoroquinolone ciprofloxacin was higher for the Δ*cdaA* mutant compared to the WT and reduced for both Δ*pde1* and Δ*pde2* mutants. UV light and fluoroquinolones affect DNA integrity differently (Table 2 and Figure 2g–i). The energy in UV radiation causes the formation of cyclobutane pyrimidine dimers and 6–4 photoproducts that in bacteria can be corrected by the photolyase or nucleotide excision repair systems (Goosen & Moolenaar, 2008). However, unmended pyrimidine dimers can result in replication fork collapse and chromosome fragmentation (Khan & Kuzminov, 2012). The fluoroquinolones target the bacterial type II topoisomerases, inhibit replication, and cause double-strand breaks in DNA. Double-strand breaks are repaired by recombinational DNA damage repair systems (Sinha et al., 2020). The exact role of c-di-AMP in DNA damage repair is unclear. It has been shown that DisA-type DACs form assemblies that dynamically move along DNA until it encounters DNA lesions and pauses (Oppenheimer-Shaanan et al., 2011). In vitro, DisA preferentially binds to three-way or four-way junctions which lead to inhibition of the DAC activity. It has therefore been speculated that DisA utilizes c-di-AMP as a reporter for DNA integrity and reduced c-di-AMP concentration signals a pause in growth or development that allows time for DNA repair (Oppenheimer-Shaanan et al., 2011). Of note, a DisA-type DAC is not encoded in *S. mitis*. In *M. smegmatis*, c-di-AMP can interact directly with RecA, which results in disassembly of the RecA-nucleoprotein filament and reduced DNA strand exchange, suggesting a direct role for c-di-AMP in DNA repair (Manikandan et al., 2018); however, this does not seem to be the case in *B. subtilis* (Torres et al., 2019). Interestingly, in *B. subtilis*, *M. smegmatis*, and *D. radiodurans*, reduced c-di-AMP concentration sensitizes the bacteria to DNA damaging stress (Dulermo et al., 2015; Manikandan et al., 2018; Rao et al., 2010). This is in contrast to the *S. mitis* group of bacteria in which increased c-di-AMP concentration reduces survival under DNA stress ((Bai et al., 2013) and this study). Future studies that identify the relevant c-di-AMP effectors involved in DNA damage repair will clarify the diverging c-di-AMP-mediated mechanisms involved in coping with DNA stress in different bacteria.

4.2 | The cellular c-di-AMP concentration affects cell envelope stress survival

Disruption of intracellular c-di-AMP homeostasis can result in changes in the cell membrane and/or the cell wall of bacteria indicating a role for c-di-AMP in maintaining the integrity of the cell envelope. Deletion of *pde1* resulted in significantly reduced survival of *S. mitis* in presence of the detergent Triton X-100 (Figure 1d). This is in agreement with studies reporting that increased c-di-AMP concentrations in *B. anthracis* and *S. pneumoniae* sensitized the bacteria to the detergent Triton X-100 (Hu et al., 2020; Kuipers et al., 2016) and reduced concentration of c-di-AMP in *S. aureus* resulted in decreased spontaneous autolysis and increased Triton X-100 tolerance (Dengler et al., 2013). Interestingly, we did not observe differences in MIC of chlorhexidine (Table 2), which also disrupts the membrane integrity, but may have additional effects on the cellular physiology (Russell & Day, 1993).

C-di-AMP signaling is frequently associated with regulation of cell wall homeostasis and altered susceptibility to cell wall-targeting antibiotics (Bowman et al., 2016; Cheng et al., 2016; Cho & Kang, 2013; Corrigan et al., 2011; Dengler et al., 2013; Fahmi et al., 2019; Griffiths & O'Neill, 2012; Massa et al., 2020). It is often reported that there is a direct correlation between cellular c-di-AMP concentration and resistance to β-lactam and/or glycopeptide antibiotics (Corrigan et al., 2011; Dengler et al., 2013; Fahmi et al., 2019; Griffiths & O'Neill, 2012; Luo & Helmann, 2012). However, it has been shown that deletion of c-di-AMP-specific phosphodiesterases in *Lactococcus lactis* and *Borrelia burgdorferi* does not influence the MIC of β-lactam antibiotics (Smith et al., 2012; Ye et al., 2014), or even increases the susceptibility to most cell wall-disrupting antibiotics in *B. burgdorferi* (Ye et al., 2014). Furthermore, conflicting results regarding the c-di-AMP-mediated effect on susceptibility to cell wall-targeting antibiotics have been reported for both *L. monocytogenes* and *B. subtilis* (Luo & Helmann, 2012; Massa et al., 2020; Witte et al., 2013).

We found that the MIC of ampicillin was twofold reduced in the Δ*cdaA* and Δ*pde2* mutants, but unaffected in the Δ*pde1* mutant (Table 2). These data indicate that the c-di-AMP concentration does

not directly control susceptibility to beta-lactam antibiotics in *S. mitis*. Deletion of *cdaA* or *pde2* negatively affects the growth of *S. mitis* in rich medium, and these phenotypes are exacerbated in the presence of sub-lethal concentrations of ampicillin (Figure 2d–f). Based on this, we interpret the MIC data for ampicillin as a general antibacterial effect that aggravates the intrinsic growth defect of the Δ*cdaA* and Δ*pde2* mutants, rather than a direct c-di-AMP-mediated susceptibility to cell wall stress. A model has been proposed suggesting that the susceptibility to cell wall-disrupting agents is an indirect consequence of c-di-AMP-mediated regulation of osmolyte transport and turgor pressure (Commichau et al., 2018). The correlation between c-di-AMP signaling and osmolyte homeostasis in *S. mitis* will be addressed in future studies.

4.3 | c-di-AMP signaling does not regulate susceptibility to heat stress, xylitol, or fluoride

Increased c-di-AMP concentration has been shown to reduce heat tolerance of *S. pneumonia* (Zarrella et al., 2018). In contrast, in *L. lactis*, increased heat resistance has been reported in response to deletion of its GdpP-type phosphodiesterase (Smith et al., 2012). However, we did not detect a statistically significant effect on the survival of bacteria undergoing heat treatment for any of our tested mutants compared to the WT (Figure 1c).

Xylitol, a commonly used sweetener, is thought to prevent caries as it is not fermented to produce organic acids (Grenby et al., 1989). Fluoride on the other hand affects bacterial growth and fermentation of carbohydrates by inhibiting the glycolytic enzyme enolase, and/or by acidification of the bacterial cytoplasm (Marquis et al., 2003). The MIC of xylitol was reduced in the Δ*pde2* mutant and the MIC of fluoride was reduced for the Δ*cdaA* and Δ*pde2* mutants (Table 2). Both of the mutants had decreased growth in sub-lethal concentrations of the two compounds (Figure 3a-c and g-i). However, since similar phenotypes are seen in the absence of antibacterial compounds, this may be caused by exacerbation of the abovementioned intrinsic slower growth of these two mutants, and not a specific c-di-AMP-mediated regulation of xylitol-or fluoride-susceptibility.

4.4 | Increased c-di-AMP concentrations may sensitize *S. mitis* to acid stress

The environment of the oral cavity fluctuates between acidic and alkaline pH. Successful colonizers of the oral cavity must be able to survive and adapt to these environmental changes. C-di-AMP signaling has been implicated in acid stress tolerance (Bowman et al., 2016; Corrigan et al., 2011; Rao et al., 2010; Witte et al., 2013; Zarrella et al., 2018). Our data suggest that increased c-di-AMP concentration may reduce the ability of *S. mitis* to survive acidic conditions (Figure 1a). Consistent with our results, reduced acid tolerance was observed in mutants of *pde1* and/or *pde2* in *S. pneumoniae* (Zarrella et al., 2018). In contrast, a c-di-AMP-deficient mutant of *Streptococcus pyogenes* did not grow at pH 6, whereas deletion mutants of the Pde1 or Pde2 homologs grew similar to the WT (Fahmi et al., 2019). In *B. subtilis* and *L. monocytogenes*, deletion mutants of their respective Pde1 homologs displayed increased survival in acidic conditions (Rao et al., 2010; Witte et al., 2013) and *S. aureus* acid tolerance at pH 4.5 directly correlated with c-di-AMP concentration (Bowman et al., 2016). The deviating results of c-di-AMP signaling in acid stress tolerance can result from differences in experimental design. Our results suggest that the survival of *S. mitis* is reduced when the external environment is acidified by acetic acid, but growth in medium acidified with HCl does not affect survival. The exact mechanism behind this is not known, but the presence of acetic acid likely results in intracellular acidification of the bacterial cytoplasm and/or toxic intracellular accumulation of the acid anion (Guan & Liu, 2020). Interestingly, growth of *S. mitis* in TSB acidified to pH 5.5 using HCl (Figure 4) displayed reduced growth of the Δ*cdaA* and Δ*pde2* mutants, indicating exacerbation of the inherent growth defects. These results suggest that the c-di-AMP signaling can have a direct role in organic acid tolerance of *S. mitis*, but may not be involved in survival in acidic environments in general.

4.5 | Biofilm formation of *S. mitis* is influenced by c-di-AMP signaling

Although the OD of the Δ*cdaA* mutant was lower compared to the WT in shaken culture, it was increased compared to the WT in static culture (Figure 4). This phenotype was more pronounced at pH 5.5 compared to 7.2 suggesting that c-di-AMP signaling influences adhesive properties of *S. mitis*, possibly in response to an acidic environment. This is in agreement with observations described for *S. mutans*, where the Δ*cdaA* mutant displays a clumping phenotype that sediments at the bottom of culturing vessels (Cheng et al., 2016), and for the cyanobacterium *Synechocystis* that aggregated upon interference with the c-di-AMP signaling system (Agostoni et al., 2018). Based on this, we hypothesized that the Δ*cdaA* mutant would be a strong biofilm former and quantified the mass of biofilm formed under different conditions. The biofilm mass produced by *S. mitis* was similar under all atmospheric conditions tested but varied depending on the pH of the growth medium (Figure 5). More biofilm was produced in TSB buffered at pH 5.5 than TSB buffered at 7.2. However, contrary to our assumption, the Δ*cdaA* mutant produced less biofilm mass compared to the WT, suggesting that auto-aggregation of bacterial cells was increased in the Δ*cdaA* mutant compared to the WT, but adhesion to the abiotic surface of the assay plate was reduced. This was in agreement with our visual observation of the static culture, as the Δ*cdaA* mutant produced an aggregating growth phenotype in unmodified TSB and TSB adjusted to pH 5.5, but not in TSB buffered to pH 7.2 (Figure 5h).

The difference in the biofilm-forming ability of the Δ*cdaA* mutant compared to the WT was bigger at acidic pH compared to neutral pH. It is likely that c-di-AMP is upregulated under acidic conditions and that increased c-di-AMP levels are positively associated with biofilm formation.

The role of c-di-AMP signaling in biofilm formation has been studied in several species of streptococci with varying outcomes. Cheng et al.

reported that a *S. mutans* Δ*cdaA* mutant produced more extracellular polysaccharides leading to a thicker biofilm (Cheng et al., 2016). This correlated with a study in *Streptococcus gallolyticus*, where an increase in c-di-AMP resulted in less biofilm (Teh et al., 2019). However, these results are contradictory to two studies published by Peng et al. where the deletion of *cdaA* in *S. mutans* resulted in diminished biofilm, while deletion of the Pde1 homolog resulted in increased biofilm formation (Peng et al., 2016; Peng, Zhang, et al., 2016). However, Konno et al. used single and double mutants of the Pde1 and Pde2 homologs in *S. mutants* and showed that it was only the mutants of the *pde2* gene that displayed increased biofilm production (Konno et al., 2018). Furthermore, the biofilm production of *S. pyogenes* directly correlated with the c-di-AMP concentration (Fahmi et al., 2019). This was in agreement with observations in *Streptococcus suis*, where increased cellular c-di-AMP concentration correlated with increased biofilm production (Du et al., 2014). Taken together, there seem to be great variations in the effect of c-di-AMP signaling on biofilm production in streptococci. Similar discrepancies have been reported for the role of c-di-AMP signaling in biofilm formation of *B. subtilis* (Gundlach et al., 2016; Townsley et al., 2018) and *S. aureus* (Corrigan et al., 2011; DeFrancesco et al., 2017).

There was a consistent but statistically nonsignificant trend toward reduced adhesion of the Δ*pde2* mutant to oral keratinocytes compared to the WT, but the Δ*cdaA* mutant that displayed increased auto-aggregation, adhered to oral keratinocytes with similar efficiency as the WT, and so did the Δ*pde1* mutant (Figure 6). Our data suggest that c-di-AMP signaling may impact adhesion to eukaryotic cells, which is in agreement with observations reported for deletion mutants of the Pde1 homolog in *S. suis*, *S. gallolyticus*, and *B. subtilis* (Du et al., 2014; Teh et al., 2019; Townsley et al., 2018). However, increased c-di-AMP concentration influences adhesion to mammalian cells negatively in streptococci ((Figure 6) (Du et al., 2014; Teh et al., 2019)), whereas reduced c-di-AMP levels reduce adhesion of *B. subtilis* to plant cells (Townsley et al., 2018).

In several of the experiments performed in this study, we observed partial complementation of mutant phenotypes and relatively large variations between biological replicates. It is possible that this can be explained by the selection of suppressor mutations that counteract the potentially toxic effect of disturbed c-di-AMP homeostasis, as previously described for several bacterial species (Devaux, Sleiman, et al., 2018; Whiteley et al., 2015). This hypothesis will be pursued in a future study.

Although deletion of genes encoding c-di-AMP turnover proteins influences bacterial biofilm formation and adhesion to eukaryotic cells, the details of the regulatory mechanisms involved are still poorly understood. Identification and characterization of c-di-AMP effectors and comprehensive analyses of the role and regulation of these signaling components will improve our understanding of the role of c-di-AMP signaling in colonization and virulence of *S. mitis*.

ACKNOWLEDGEMENT

The authors would like to thank Benoit Follin-Arbelet and Agnieszka Rogala for the technical assistance in the maintenance and care of the oral squamous cells used in this study.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Gro Herredsvela Rørvik: Conceptualization (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Visualization (lead); Writing-original draft (equal); Writing-review & editing (equal). **Ali-Oddin Naemi:** Investigation (supporting); Methodology (supporting); Validation (supporting); Writing-review & editing (supporting). **Per Kristian Thorén Edvardsen:** Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Roger Simm:** Conceptualization (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Project administration (lead); Resources (lead); Validation (lead); Visualization (lead); Writing-original draft (equal); Writing-review & editing (equal).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

ORCID

Gro Herredsvela Rørvik https://orcid.org/0000-0002-1650-1980
Roger Simm https://orcid.org/0000-0001-5187-2456

REFERENCES

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, *43*(11), 5721–5732. https://doi.org/10.1128/jcm.43.11.5721-5732.2005

Agostoni, M., Logan-Jackson, A. R., Heinz, E. R., Severin, G. B., Bruger, E. L., Waters, C. M., & Montgomery, B. L. (2018). Homeostasis of second messenger cyclic-di-AMP is critical for cyanobacterial fitness and acclimation to abiotic stress. *Frontiers in Microbiology*, *9*, 1121. https://doi.org/10.3389/fmicb.2018.01121

Al-Farsi, F., Al-Busaidi, I., & Al-Zeedi, K. (2018). Acute *Streptococcus mitis* Sacroiliitis in a teenager with unclear source of bacteremia: A case report and literature review. *Case Reports in Infectious Diseases*, *2018*, 2616787. https://doi.org/10.1155/2018/2616787

Andrade, W. A., Firon, A., Schmidt, T., Hornung, V., Fitzgerald, K. A., Kurt-Jones, E. A., Trieu-Cuot, P., Golenbock, D. T., & Kaminski, P.-A. (2016). Group B streptococcus degrades cyclic-di-AMP to modulate STING-dependent type I interferon production. *Cell Host & Microbe*, *20*(1), 49–59. https://doi.org/10.1016/j.chom.2016.06.003

Bai, Y., Yang, J., Eisele, L. E., Underwood, A. J., Koestler, B. J., Waters, C. M., Metzger, D. W., & Bai, G. (2013). Two DHH subfamily 1 proteins in *Streptococcus pneumoniae* possess cyclic di-AMP phosphodiesterase activity and affect bacterial growth and virulence. *Journal of Bacteriology*, *195*(22), 5123–5132. https://doi.org/10.1128/jb.00769-13

Barker, J. R., Koestler, B. J., Carpenter, V. K., Burdette, D. L., Waters, C. M., Vance, R. E., & Valdivia, R. H. (2013). STING-dependent recognition of cyclic di-AMP mediates type I interferon responses during *Chlamydia trachomatis* infection. *MBio*, *4*(3), e00018-00013. https://doi.org/10.1128/mBio.00018-13

Basaranoglu, S. T., Ozsurekci, Y., Aykac, K., Aycan, A. E., Bicakcigil, A., Altun, B., & Ceyhan, M. (2019). *Streptococcus mitis/oralis* causing blood stream infections in pediatric patients. *Japanese Journal of Infectious Diseases*, *72*(1), 1–6. https://doi.org/10.7883/yoken.JJID.2018.074

Bowman, L., Zeden, M. S., Schuster, C. F., Kaever, V., & Grundling, A. (2016). New insights into the cyclic di-adenosine monophosphate (c-di-AMP) degradation pathway and the requirement of the cyclic dinucleotide for acid stress resistance in *Staphylococcus aureus*. *Journal of Biological Chemistry*, *291*(53), 26970–26986. https://doi.org/10.1074/jbc.M116.747709

Bremer, E., & Krämer, R. (2019). Responses of microorganisms to osmotic stress. *Annual Review of Microbiology*, *73*, 313–334. https://doi.org/10.1146/annurev-micro-020518-115504

Byrd, V. S., & Nemeth, A. S. (2017). A case of infective endocarditis and spinal epidural abscess caused by *Streptococcus mitis* Bacteremia. *Case Reports in Infectious Diseases*, *2017*, 7289032. https://doi.org/10.1155/2017/7289032

Cheng, X., Zheng, X., Zhou, X., Zeng, J., Ren, Z., Xu, X., & Li, Y. (2016). Regulation of oxidative response and extracellular polysaccharide synthesis by a diadenylate cyclase in *Streptococcus mutans*. *Environmental Microbiology*, *18*(3), 904–922. https://doi.org/10.1111/1462-2920.13123

Cho, K. H., & Kang, S. O. (2013). *Streptococcus pyogenes* c-di-AMP phosphodiesterase, GdpP, influences SpeB processing and virulence. *PLoS One*, *8*(7), e69425. https://doi.org/10.1371/journal.pone.0069425

Commichau, F. M., Gibhardt, J., Halbedel, S., Gundlach, J., & Stulke, J. (2018). A delicate connection: c-di-AMP affects cell integrity by controlling osmolyte transport. *Trends in Microbiology*, *26*(3), 175–185. https://doi.org/10.1016/j.tim.2017.09.003

Corrigan, R. M., Abbott, J. C., Burhenne, H., Kaever, V., & Grundling, A. (2011). c-di-AMP is a new second messenger in Staphylococcus aureus with a role in controlling cell size and envelope stress. *PLoS Path*, *7*(9), e1002217. https://doi.org/10.1371/journal.ppat.1002217

DeFrancesco, A. S., Masloboeva, N., Syed, A. K., DeLoughery, A., Bradshaw, N., Li, G. W., & Losick, R. (2017). Genome-wide screen for genes involved in eDNA release during biofilm formation by *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(29), E5969–E5978. https://doi.org/10.1073/pnas.1704544114

Dengler, V., McCallum, N., Kiefer, P., Christen, P., Patrignani, A., Vorholt, J. A., Berger-Bächi, B., & Senn, M. M. (2013). Mutation in the C-di-AMP cyclase dacA affects fitness and resistance of methicillin resistant *Staphylococcus aureus*. *PLoS One*, *8*(8), e73512. https://doi.org/10.1371/journal.pone.0073512

Devaux, L., Kaminski, P. A., Trieu-Cuot, P., & Firon, A. (2018). Cyclic di-AMP in host-pathogen interactions. *Current Opinion in Microbiology*, *41*, 21–28. https://doi.org/10.1016/j.mib.2017.11.007

Devaux, L., Sleiman, D., Mazzuoli, M.-V., Gominet, M., Lanotte, P., Trieu-Cuot, P., Kaminski, P.-A., & Firon, A. (2018). Cyclic di-AMP regulation of osmotic homeostasis is essential in Group B Streptococcus. *PLoS Genetics*, *14*(4), e1007342. https://doi.org/10.1371/journal.pgen.1007342

Du, B., Ji, W., An, H., Shi, Y., Huang, Q., Cheng, Y., Fu, Q., Wang, H., Yan, Y., & Sun, J. (2014). Functional analysis of c-di-AMP phosphodiesterase, GdpP, in *Streptococcus suis* serotype 2. *Microbiological Research*, *169*(9–10), 749–758. https://doi.org/10.1016/j.micres.2014.01.002

Dulermo, R., Onodera, T., Coste, G., Passot, F., Dutertre, M., Porteron, M., Confalonieri, F., Sommer, S., & Pasternak, C. (2015). Identification of new genes contributing to the extreme radioresistance of *Deinococcus radiodurans* using a Tn5-based transposon mutant library. *PLoS One*, *10*(4), e0124358. https://doi.org/10.1371/journal.pone.0124358

Fahmi, T., Faozia, S., Port, G. C., & Cho, K. H. (2019). The second messenger c-di-AMP regulates diverse cellular pathways involved in stress response, biofilm formation, cell wall homeostasis, SpeB expression, and virulence in *Streptococcus pyogenes*. *Infection and Immunity*, *87*(6), https://doi.org/10.1128/iai.00147-19

Goosen, N., & Moolenaar, G. F. (2008). Repair of UV damage in bacteria. *DNA Repair (Amst)*, *7*(3), 353–379. https://doi.org/10.1016/j.dnarep.2007.09.002

Grenby, T. H., Phillips, A., & Mistry, M. (1989). Studies of the dental properties of lactitol compared with five other bulk sweeteners in vitro. *Caries Research*, *23*(5), 315–319. https://doi.org/10.1159/000261199

Griffiths, J. M., & O'Neill, A. J. (2012). Loss of function of the gdpP protein leads to joint beta-lactam/glycopeptide tolerance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *56*(1), 579–581. https://doi.org/10.1128/AAC.05148-11

Guan, N., & Liu, L. (2020). Microbial response to acid stress: Mechanisms and applications. *Applied Microbiology and Biotechnology*, *104*(1), 51–65. https://doi.org/10.1007/s00253-019-10226-1

Gundlach, J., Rath, H., Herzberg, C., Mader, U., & Stulke, J. (2016). Second messenger signaling in *Bacillus subtilis*: Accumulation of cyclic di-AMP inhibits biofilm formation. *Frontiers in Microbiology*, *7*, 804. https://doi.org/10.3389/fmicb.2016.00804

Hu, J., Zhang, G., Liang, L., Lei, C., & Sun, X. (2020). Increased intracellular cyclic di-AMP levels impair growth and virulence of *Bacillus anthracis*. *Journal of Bacteriology*, https://doi.org/10.1128/jb.00653-19

Kamegaya, T., Kuroda, K., & Hayakawa, Y. (2011). Identification of a Streptococcus pyogenes SF370 gene involved in production of c-di-AMP. *Nagoya Journal of Medical Science*, *73*(1–2), 49–57.

Khan, S. R., & Kuzminov, A. (2012). Replication forks stalled at ultraviolet lesions are rescued via RecA and RuvABC protein-catalyzed disintegration in *Escherichia coli*. *Journal of Biological Chemistry*, *287*(9), 6250–6265. https://doi.org/10.1074/jbc.M111.322990

Konno, H., Yoshida, Y., Nagano, K., Takebe, J., & Hasegawa, Y. (2018). Biological and biochemical roles of two distinct cyclic dimeric adenosine 3',5'-monophosphate- associated phosphodiesterases in *Streptococcus mutans*. *Frontiers in Microbiology*, *9*, 2347. https://doi.org/10.3389/fmicb.2018.02347

Kontiokari, T., Uhari, M., & Koskela, M. (1995). Effect of xylitol on growth of nasopharyngeal bacteria in vitro. *Antimicrobial Agents and Chemotherapy*, *39*(8), 1820–1823. https://doi.org/10.1128/aac.39.8.1820

Kuipers, K., Gallay, C., Martinek, V., Rohde, M., Martinkova, M., van der Beek, S. L., & de Jonge, M. I. (2016). Highly conserved nucleotide phosphatase essential for membrane lipid homeostasis in *Streptococcus pneumoniae*. *Molecular Microbiology*, *101*(1), 12–26. https://doi.org/10.1111/mmi.13312

Lu, H.-Z., Weng, X.-H., Zhu, B., Li, H., Yin, Y.-K., Zhang, Y.-X., Haas, D. W., & Tang, Y.-W. (2003). Major outbreak of toxic shock-like syndrome caused by *Streptococcus mitis*. *Journal of Clinical Microbiology*, *41*(7), 3051–3055. https://doi.org/10.1128/jcm.41.7.3051-3055.2003

Luo, Y., & Helmann, J. D. (2012). Analysis of the role of *Bacillus subtilis* σ(M) in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Molecular Microbiology*, *83*(3), 623–639. https://doi.org/10.1111/j.1365-2958.2011.07953.x

Manikandan, K., Prasad, D., Srivastava, A., Singh, N., Dabeer, S., Krishnan, A., & Sinha, K. M. (2018). The second messenger cyclic di-AMP negatively regulates the expression of *Mycobacterium smegmatis* recA and attenuates DNA strand exchange through binding to the C-terminal motif of mycobacterial RecA proteins. *Molecular Microbiology*, *109*(5), 600–614. https://doi.org/10.1111/mmi.13991

Marquis, R. E., Clock, S. A., & Mota-Meira, M. (2003). Fluoride and organic weak acids as modulators of microbial physiology. *FEMS Microbiology Reviews*, *26*(5), 493–510. https://doi.org/10.1111/j.1574-6976.2003.tb00627.x

Marron, A., Carratala, J., Gonzalez-Barca, E., Fernandez-Sevilla, A., Alcaide, F., & Gudiol, F. (2000). Serious complications of bacteremia caused by *Viridans streptococci* in neutropenic patients with

cancer. *Clinical Infectious Diseases*, *31*(5), 1126–1130. https://doi.org/10.1086/317460

Massa, S. M., Sharma, A. D., Siletti, C., Tu, Z., Godfrey, J. J., Gutheil, W. G., & Huynh, T. N. (2020). c-di-AMP accumulation impairs muropeptide synthesis in listeria monocytogenes. *Journal of Bacteriology*, *202*(24), https://doi.org/10.1128/jb.00307-20

McFarland, A. P., Luo, S., Ahmed-Qadri, F., Zuck, M., Thayer, E. F., Goo, Y. A., & Woodward, J. J. (2017). Sensing of bacterial cyclic dinucleotides by the oxidoreductase RECON promotes NF-kappaB activation and shapes a proinflammatory antibacterial state. *Immunity*, *46*(3), 433–445. https://doi.org/10.1016/j.immuni.2017.02.014

Nygren, D., Nilson, B., & Rasmussen, M. (2018). A case of recurrent erysipelas caused by *Streptococcus mitis* group. *Case Reports in Infectious Disease*, *2018*, 5156085. https://doi.org/10.1155/2018/5156085

Oppenheimer-Shaanan, Y., Wexselblatt, E., Katzhendler, J., Yavin, E., & Ben-Yehuda, S. (2011). c-di-AMP reports DNA integrity during sporulation in *Bacillus subtilis*. *EMBO Reports*, *12*(6), 594–601. https://doi.org/10.1038/embor.2011.77

Parvatiyar, K., Zhang, Z., Teles, R. M., Ouyang, S., Jiang, Y., Iyer, S. S., Zaver, S. A., Schenk, M., Zeng, S., Zhong, W., Liu, Z.-J., Modlin, R. L., Liu, Y.-J., & Cheng, G. (2012). The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. *Nature Immunology*, *13*(12), 1155–1161. https://doi.org/10.1038/ni.2460

Peng, X., Michalek, S., & Wu, H. (2016). Effects of diadenylate cyclase deficiency on synthesis of extracellular polysaccharide matrix of *Streptococcus mutans* revisit. *Environmental Microbiology*, *18*(11), 3612–3619. https://doi.org/10.1111/1462-2920.13440

Peng, X., Zhang, Y., Bai, G., Zhou, X., & Wu, H. (2016). Cyclic di-AMP mediates biofilm formation. *Molecular Microbiology*, *99*(5), 945–959. https://doi.org/10.1111/mmi.13277

Rao, F., See, R. Y., Zhang, D., Toh, D. C., Ji, Q., & Liang, Z. X. (2010). YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *Journal of Biological Chemistry*, *285*(1), 473–482. https://doi.org/10.1074/jbc.M109.040238

Rørvik, G. H., Liskiewicz, K. A., Kryuchkov, F., Naemi, A.-O., Aasheim, H.-C., Petersen, F. C., Küntziger, T. M., & Simm, R. (2020). Cyclic Di-adenosine monophosphate regulates metabolism and growth in the oral commensal *Streptococcus mitis*. *Microorganisms*, *8*(9), 1269. https://doi.org/10.3390/microorganisms8091269

Rukke, H. V., Engen, S. A., Schenck, K., & Petersen, F. C. (2016). Capsule expression in *Streptococcus mitis* modulates interaction with oral keratinocytes and alters susceptibility to human antimicrobial peptides. *Molecular Oral Microbiology*, *31*(4), 302–313. https://doi.org/10.1111/omi.12123

Russell, A. D., & Day, M. J. (1993). Antibacterial activity of chlorhexidine. *Journal of Hospital Infection*, *25*(4), 229–238. https://doi.org/10.1016/0195-6701(93)90109-d

Shelburne, S. A., Sahasrabhojane, P., Saldana, M., Yao, H., Su, X., Horstmann, N., & Flores, A. R. (2014). *Streptococcus mitis* strains causing severe clinical disease in cancer patients. *Emerging Infectious Diseases*, *20*(5), 762–771. https://doi.org/10.3201/eid2005.130953

Sinha, A. K., Possoz, C., & Leach, D. R. F. (2020). The roles of bacterial DNA double-strand break repair proteins in chromosomal DNA replication. *FEMS Microbiology Reviews*, *44*(3), 351–368. https://doi.org/10.1093/femsre/fuaa009

Smith, W. M., Pham, T. H., Lei, L., Dou, J., Soomro, A. H., Beatson, S. A., & Turner, M. S. (2012). Heat resistance and salt hypersensitivity in *Lactococcus lactis* due to spontaneous mutation of llmg_1816 (gdpP) induced by high-temperature growth. *Applied and Environment Microbiology*, *78*(21), 7753–7759. https://doi.org/10.1128/aem.02316-12

Sulyanto, R. M., Thompson, Z. A., Beall, C. J., Leys, E. J., & Griffen, A. L. (2019). The predominant oral microbiota is acquired early in an organized pattern. *Scientific Reports*, *9*(1), 10550. https://doi.org/10.1038/s41598-019-46923-0

Teh, W. K., Dramsi, S., Tolker-Nielsen, T., Yang, L., & Givskov, M. (2019). Increased intracellular cyclic di-AMP levels sensitize *Streptococcus gallolyticus* subsp. gallolyticus to osmotic stress and reduce biofilm formation and adherence on intestinal cells. *Journal of Bacteriology*, *201*(6), https://doi.org/10.1128/jb.00597-18

Torres, R., Carrasco, B., Gándara, C., Baidya, A. K., Ben-Yehuda, S., & Alonso, J. C. (2019). Bacillus subtilis DisA regulates RecA-mediated DNA strand exchange. *Nucleic Acids Research*, *47*(10), 5141–5154. https://doi.org/10.1093/nar/gkz219

Townsley, L., Yannarell, S. M., Huynh, T. N., Woodward, J. J., & Shank, E. A. (2018). Cyclic di-AMP acts as an extracellular signal that impacts *Bacillus subtilis* biofilm formation and plant attachment. *MBio*, *9*(2), https://doi.org/10.1128/mBio.00341-18

Whiteley, A. T., Pollock, A. J., & Portnoy, D. A. (2015). The PAMP c-di-AMP Is essential for *Listeria monocytogenes* growth in rich but not minimal media due to a toxic increase in (p)ppGpp. [corrected]. *Cell Host & Microbe*, *17*(6), 788–798. https://doi.org/10.1016/j.chom.2015.05.006

Witte, C. E., Whiteley, A. T., Burke, T. P., Sauer, J. D., Portnoy, D. A., & Woodward, J. J. (2013). Cyclic di-AMP is critical for Listeria monocytogenes growth, cell wall homeostasis, and establishment of infection. *MBio*, *4*(3), e00282-00213. https://doi.org/10.1128/mBio.00282-13

Witte, G., Hartung, S., Buttner, K., & Hopfner, K. P. (2008). Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. *Molecular Cell*, *30*(2), 167–178. https://doi.org/10.1016/j.molcel.2008.02.020

Woodward, J. J., Iavarone, A. T., & Portnoy, D. A. (2010). c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. *Science*, *328*(5986), 1703–1705. https://doi.org/10.1126/science.1189801

Xia, P., Wang, S., Xiong, Z., Zhu, X., Ye, B., Du, Y., Meng, S., Qu, Y., Liu, J., Gao, G., Tian, Y., & Fan, Z. (2018). The ER membrane adaptor ERAdP senses the bacterial second messenger c-di-AMP and initiates antibacterial immunity. *Nature Immunology*, *19*(2), 141–150. https://doi.org/10.1038/s41590-017-0014-x

Xiong, Z. Q., Fan, Y. Z., Song, X., Liu, X. X., Xia, Y. J., & Ai, L. Z. (2020). The second messenger c-di-AMP mediates bacterial exopolysaccharide biosynthesis: A review. *Molecular Biology Reports*, *47*(11), 9149–9157. https://doi.org/10.1007/s11033-020-05930-5

Ye, M., Zhang, J.-J., Fang, X., Lawlis, G. B., Troxell, B., Zhou, Y., Gomelsky, M., Lou, Y., & Yang, X. F. (2014). DhhP, a cyclic di-AMP phosphodiesterase of *Borrelia burgdorferi*, is essential for cell growth and virulence. *Infection and Immunity*, *82*(5), 1840–1849. https://doi.org/10.1128/iai.00030-14

Zarrella, T. M., & Bai, G. (2020). The many roles of the bacterial second messenger cyclic di-AMP in adapting to stress cues. *Journal of Bacteriology*, *203*(1), https://doi.org/10.1128/jb.00348-20

Zarrella, T. M., Metzger, D. W., & Bai, G. (2018). stress suppressor screening leads to detection of regulation of cyclic di-AMP homeostasis by a Trk family effector protein in *Streptococcus pneumoniae*. *Journal of Bacteriology*, *200*(12), https://doi.org/10.1128/jb.00045-18

How to cite this article: Rørvik, G. H., Naemi, A.-O., Edvardsen, P. K. T., & Simm, R. (2021). The c-di-AMP signaling system influences stress tolerance and biofilm formation of *Streptococcus mitis*. *MicrobiologyOpen*, 10, e1203. https://doi.org/10.1002/mbo3.1203