Inhibition of *Enterococcus faecalis* Growth and Biofilm Formation by Molecule Targeting Cyclic di-AMP Synthetase Activity

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Abstract

Introduction: *Enterococcus faecalis* is correlated with oral diseases including recurrent root canal treatment failure because of its biofilm formation ability and various virulence factors. Cyclic di-AMP (c-di-AMP) is an omnipresent second messenger involved in many crucial cellular physiological processes, including biofilm formation. ST056083 is a small molecule working as an inhibitor of the c-di-AMP synthetase DNA integrity scanning protein (DisA) *in vitro*. In this study, the impact of ST056083 on *E. faecalis* DisA activity, bacterial growth, and biofilm formation was tested. **Methods:** The binding affinity between the protein and ligand was evaluated using the Amber score, and the binding mode was analyzed and visualized using UCSF Chimera (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, San Francisco, CA). The effect of ST056083 on *E. faecalis* DisA was evaluated using the coralyne assay. The effect of ST056083 on *E. faecalis* biofilm formation was determined by the biofilm quantification assay, scanning electron microscopic examination, and 3-dimensional confocal laser scanning microscopic assay. The effect of ST056083 on *E. faecalis* exopolysaccharide synthesis was measured by the anthrone-sulfuric method. **Results:** We expressed and purified *E. faecalis* DisA *in vitro* and confirmed the inhibitory effect of ST056083 on its biological activity. In addition, we showed the inhibitory effect of ST056083 on *E. faecalis* growth, biofilm formation, and exopolysaccharide synthesis. **Conclusions:** Our findings enhance the understanding of the physiological role of c-di-AMP in *E. faecalis* and represent a preliminary study on the ST056083 inhibitory effect and mechanism. *(J Endod 2018;■:1–7)*

Key Words

Biofilm, cyclic di-AMP, *Enterococcus faecalis*, ST056083

Significance

Enterococcus faecalis is considered the primary causal agent in failed root canal therapy. ST056083 is proved to effectively inhibit *E. faecalis* biofilm formation by targeting c-di-AMP synthetase and has the potential to be exploited in dental treatment.

Enterococcus faecalis, a gram-positive facultative bacterium, is considered a predominant bacterium that is identified from persistently infected root canals and other oral diseases, such as dental caries, periodontitis, and peri-implantitis (1). The ability of *E. faecalis* to cause infections is associated with biofilm formation in which bacteria are approximately 100 to 1000 times more resistant to antibiotics and antibodies than when they are in their planktonic state (2) because of several factors, including the presence of an extracellular polymeric matrix, quorum-sensing signal transduction systems, and sugar-binding transcriptional regulators (3).

Clinical medicaments in eradicating bacteria from the root canal system include calcium hydroxide, phenolic and nonphenolic biocides, antibiotics, and iodine compounds (4). Sodium hypochlorite has been applied extensively in root canal treatment with its potent antibiofilm activity and capacity to dissolve organic tissues (5); however, several studies have shown that a certain amount of contamination still remains in root canals (6), and its toxicity is likely to cause allergic reactions (7). Calcium hydroxide is commonly used to kill bacteria, in part by the release of hydroxyl ions (OH^-) resulting in an alkaline environment that is lethal to some organisms. However, the use of calcium hydroxide as an antimicrobial medicament to eliminate *E. faecalis* has been considered inefficient because of high resistance (8). Phenol compounds such as camphorated monochlorophenol have been used for decades, but both the problematic biocompatibility and the loss through evaporation are nonnegligible (9, 10). Some more recent studies have shown the combination of calcium hydroxide and camphorated monochlorophenol is likely to compensate for the weaknesses and increase the efficiency in eliminating the bacteria (11), but the antimicrobial action still needs long-term observation.

Cyclic di-GMP (c-di-GMP) synthesized from 2 molecules of guanosine triphosphate via diguanylate cyclase has been shown to play a key role in multiple cellular functions since 1987, including biofilm formation, virulence, and other processes (12). Another

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cyclic dinucleotide, cyclic di-AMP (c-di-AMP), was not discovered until 2008 (13); c-di-AMP is characterized as an essential second messenger because few c-di-AMP–producing bacteria can survive without it (14). Initially, c-di-AMP was identified in the crystallization research of a DNA integrity scanning protein (DisA) (13). Since then, c-di-AMP has been detected among microbiota, including *Staphylococcus aureus*, *Chlamydia trachomatis*, *Streptococcus pyogenes*, and even in a subset of archaea (15). Previous studies have uncovered that c-di-AMP is synthesized from 2 molecules of adenosine triphosphate (ATP) or adenosine diphosphate (ADP) by diadenylyl cyclase (DAC) enzymes and degraded to pApA or adenosine monophosphate by phosphodiesterase enzymes (16). As a crucial intracellular signaling molecule, c-di-AMP plays a major role in bacterial growth and virulence, biofilm formation, cellular morphology, regulation of fatty acid synthesis, and host immune response through activating signal transduction by binding to and modulating nucleic acid or protein at a translational or transcriptional level (16). As 1 of the commonest groups of DAC domain–containing proteins, DisA is able to convert ATP into c-di-AMP (17). A prior study has reported that enhanced c-di-AMP levels contributed to the promoted biofilm formation in *Streptococcus mutans* via up-regulating the expression of *gtfB*, the gene coding for a major enzyme relevant to biofilm formation (18). More recently, the presence of intracellular c-di-AMP signaling has been confirmed in Enterococci and a novel phosphodiesterase of the GdpP family has been determined to be responsible for the regulation of c-di-AMP in response to the antibiotic-induced cell membrane stress (19).

5-([3,5-dibromo-2- hydroxyphenyl] methylene)-2-thioxo-1,3-diazolidin-4-one (ST056083, Supplemental Fig. S1B is available online at www.jendodon.com) was the first DisA inhibitor reported through high-throughput screening using the coralyne assay in 2014 (20). The mechanism behind the coralyne assay is simply based on c-di-AMP protecting coralyne from quenchers. Even though ST056083 has been screened using the coralyne assay and proved as an inhibitor of c-di-AMP synthetase, no further studies have been reported since then. Therefore, the impact of ST056083 on pathogens still remains unknown.

Taken together, we hypothesized that the inhibitor ST056083 was presumably able to inhibit the growth and biofilm formation of *E. faecalis* by targeting c-di-AMP synthetase activity. In the present study, we used the sequence alignment function and molecular docking to explore if ST056083 was able to affect c-di-AMP synthetase in *E. faecalis* as our first step. To verify the hypothesis, we identified and characterized the inhibitory activity of ST056083 on c-di-AMP synthetase DisA in *E. faecalis* ATCC 29212 (American Type Culture Collection, Manassas, VA) extracellularly and intracellularly. Furthermore, we performed serial phenotypic assays to confirm the ST056083 inhibitory effect on *E. faecalis* growth and biofilm formation. Our findings suggested that this molecule could suppress *E. faecalis* presumably by targeting c-di-AMP synthetase activity.

Materials and Methods

Sequence Alignment

The Research Collaboratory for Structural Bioinformatics Protein Data Bank (21) search revealed that only the crystal structure of DisA from *Thermotoga maritima* is publicly available among all DisA proteins. *T. maritima* DisA at different states was downloaded from The Research Collaboratory for Structural Bioinformatics Protein Data Bank with the accession number 3C1Z (apoDisA), 3C21 (apoDisA-ATP), 3C23 (apoDisA-Cordycepin-Triphosphate), and 3C1Y (apoDisA-c-di-AMP). All structures were originally obtained using X-ray diffraction with high resolution (<3 Å) (22).

Using the sequence alignment function in ClustalX2 (University College Dublin, Dublin, Ireland) (23), we aligned the sequences of *T. maritima* DisA and *Bacillus subtilis* DisA with *E. faecalis* EF2157 protein, and we found the conservation of *E. faecalis* (residues 133–250) with *T. maritima* (residues 1–146) and *B. subtilis* (residues 1–148). Then, the 3-dimensional structures of *B. subtilis* DisA (aa 1–148) and *E. faecalis* DisA (aa 133–250) were acquired using the protein structure homology modeling server SWISS-MODEL (University of Basel, Basel, Switzerland) (23) with *T. maritima* DisA (aa 1–146) as templates.

Protein-ligand Docking

Before docking, proteins and ligands were prepared with energy minimized and water removal using the Dock prep tool of the University of California, San Francisco (UCSF) Chimera (24), a molecular modeling system available online. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at UCSF. The docking site in each protein was defined to precisely orient ST056083 to the active site of DisA. Regarding apoDisA, the largest sphere was calculated over the entire sphere using UCSF DOCK6 (25) as the docking site; for complexed DisA, *B. subtilis* DisA and *E. faecalis* DisA were first matched to *T. maritima* DisA using the matchmaker function in UCSF Chimera (24), and spheres within 8.0 Å root mean square deviation from every atom of the original ligand were used as the docking site. Molecular docking was first performed using the grid dock where the flexible ligand docked to a rigid receptor using the anchor-and-grow algorithm and then using amber dock where both the ligand and receptor were allowed to be flexible to reproduce the so-called induced fit (25). The binding affinity between the protein and ligand was evaluated using the Amber score, and the binding mode was analyzed and visualized using UCSF Chimera.

Chemicals, Test Bacteria, and Growth Conditions

The test bacteria and plasmids used in our study are listed in Supplemental Table S1 (available online at www.jendodon.com). The test bacterium *E. faecalis* ATCC 29212 in the study was purchased from American Type Culture Collection. The compound ST056083 (Supplemental Fig. S1B is available online at www.jendodon.com) powder was purchased from TimTec (Newark, DE) and was dissolved and subsequently diluted in dimethyl sulfoxide (DMSO; Sigma, Tuckerton, Germany). *E. faecalis* was grown in brain-heart infusion (BHI) broth (Difco, Sparks, MD) in an anaerobic chamber (10% H_2, 5% CO_2, and 85 % N_2; Thermo Fisher Scientific, Inc, Waltham, MA) at 37°C. For all experiments, overnight inoculum was adjusted to 1×10^7 colony-forming units/mL with fresh BHI medium and diluted 1:100 in the growth culture. For the planktonic growth assay (Supplemental Fig. S2), biofilm quantification, extracellular polysaccharide production assays, and the coralyne assay, polystyrene 96-well (flat-bottom) cell culture clusters (Corning Inc, Corning, NY) were used, and the final volume of each individual well was 200 μL. For scanning electron microscopic examination and confocal laser scanning microscopic examination, polystyrene 24-well (flat-bottom) cell culture clusters (Corning Inc) were used, and the final volume of each individual well was 3 mL. The final concentration of DMSO in each well was maintained at 1%. Medium with or without 1% DMSO was used as the control to ensure DMSO had no effect on the test bacteria. Each assay was performed in quadruplicate on 3 occasions.

Cloning, Expression, and Purification of Recombinant Protein

The *E. faecalis EF2157* gene was amplified from the genomic DNA of strain *E. faecalis* ATCC 29212 by appropriate primers (Supplemental Table S2 is available online at www.jendodon.com) containing designed restriction sites using the high-fidelity polymerase chain reaction system