The Effect of Benzyl Isothiocyanate On The Expression of Genes Encoding NADH Oxidase And Fibronectin-Binding Protein In Oral Streptococcal Biofilms

Hawraa Alhandal

Kuwait University

Esraa Almesaileikh

Kuwait University

Radhika G Bhardwaj

Kuwait University

Areej Al Khabbaz

Kuwait University

Maribasappa Karched (✉ maribasappa.karched@ku.edu.kw)

Kuwait University

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Abstract

Background: Recent studies have shown that antibiotic treatment results in up- or down regulation of several virulence-associated genes. The genes encoding NADH oxidase (*nox*) and fibronectin-binding protein (*fbp*) are known to play important roles in biofilms of some oral bacterial species. The objective was to study the effect of benzyl isothiocyanate (BITC), an antimicrobial agent from Miswak plant, on the expression of *nox* and *fbp* genes in some oral streptococci.

Methods: Bacterial strains were grown as biofilms in brucella broth. The crystal violet stained biofilms were quantified by optical density measurements at 590 nm. The biofilms were treated with an antimicrobial agent (BITC) for 2 h and fold change in mRNA expression of *nox* and *fbp* genes in BITC treated selected oral streptococci was measured by comparative $\Delta\Delta C_t$ method on Real-Time PCR machine.

Results: The highest amount of biofilm mass was produced by *A. defectiva*, followed by *S. gordonii*, *S. mutans*, *G. elegans* and *G. adiacens*. Upon treatment with BITC, *S. gordonii* biofilms showed highest mRNA expression for both *fbp* and *nox* genes. Mean (SE) folds increase in the expression of *nox* mRNA: *S. gordonii* 2 (0.30), followed by *S. mutans* 1.25 (0.18), *A. defectiva* 1.03 (0.09), *G. adiacens* 0.7 (0.03). Similarly for *fbp*, folds increase in mRNA expression was: *S. gordonii* 2.65 (0.03), followed by *A. defectiva* 2.09 (0.60), *G. elegans* 1.61 (0.40), *S. mutans* 1.57 (0.20), and *G. adiacens* 0.58 (0.06). *G. elegans* mRNA levels for *nox* were extremely low (0.006-fold).

Conclusion: BITC treatment of the biofilms caused an upregulation of biofilm-associated genes *fbp* and *nox* genes in most of the tested species suggesting the significance of *these* genes in biofilm lifestyle of these oral bacteria. Increased expression of *nox* and *fbp* genes in the biofilm lifestyle of these species needs further investigation to understand if it contributes to antimicrobial resistance.

Background

Biofilms are a complex and functional community of microorganisms encased in a polymeric matrix and attached to one another or to a solid surface. A characteristic feature of microbial biofilms is the secretion of an adhesive matrix of highly hydrated extracellular polymeric substances (EPSs), comprised of polysaccharides, proteins, lipids, and nucleic acids [3]. Biofilms are structurally and physiologically complex entities. Within the biofilms, microorganisms coordinate and cooperate like multicellular organisms [4]. Ability of bacterial species to form biofilm is regarded a key virulence factor. Biofilm-associated bacterial infections in medicine have been extensively studied [5, 6].

Dental plaque develops naturally, but it is also associated with two of the most prevalent diseases affecting the people worldwide, which are caries and periodontal diseases [7]. Dental plaque is the diverse microbial community that develops on the tooth surface embedded in a matrix of polymers of bacterial and salivary origin [8]. Plaque formation starts once a tooth surface is cleaned and the early colonizers adhere to the acquired enamel pellicle, which is a thin layer of salivary glycoproteins deposited on tooth

surface. Subsequently, secondary colonizers attach to the early colonizers through specific molecular interactions known as co-aggregation [8, 9]. These can involve protein-protein or carbohydrate-protein interactions, and this process determines the pattern of bacterial succession.

The oral cavity contains complex, multispecies microbial communities [10, 11]. This indicates that the residents in this community should exhibit extensive interactions while forming biofilm structures, carrying out physiological functions, and inducing microbial pathogenesis. Interspecies interactions in biofilms are competitive, cooperative and antagonistic [12]. In addition, such interactions may have specific effects in terms of the virulence properties of biofilm residents, which could influence the overall pathogenicity of biofilms.

Even though it is known that biofilms generally are more resistant to antibiotics, antibiotics can affect bacterial biofilms at different levels, including changes in the overall structure of the population, selection of resistant organisms, or alterations in bacterial physiology. Recent studies have shown that antibiotic treatment results in up- or down regulation of several genes [1]. The genes encoding NADH oxidase (*nox*) [13] and fibronectin-binding protein (*fbp*) [2] are known to play important roles in biofilms of some oral bacterial species. In this study, we aim to study the effect of benzyl isothiocyanate [14], an antimicrobial agent from Miswak plant, on the expression of *nox* and *fbp* genes in the following oral streptococci: *Granulicatella elegans*, *Granulicatella adiacens*, *Abiotrophia defectiva*, *Streptococcus mutans*, and *Streptococcus gordonii*.

Results

PCR optimization for the target genes *nox* and *fbp*

Prior to quantification of mRNA expression from *nox* and *fbp* genes, PCR conditions and the amplicon sizes were standardized. After the PCR run was complete, the PCR products were analyzed using agarose gel electrophoresis which showed single bands for all the five species. Expected sizes of the amplicons for the *fbp* gene visualized on the agarose gels were for *G. elegans* 127 bp, *G. adiacens* 118 bp, *A. defectiva* 137 bp, *S. mutans* 106 bp, and *S. gordonii* 102 bp. Similarly, expected sizes of the amplicons for the *nox* gene were for *G. elegans* 127 bp, *G. adiacens* 109 bp, *A. defectiva* 108 bp, *S. mutans* 127 bp, and *S. gordonii* 120 bp (Table 1). The absence of non-specific amplifications in all the tested species was evident from the single bands (Fig. 1).

Table 1
Primers used in this study.

Gene	Species	Sequence	Annealing temperature (°C)	Product size (bp)	Reference
<i>fbp</i>	<i>G. elegans</i>	F: AGGCTTTTGCGATTGCTGCG R: TGAGGAGTTGCGCCACGAAT	52	127	This study
	<i>G. adiacens</i>	F: AGGGGAGCTCTGGTTCCATGT R: TGCTAATTCGGCAGCATCCGT	55	118	This study
	<i>A. defectiva</i>	F: GCCAGTCGACCCAGTCCTTG R: CGGCCACCTCAAGATGAGCA	55	137	This study
	<i>S. mutans</i>	F: GTGGCGCCTCAATTAGTGGGA R: GGTGTGCCGAACAGACAAGC	55	106	This study
	<i>S. gordonii</i>	F: ACCGATGAAACGATTGGGCA R: CCGGTTTAGCTCCATTTGGC	55	102	This study
<i>nox</i>	<i>G. elegans</i>	F: GAACTTTGTCGCCCCGCGATG R: CAGGTGACTCCTGTGCGGTTA	55	127	This study
	<i>G. adiacens</i>	F: ACCGATGTATCCACCACCGA R: TCCTCCAATTCCAGGACGTGA	55	109	This study
	<i>A. defectiva</i>	F: TGGTGGCGAATACGAAGCTGA R: ACGATGATAGCACCGTTAGGCA	55	108	This study
	<i>S. mutans</i>	F: AACCAAGGAAAGAGATGTTTGA R: GGTGCTAACCACGCAGGTA	50	127	[30]
	<i>S. gordonii</i>	F: GGGTTGTGGAATGGCACTTTGG R: CAATGGCTGTCACTGGCGATTC	50	120	[25]

Gene	Species	Sequence	Annealing temperature (°C)	Product size (bp)	Reference
16S rRNA	All bacteria	F: GTGSTGCAYGGYTGTCGTCA R: ACGTCRTCCMCACCTTCCTC	50, 55	147	[31]

Effect of BITC on biofilm formation ability of the bacterial species

As evident from confocal microscopy and visual inspection of crystal violet stained biofilm preparations (Fig. 2), all five bacterial species formed biofilm after 3 days of incubation. *G. adiacens* and *A. defectiva* formed lowest and highest amounts of biofilm, respectively (Fig. 3). Further, *A. defectiva* biofilm mass was significantly higher than that of all other species except *S. gordonii*. Interestingly, crystal violet staining results were different from Syto-9 staining. This discrepancy is probably because crystal violet mainly stains complete EPS in the biofilm matrix while Syto-9 is a nucleic acid stain. Even though our initial disk diffusion screening studies revealed that all tested species showed an inhibition zone in the range of 10–15 mm, the inhibitory effect was not evident on the biofilms (Fig. 3). No significant difference was seen between the quantities of BITC-treated and -untreated biofilms.

Effect of BITC treatment on the expression of biofilm-associated genes.

Following treatment of the biofilms with BITC, *nox* mRNA expression in *A. defectiva*, *S. mutans*, and *S. gordonii* increased by 1.03, 1.25, 1.98 folds respectively, compared to the untreated control after normalization with the housekeeping gene 16S rRNA. However, in *G. adiacens* and *G. elegans*, the expression decreased by 0.73 and 0.06 fold respectively (Fig. 4A). *nox* mRNA expression in *S. gordonii* and *S. mutans* was significantly higher than in *G. adiacens* and *G. elegans* ($P < 0.05$). The expression *fbp* mRNA was 1.61, 1.57, 2.09 and 2.65-folds higher in *G. elegans*, *S. mutans*, *A. defectiva*, and *S. gordonii*, respectively, when compared to the untreated control.

Similar to *nox*, *fbp* expression decreased 0.58-folds in *G. adiacens* (Fig. 4B). The *fbp* mRNA levels in *A. defectiva*, *S. mutans*, and *S. gordonii* were significantly ($P < 0.05$) higher than in *G. adiacens*.

Discussion

Dental plaque biofilms are the important etiologic factor in oral infectious diseases. Bacteria embedded in multilayered biofilms are difficult to kill because of the inherent resistance of the biofilm matrix towards antimicrobials. The present study demonstrated that the biofilms of three of the five bacterial species tested showed an increase in expression of the *nox* gene, which encodes for NADH oxidase and four bacterial species showed an increase of the *fbp* gene, encoding fibronectin binding protein following treatment with BITC. Altered gene expression due to antimicrobial treatment of biofilms has been reported previously [1, 16, 17]. The natural antimicrobial BITC has been shown to exert potential health benefits to humans [17, 18] in addition to its antimicrobial activity [19, 20]. Recently, the expression differences of

pathogenicity-related genes in *Salmonella typhimurium* upon BITC treatment was explored using transcriptome analysis and phenotypic validation like motility and biofilm formation characteristics. Differentially expressed genes including down- and upregulated were noticed in BITC treated group in comparison to the control group [21]. Similarly, downregulated expression of virulence factors was also observed in BITC treated *Staphylococcus aureus* experimental group [22].

We subjected all strains to the same concentration (10 μ M) of BITC. Even though our screening experiments showed that the inhibitory zones ranged 10–15 mm, no significant reduction in biofilm mass was seen at this concentration of BITC (Fig. 3). Thus, we were prompted to study the expression of the biofilm-associated genes *nox* and *fbp* when the biofilm growth was not inhibited. Importantly, this eliminates the possibility of reduced gene expression in some species due to cell death.

We chose NADH oxidase (*nox*) and Fibronectin (*fbp*) since their role in biofilm formation and virulence has been known in some oral streptococci [2, 13]. In mutants lacking *nox* and *fbp*, biofilm formation was significantly decreased, which appeared to be due to reduced expression of a certain biofilm-associated genes [13]. In our study, when biofilms were treated with BITC, the overall structure of the biofilms was intact and no signs of biofilm detachment were seen upon visual inspection by stereo microscopy. While the expression of *nox* genes increased in *S. gordonii*, *S. mutans* and *A. defectiva*, a decreased expression was found in *G. adiacens* and *G. elegans* compared to the untreated control. In case of *fbp* genes, the increased expression was noticed in *S. gordonii*, *S. mutans*, *A. defective* and *G. elegans* while the expression was found to be decreased in *G. adiacens* alone compared to the untreated control. In the human oral cavity, bacteria need to cope with oxidative stress caused by diatomic oxygen as well as large amounts of H₂O₂ produced by competitor species. NADH oxidase is an important oxygen-metabolizing enzyme that plays a critical role in reducing dissolved oxygen in dental plaque [23]. Further, *nox* is also involved in the regulation of competence in some streptococci [24]. The increased expression of *nox* genes was reported in biofilm cells and under aerated and static aerobic conditions when compared to planktonic cells and anaerobic conditions [25]. Thus, our results suggest that *S. gordonii*, *S. mutans* and *A. defectiva* and *G. elegans* in which the gene expressions were increased, are possibly better equipped against antimicrobial assault. Increased expression of *nox* may indicate that these species produce higher amounts of NADH oxidase to combat an increased oxygen stress upon treatment with antimicrobials [26, 27]. Similarly, antibiotic treatment is also known to cause an increased expression of *fbp* genes in some bacterial species, enhancing adhesion efficacy of the bacterial cells [28, 29]. On the contrary, *G. elegans* and *G. adiacens*, which showed reduced expression of *nox* and *G. adiacens* of *fbp* upon BITC treatment, possibly are less capable of tailoring their gene expression upon treatment with antimicrobials.

A major limitation of this study is the lack of overall effect of BITC treatment on gene expression by microarray or transcriptome analysis, which may throw light on more interesting genes. Nevertheless, the two genes we studied, *nox* and *fbp* are known to be important for biofilm life in other species. In our ongoing study, we are aiming to show causality between upregulation of the expression of these two and several other genes upon antimicrobial treatment by constructing specific deletion mutants.

Conclusion

Biofilms of the studied oral bacteria were not significantly inhibited in the presence of BITC.

Increased expression of the biofilm-associated genes *nox* and *fbp* suggests a possible biological significance of the genes in the biofilm life style of these species. Further investigation is needed to understand the role of *nox* and *fbp* genes when oral bacterial biofilms are exposed to antimicrobial treatments. Further, molecular mechanisms of species-specific regulatory effects of BITC treatment on the expression of the biofilm-associated genes may offer a greater insight.

Methods

Bacterial strains and culture conditions

Granulicatella elegans CCUG 38949, *Granulicatella adiacens* CCUG 27809, and *Abiotrophia defectiva* CCUG 27639 were cultured on chocolate blood agar with 0.001 % pyridoxal HCl [15] for 2 days at 37 °C and 5 % CO₂ in air. While *Streptococcus mutans* CCUG 11877 and *Streptococcus gordonii* CCUG 33482 were cultured on brucella blood agar containing 5% sheep blood and incubated as above. Prior to harvesting the growth for each experiment, purity of cultures was checked by observing the plates under stereo microscope.

In silico- and PCR-confirmation of the target genes

Presence of the target genes encoding NADH oxidase and fibronectin binding protein was checked in the whole genome sequences of each species by using NCBI BLAST. Once confirmed, the two genes were PCR-amplified from each species. For PCR, primers were designed based on the obtained nucleotide sequences using the Bioinformatics Software Laser Gene Core Suite vs 12 (Table 1). PCR reactions were performed on Veriti™ PCR machine (Applied Biosystems) using Ready-To-Go PCR beads (GE Life Sciences) and reaction conditions mentioned as earlier [15]. Agarose gel electrophoresis of the PCR amplicons was performed as per standard protocol (Karched et al. 2015) and the amplicons were finally visualized on a G:Box Imaging system (SynGene).

Biofilm culture and quantification

Biofilm cultures were set up as described previously [15]. Briefly, from the agar-grown cultures, colonies were harvested, suspended in brucella broth and the cell suspensions adjusted to OD₆₀₀ = 1. A 100-μl aliquot from each bacterial cell suspension was added separately into wells of a 24-well cell culture plate containing 900 μl brucella broth. For *Granulicatella* and *Abiotrophia* species, 0.001% pyridoxal was added. Wells containing only broth was served as a negative control. Biofilm cultures were incubated for 3 days in 5% CO₂ atmosphere at 37 °C.

Quantification of biofilms

After incubating for 3 days, broth supernatant was removed and biofilms were washed twice gently with 1 ml sterile Phosphate Buffer Saline (PBS). One ml of methanol was added to each well and the plate was kept undisturbed for 15 minutes at room temperature for fixing of the biofilms. Methanol was removed and the plate was air-dried for 45 minutes at room temperature. One ml of 0.1% aqueous crystal violet stain was added to each well and allowed to stay at room temperature for 20 min. After removing the crystal violet stain, the wells were washed 7-8 times with distilled water till the supernatant was transparent without any traces of crystal violet stain. Excess water was removed from the wells by tapping the plate upside down on a dry tissue paper. The plate was completely dried by keeping it open for 5-10 minutes in the fume hood. To each well, 500 µl 33 % acetic acid was added and incubated at room temperature on a shaker for 5 minutes. One hundred µl from each well was added into wells of a 96-well plate in triplicates and the absorbance was read at 590 nm using a microplate reader.

For parallel duplicate wells, confocal microscopy was done. One ml of 4% paraformaldehyde solution (pH= 7.4) was added to each well and incubated for 30 minutes at room temperature for fixation. Supernatant was removed and the wells were washed twice with 1 ml of sterile PBS. To each well, 500 µl of a mixture containing 1.5 ml PBS and 4.5 µl SYTO-9 dye was added. The plate was incubated in dark for 15 minutes and then the supernatant was removed and washed with PBS. The plate was dried and a drop of mounting oil was added to each well and covered with 24x50 mm coverslip. Biofilms were visualized by confocal microscopy.

Treatment of biofilms with antimicrobials

After growing biofilms of each bacterial species as above, benzyl isothiocyanate (Sigma) was added at a concentration of 10 µM and allowed for 2 h at the same incubation conditions as above. After the incubation, broth supernatants were removed and the biofilms were scrapped off and collected into sterile micro centrifuge tubes. Immediately, RNA *later*[®] (~10 volumes of the approximate weight of biofilms) was added and all samples were stored at -80 °C until used.

RNA isolation

For RNA isolation, the samples were taken out from the freezer and thawed on ice. After centrifuging at 5000 ´ g for 5 min, the pellets were subjected to RNA isolation. After adding 350 µl of RLT buffer, 1 volume (350 µl) of 70% ethanol was added and mixed gently with a pipet tip without vortexing. All the contents were transferred to mini spin columns and centrifuged at 8000 ´ g for 15 seconds. The flow-through was discarded and the RNA bound to the membrane was washed with 700 µl wash buffers RW1. Five hundred µl elution buffer RPE was added and centrifuged at 8000 ´ g for 15 seconds and the flow-through was discarded. Another 500 µl elution buffer RPE was also added and centrifuged at 8000 x g for 2 minutes. Finally, to elute the bounded RNA, 50 µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at 8000 ´ g. The concentration of RNA was determined by Nanodrop and the purity was assessed by A₂₆₀/A₂₈₀ ratio.

Reverse transcription Real-time PCR

From the purified RNA stored at -20 °C, cDNA was synthesized by using a High-Capacity cDNA Reverse Transcription Kit (ABI Systems) according to manufacturer's instructions. Expression levels of all target genes were normalized using the 16S rRNA gene. The reactions were performed on ABI 7500 Fast Real-Time PCR machine. The reaction mixture was as follows: 10 µl SYBR Green master mix (Power SYBR Green® Kit, Applied Biosystems), 1 µl each of forward and reverse primer (0.2 µM), 7 µl H₂O and 1 µl DNA template. Temperature profile was as follows: a 10-min initial denaturation at 95 °C followed by 40 cycles of 95 °C for 15 sec, 50-60 °C (depending on the primer pair) for 30 sec and 72 °C for 30 sec. Primers and conditions used in real-time PCR for mRNA gene expression analysis are mentioned in Table 1. Data from fluorescent signal was acquired at the elongation step and analyzed using the software SDS 2.3.0v. Analysis of the expression of *nox* and *fbp* genes was performed on cells from untreated control and biofilm treated groups by comparative $\Delta\Delta C_t$ method in ABI 7500 SDS system (Applied Biosystems). The amount of target, normalized to endogenous (16S rRNA gene) and relative to calibrator (untreated), by $2^{-\Delta\Delta C_t}$ and the expression fold change was presented graphically.

Statistics

All experiments were repeated 3 times. Biological and technical replicates were included in each experiment. Differences between groups were compared by Mann Whitney U test. A P value <0.05 was regarded significant. SPSS vs25 for windows was used for statistical analysis.

Abbreviations

BITC: Benzyl isothiocyanate

nox: The gene encoding for NADH oxidase

fbp: The gene encoding for fibronectin-binding protein

EPS: Extracellular polymeric substances

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they do not have any competing interest.

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Author contribution

HA: Investigation, Formal analysis, original draft

EA: Investigation, Formal analysis, original draft-review and editing

RGB: Methodology, Data curation, Formal analysis

AA: Project administration, Resources, Original draft-review and editing

MK: Conceptualization, Funding acquisition, Supervision, Original draft-review and editing

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Figures

Figure 1

PCR optimization of *nox* (A) and *fbp* (B) genes. To confirm the amplification of *nox* and *fbp* genes from each strain, PCR and agarose gel electrophoresis were performed prior to RT-PCR experiments. bp = base pair; Ga = *G. adiacens*; Ge = *G. elegans*; Ad = *A. defectiva*; Sm = *S. mutans*; Sg = *S. gordonii*.

CV staining Confocal microscopy

Figure 2

Confocal microscopy and crystal violet staining of monospecies biofilms of five oral streptococcal species. For confocal microscopy, bacteria were cultured in brucella broth in the wells of Millicell® EZ slides (Millipore) and stained with Syto9®. Images were acquired on Carl-Zeiss LSM 700 at 630x magnification and 3D view reconstructed using the software ZEN 2012. The bacteria were grown similarly for crystal violet staining except that 24-well cell culture plates (BD Bioscience) were used.

Figure 3

Effect of BITC treatment on the biofilms. The biofilm biomass was quantified by crystal violet staining after treating with BITC for 2 h in 5% CO₂ at 37°C. The absorbance was measured at 590 nm wavelength.

Figure 4

mRNA expression of *nox* and *fbp* genes. Mature biofilms of all strains were treated with BITC (10 μ M) for 2 h in 5% CO₂ at 37°C. The biofilms were scraped off from the wells, RNA later® was added to the biofilms and preserved at -80 °C until used. RNA purified from the samples was converted to cDNA and expression of *nox* (panel A) and *fbp* (panel B) genes was assessed by Real-time PCR quantification.