Oral Microbiology Immunology 2008: 23: 315–319
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Regulation of urease expression of *Actinomyces naeslundii* in biofilms in response to pH and carbohydrate

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Yaling L, Dan J, Tao H, Xuedong Z. Regulation of urease expression of Actinomyces naeslundii *in biofilms in response to pH and carbohydrate.*
Oral Microbiol Immunol 2008: 23: 315–319. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction: The hydrolysis of urea by the urease enzymes of oral bacteria is believed to have a major impact on oral microbial ecology and to be intimately involved in oral health and diseases. *Actinomyces naeslundii* is a ureolytic bacterium that is adapted to tolerate the rapid and dramatic fluctuations in nutrient availability, carbohydrate source, and pH in dental biofilms. Our research objectives were to better understand the regulation of the expression of urease under environmental conditions that closely mimic those in dental biofilms.
Methods: *A. naeslundii* ATCC12104 were grown in a chemostat biofilm reactor with carbohydrate-limited medium for 3 days followed by a carbohydrate pulse, at pH 7.0 and at pH 5.5. Urease activities and *ureC* gene messenger RNA levels of cells in the biofilm were measured before and after the carbohydrate pulse.
Results: We found that the neutral pH environments and excess carbohydrate availability could both result in enhancement of urease activity in biofilm cells. The *ureC* messenger RNA level of *A. naeslundii* biofilm cells cultivated at pH 7.0 was approximately 10-fold higher than that of cells grown at pH 5.5, but no changes in *ureC* gene expression were detected after the carbohydrate pulse.
Conclusions: Neutral pH environments and excess carbohydrate availability could promote urease expression of *A. naeslundii* in biofilms, but only neutral pH environments could up-regulate the *ureC* gene expression and the pH regulates *ureC* gene expression at a transcriptional level.

Key words: Actinomyces naeslundii; biofilm; urease activity; ureC gene expression

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Accepted for publication December 3, 2007

Substantial amounts of urea are secreted continuously in saliva and gingival crevicular fluid, at concentrations in healthy individuals of 3–10 mM (11). Urea is rapidly hydrolyzed by urease from ureolytic bacteria in the oral cavity to produce two molecules of ammonia and one molecule of carbon dioxide. This could neutralize the acid end products from carbohydrate metabolism and so help to inhibit enamel demineralization, creating an environment that is less conducive to the emergence of an aciduric, acidogenic microflora (5, 9), and could stabilize the microflora diversity in the face of a strong carbohydrate challenge (18). For these reasons, ureolysis is considered to be an important factor in the prevention of caries formation. Conversely, the elevated plaque pH resulting from ureolysis is considered to be necessary for mineral precipitation and calculus formation (2). Despite much previous research shedding light on the critical role of ureolysis in the ecological balance of dental biofilms and pH homeostasis in the oral cavity, molecular information on the urease of oral bacteria is still very limited.

Actinomyces naeslundii, one of the few species in dental biofilms confirmed to be ureolytic (19), is an early colonizer of the oral cavity and forms a significant

portion of both supragingival and subgingival dental biofilms (21). Nucleotide sequence analysis has demonstrated that the *ure* gene cluster of *A. naeslundii* is comprised of seven contiguously arranged open reading frames with significant homologies at the protein and nucleotide sequence levels to the *ureABCEFGD* genes from other organisms (16). Cells in dental biofilms are subjected to a variety of environmental conditions, most notably sudden changes in the type and amount of carbohydrate, as well as rapid and significant fluctuations in pH (5, 7). How bacteria tolerate the fluctuations of carbohydrate source and environmental pH is very important for the survival of these organisms, and ureolysis is known to be an important pathway of alkali production. Research on the response of *A. naeslundii* urease expression to carbohydrate and pH fluctuation in biofilms may allow us to understand the role that ureolysis plays in the survival of *A. naeslundii* in acidic environments, and indicate its role in the overall alkali production of oral biofilms.

Material and methods

Bacterial strains, growth media, and biofilm reactor conditions

The bacteria used in this study were *A. naeslundii* ATCC12104. Monospecies biofilms were cultivated in a chemostat (12) with modification to a chemostat biofilm reactor according to the method used by Burne for biofilm fermentation as described previously (6). For the development of biofilms, an *Actinomyces*-defined medium (4) was used with a working volume of 0.6 l; 10 mM sucrose was used as the carbohydrate-limited source with no free glucose in the culture (tested using the phenol–sulphuric acid method with glucose as a standard). Cultures were maintained at pH 5.5 or 7.0 by the addition of 2 M KOH. Biofilms formed on six glass sheets, each with a mean exposed surface area of 30 cm^2 for biofilm accumulation. After inoculation with 50 ml of an overnight culture of *A. naeslundii* ATCC12104, medium was pumped into the vessel at a constant rate of 60 ml/h ($D = 0.1$/h). The speed of the rotating inner drum of the chemostat was kept constant at 75 r.p.m., and the temperature was maintained by immersion of the vessel in a 37°C circulating water bath. Analyses of biofilm cells were conducted 3 days after inoculation.

Physical characterization of the 3-day biofilms

Slides with biofilms were removed and briefly dipped in deionized H_2O to remove adventitiously bound material. To observe the spatial distribution of colonies in the biofilms, the samples were stained with BODIPY™ FL phallacidin (Invitrogen, Carlsbad, CA), gently washed again, and then dried for 2 min at 37°C. Using an MRC-1024 Confocal Laser Scanning Microscope (BioRad, Hercules, CA) equipped with an argon–krypton laser, the biofilms were excited at 504 nm. Optical sections of 1 μm each were recorded from the center of the specimen. The biofilm thickness was determined by counting the number of sections in which bacteria could be visualized.

Immediately after removal from the vessel, the biofilms were mechanically dissociated from the glass sheets by gentle scraping with a sterile cell scraper (Corning, New York, NY) into tubes containing 40 ml sterile ice-cold deionized H_2O, and the tubes were turbo-mixed to disperse the cells. Insoluble material was collected by centrifugation at 1500 ***g*** for 10 min at 4°C. Samples were lyophilized to determine the dry weight of the biomass. The biofilms were resuspended in 5 ml deionized H_2O to assay the total carbohydrates in the biofilms. Carbohydrate content was estimated by using the phenol–sulphuric acid method (10) with glucose as an internal standard. Bacterial viability in biofilms was estimated by comparing viable cell counts with the cell numbers obtained by microscopic enumeration of cells in a Petroff–Hauser counter. To count viable cells, the biofilms and planktonic phase were collected, washed once, and resuspended in 5 ml reduced transport fluid pH 7.2 (20). The samples were then subjected to gentle sonication at 150 W for 20 s to break the bacterial chains using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA). A serial dilution was performed by plating on six brain–heart infusion (Difco, Lawrence, KS) plates. Viable cells were counted after the plates had been cultivated at 37°C for 24 h in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2.

In situ measurement of biofilm pH

The pH of the biofilms was measured as previously detailed (13) using a superminiature, Beetrode pH electrode (World Precision Instruments, Berlin, Germany). Briefly, after the biofilms had been cultured for 3 days, glass slides bearing biofilms were removed from the vessel and placed on end on a paper towel to allow excess medium to be absorbed from the ends of the slides. The microreference electrode, which was connected through the Bee-Cal adapter and two cables to the pH probe and a standard pH meter, was positioned in the biofilm to be partially immersed in the biomass. *In situ* measurement of pH was conducted immediately by placing the tip of the pH probe into the biofilms and a series of pH readings was recorded from a minimum of 30 different sites selected at random.

Carbohydrate pulsing and biofilm sampling

After the biofilms had been cultured for 3 days, 25 mM sucrose or glucose was pulsed as excess carbohydrate source, with free glucose detected in the culture using the phenol–sulphuric acid method. The *in situ* pH in the biofilm was recorded as described above. Urease specific activity and *ureC* gene messenger RNA (mRNA) levels of cells in the biofilm were examined 60 min after the carbohydrate pulse. The biofilms were mechanically dissociated from the slides by scraping with a sterile razor blade into 40 ml ice-cold phosphate-buffered saline (pH 6.8) and centrifuged at 4000 ***g*** for 10 min at 4°C. The cells were washed twice with phosphate-buffered saline and resuspended in the same buffer.

Enzyme assays

Urease activity was quantified by measuring the amount of ammonia released from urea by intact cells using the Ammonia Color Reagent (Sigma, St. Louis, MO) with ammonium sulfate as the standard. The Bradford method was used to measure intact cell protein with bovine serum albumin as standard. Urease activity in the intact cells was expressed as nanomoles of ammonia produced per minute per milligram of protein.

Transcriptional analysis

The *UreC* gene, which encodes the largest structure subunit (α) of urease, has been used to analyze urease gene expression at transcriptional levels (8). Levels of *ureC* mRNA were quantified using a quantitative real-time polymerase chain reaction (PCR). The biofilms were dissociated and cells were collected in the same way as for the enzyme assays. Extraction of RNA and reverse transcriptase-PCR were performed

following the protocol described by Ahn et al. (1), the complementary DNA templates were created from 1 μg RNA. On the basis of the *ureC* gene sequence (GenBank accession no. AF048780), primers 5′-GAGCACGCCGCTCTGTA-3′ and 5′-ACCTTGCCGCCTCCGAA-3′ were designed to amplify the *ureC* gene. The PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). A 128-base-pair fragment was amplified and cloned onto pMD18-T (TaKaRa, Otsu, Shiga, Japan). Pure plasmid DNA was extracted and its identity was confirmed by DNA sequencing.

Gene quantification was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Taqman primers were designed using PRIMER EXPRESS 1.0 (Applied Biosystems); *Ure*C forward primer: 5′-GAGCACGCCGCTCTGTA-3′, *ureC* reverse primer: 5′-ACCTTGCCGCCTCCGAA-3′, *ureC* TM: 5′-CCTGGCCGATACCGGTCTC-3′. The TM probe consisted of an oligonucleotide labeled at the 5′ end with the reporter dye 5-carboxyfluorescein and at the 3′ end with the quencher *N*, *N*′, *N*′-tetramethyl-6-carboxyrhodamine. Quantitative PCR was performed with 2 μl complemtary DNA, 12.5 μl 2× Taqman PCR master mix (Perkin-Elmer Applied Biosystems, Walthen, MA), 900 nmol of each primer, and a 200-nmol probe in a final volume of 25 μl. Quantitative PCR conditions were as follows: one cycle at 50°C for 30 min, one cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 s and of 60°C for 1 min. Data collection was performed during each annealing phase. During each run, a standard dilution of the plasmid with a known quantity was included to permit gene quantification using the supplied software according to the instructions of the manufacturer. In each run, a negative control (distilled water) and an RNA sample without a reverse transcriptase step (to determine genomic DNA contamination) were included. For each sample, measurements of gene expression were taken three times via quantitative PCR, and the mean of these values was used for further analysis. The generation of quantitative data was based on different PCR kinetics of samples with different levels of target gene expression. We used absolute quantification in which the expression levels of *ureC* gene were compared to the data from a calibration curve, which was generated by amplifying serial dilutions of a known quantity of plasmid DNA. The gene expression was expressed as the number of mRNA copies per μg RNA.

Statistical analysis

All data expressed were derived from experiments that were performed in triplicate. Values reported represent the mean and standard error. Data about the urease specific activity and the *ureC* gene expression level of *A. naeslundii* in each group were compared using Student's *t*-test, with $P < 0.05$ considered statistically significant.

Results

Physical characterization of 3-day *A. naeslundii* biofilms

Three-day *A. naeslundii* ATCC12104 biofilms formed as compact structures; they were spatially heterogeneous and achieved wet thicknesses ranging from 20 to 260 μm on a single slide. The biofilm consisted of long chains with many microcolonies and large mats but small microcolonies, monolayers, regions of intermediate thickness, and uncolonized areas were still present on the slides (Fig. 1).

Physical characterization of 3-day *A. naeslundii* biofilms is detailed in Table 1. In the culture with pH 5.5 medium, the dry weight of the biofilms was around 121 μg/cm. When the biofilms were cultured in pH 7.0 medium, the biomass of the biofilms was almost 50% greater than the biofilms in pH 5.5 ($P < 0.05$). Analysis of total carbohydrates showed that the biofilms formed at pH 7.0 contained a higher percentage (76%) of carbohydrates than those biofilms formed in pH 5.5 (52%) ($P < 0.05$). Compared with biofilms formed at pH 5.5, the biofilms cultured at pH 7.0 had threefold higher total number of cells but sixfold higher viable cells ($P < 0.05$). The results suggest that neutral environments were of greater advantage to the growth of *A. naeslundii* in biofilms. The viable count of planktonic cells in the culture of pH 7.0 was 1.5-fold higher than that of pH 5.5 ($P < 0.05$). The result suggests that a possible different metabolic pathway exists between planktonic cells and biofilm cells.

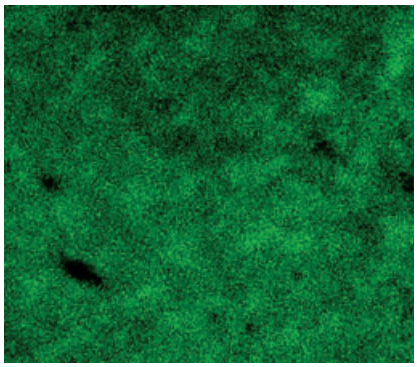

Fig. 1. The distribution of microcolonies of *Actinomyces naeslundii* ATCC12104 after 3 days of culture in biofilms stained with fluorescein, observed under CLSM at 504 nm.

Urease specific activity and *ureC* transcription of cells in 3-day biofilms and following sucrose or glucose pulse

In situ pH of biofilms, urease activities and *ureC* gene transcriptional levels of biofilm cells were measured immediately before the carbohydrate pulse and 60 min after (Table 2). When biofilms were cultured at pH 7.0 for 3 days, the biofilm pH was slightly higher than the pH of the fluid phase ($P < 0.05$). However, in the pH 5.5 culture, there was no significant pH difference between biofilms and fluid phase ($P > 0.05$). These results suggested that the alkali-producing ability of biofilms cultured in a neutral pH environment could be higher than that of biofilms cultured in an acidic environment. Another possibility, that acidogenesis by *A. naeslundii* was greater at pH 5.5 than pH 7.0, needs to be studied further. After a pulse of 25 mM sucrose or glucose for 60 min, no significant change of biofilm pH could be detected ($P > 0.05$). The data suggested that the chemostat biofilm reactor could be very useful to maintain the biofilm pH within a stable range, when the intake of carbohydrate by the biofilms increased it was followed by more acid production.

In the biofilm cultivated at pH 7.0 for 3 days, urease activity of the biofilm cells was around 10-fold higher than at pH 5.5. These data suggest that urease expression of *A. naeslundii* biofilm cells was higher in a neutral pH than in an acidic environment. When biofilms were pulsed with excess carbohydrate at pH 7.0, the urease activity was increased two-fold by 25 mM sucrose and 1.2-fold by 25 mM glucose ($P < 0.05$). After the carbohydrate pulse at pH 5.5, the urease activity of biofilm cells was increased eight-fold by 25 mM sucrose and five-fold by 25 mM glucose ($P < 0.05$). The excess carbohydrate amount could induce higher urease activity at both neutral and acidic pH, but greater induction could be found in acidic environments. *A. naeslundii* enzymatically degrade sucrose to equimolar quantities of free glucose and fructose (15), therefore it is possible that the 25 mM sucrose when degraded to 50 mM monosaccharide,

Table 1. Physical characteristics of the 3-day biofilm cell and planktonic cells of *Actinomyces naeslundii* ATCC12104

	Biofilm cells				
Culture condition	Dry weight ($\mu g/cm^2$)	Direct count ($\times 10^{-8}$, cm^{-2})[1]	Viable count ($\times 10^{-8}$, cm^{-2})[2]	Total carbohydrate (%)	Planktonic cells (CFU, $\log_{10}$/ml)[2]
pH 7.0	189 ± 36	10.76 ± 1.98	6.15 ± 1.10	76 ± 4	4.79 ± 0.70
pH 5.5	121 ± 22	2.65 ± 0.15	1.08 ± 0.25	52 ± 1	3.10 ± 0.65

[1]Direct counts were determined in a Petroff–Hauser counter.
[2]Viable counts were determined by plating on brain–heart infusion.
CFU, colony-forming units.

compared to the 25 mM glucose, gave higher urease levels. The increase in induction by 25 mM sucrose was not twice that induced by 25 mM glucose, suggesting a difference between excess glucose and excess sucrose in inducing the urease expression.

In the 3-day biofilms, the *ureC* mRNA level for *A. naeslundii* cells cultivated at pH 7.0 was approximately 10-fold higher than that for *A. naeslundii* grown at pH 5.5 ($P < 0.05$). After the carbohydrate pulse, no changes were detected in the *ureC* gene expression for biofilms cultured at pH 7.0 or pH 5.5 ($P > 0.05$). The data indicated that environmental pH has a great effect on the *ureC* gene mRNA level but carbohydrate did not influence the *ureC* gene transcription.

Discussion

When biofilms of *A. naeslundii* were cultured in a chemostat for 3 days, it was shown that neutral pH environments supported higher biomass and viable cell composition, with a higher percentage of carbohydrate content than acidic environments. In addition, neutral pH environments could promote the urease expression of *A. naeslundii* biofilm cells more than acidic environments; this was consistent with the data reported by Barboza-Silva et al. (3). One postulated role for ureolysis is to increase the pH when cells are subjected to acid stress. For example *Streptococcus salivarius*, which is another important ureolytic bacterium in the oral cavity, was found to have a higher urease activity in an acidic environment than in a neutral pH (8). Although the urease activity of *A. naeslundii* biofilm cells was higher at neutral pH than in acidic environments, it was increased with an excess carbohydrate source. Such conditions of carbohydrate excess are followed by greater acid production in the oral biofilms. Enhancement of urease activity when cells are faced with excess carbohydrate availability would be of great advantage for cells to tolerate acidic environments, so ureolysis is still very important for *A. naeslundii* biofilm cells to survive an acid stress.

However, there is a great discrepancy between the factors affecting urease activity and those regulating *ureC* gene transcriptions: only environmental pH could influence *ureC* gene mRNA levels, and carbohydrate availability was found to be influential on urease activity but had no regulatory effect on *ureC* gene transcription. One possible reason is that carbohydrate availability may affect urease activity through another route, such as modulating the expression of other genes that are involved in urease synthesis or apoenzyme activation. Nor can we exclude the possibility that additional factors, such as pretranscriptional or post-translational modifications, may also be involved. Since the urease activity and *ureC* mRNA level of biofilm cells cultivated at pH 7.0 were both almost 10-fold higher than those of biofilms formed at pH 5.5, environmental pH was suggested to induce urease expression mainly at the transcriptional level.

Another notable finding is that the urease specific activity of *A. naeslundii* cells in biofilms could be up to 512 nmol/min/mg protein, which is around 10-fold higher than the activity seen in planktonic cells when cultured under similar conditions, which has been reported elsewhere (14). This difference is possibly the result of differences in mass transport limitations, nutrition concentrations, pH, and growth rate experienced by organisms in biofilms and those in a planktonic state. The observation that biofilm cells of *A. naeslundii* can accumulate greater quantities of urease enzyme than planktonic cells growing under similar conditions was helpful to reconcile the observations that the ureolytic capacity of saliva and plaque exceeds by 60% that which can be calculated from measuring urease production in biofilm-grown microorganisms (19). Based on the greater levels of urease activity of *A. naeslundii* in biofilm cells and on the fact that a significant proportion of oral *A. naeslundii* resides in both supragingival and subgingival dental biofilms, *A. naeslundii* potentially contributes a great deal to the total ureolytic capacity of natural dental biofilms. To fully understand the contribution of *A. naeslundii* to the total ureolytic activity of natural dental biofilms, some comparison with the activity of natural plaque is needed, and this work is underway.

This work contributes to a small but growing body of knowledge on the microbiology, biochemistry, and molecular genetics of ureolysis in dental biofilms. Clearly, pH and carbohydrate could influence the urease expression of *A. naeslundii* in biofilms, but only pH regulates *ureC* gene expression on a transcriptional level. A number of other factors, such as other nutrients and oxygen, could possibly also account for the altered urease gene expres-

Table 2. Urease specific activity (nmol ammonia produced per minute per mg protein) and real-time polymerase chain reaction-based expression of *ureC* gene of *Actinomyces naeslundii* ATCC12104 growing in *in vitro* biofilms under sucrose limitation and following a 25-mM sucrose or glucose pulse

	pH 7.0			pH 5.5		
	In situ pH	urease activity[1]	*ureC* gene expression level[2]	*In situ* pH	urease activity[1]	*ureC* gene expression level[2]
Before carbohydrate pulse	7.32 ± 0.10	266.70 ± 19.43	2.10 ± 0.21	5.12 ± 0.16	22.61 ± 4.70	0.19 ± 0.04
After sucrose pulse	6.95 ± 0.25	512.52 ± 24.30	2.32 ± 0.31	4.98 ± 0.20	176.54 ± 11.12	0.21 ± 0.06
After glucose pulse	7.05 ± 0.33	345.58 ± 36.38	1.96 ± 0.08	5.04 ± 0.18	153.92 ± 37.30	0.23 ± 0.10

[1]Urease activity in the intact cells was expressed as nmol ammonia produced per minute per mg protein.
[2]The values show the number of copies of *ureC* mRNA per μg RNA.
The values shown are means from at least three separate chemostat runs and all measurements were acquired in at least triplicate.

sion in biofilms. It was reported that nitrogen availability could influence urease activity by *A. naeslundii* in batch cultures (17); research on the effect of nitrogen source on the urease expression in biofilms is underway. In summary, this study highlights the need for additional research on these factors and other urease gene expression to completely understand the regulation mechanism of urease.

Acknowledgments

This research was supported by Grants 30430800 and 30371541 from the National Natural Science Foundation of China.

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