Incorporation of Phosphorylcholine into the Lipooligosaccharide of Nontypeable *Haemophilus influenzae* Does Not Correlate with the Level of Biofilm Formation *In Vitro*

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**Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen that causes otitis media in children and community-acquired pneumonia or exacerbations of chronic obstructive pulmonary disease in adults. A large variety of studies suggest that biofilm formation by NTHi may be an important step in the pathogenesis of this bacterium. The objective of this report was to determine the relationship between the presence of phosphorylcholine in the lipooligosaccharide of NTHi and the level of biofilm formation. The study was performed on 111 NTHi clinical isolates collected from oropharyngeal samples of healthy children, middle ear fluid of children with otitis media, and sputum samples of patients with chronic obstructive pulmonary disease or community-acquired pneumonia. NTHi clinical isolates presented a large variation in the level of biofilm formation in a static assay and phosphorylcholine content. Isolates collected from the oropharynx and middle ear fluid of children tended to have more phosphorylcholine and made denser biofilms than isolates collected from sputum samples of patients with chronic obstructive pulmonary disease or community-acquired pneumonia. No correlation was observed between biofilm formation and the presence of phosphorylcholine in the lipooligosaccharide for either planktonic or biofilm growth. This lack of correlation was confirmed by abrogating phosphorylcholine incorporation into lipooligosaccharide through *lic*A gene deletion, which had strain-specific effects on biofilm formation. Altogether, we present strong evidence to conclude that there is no correlation between biofilm formation in a static assay and the presence of phosphorylcholine in lipooligosaccharide in a large collection of clinical NTHi isolates collected from different groups of patients.**

*Haemophilus influenzae* is a Gram-negative human-restricted pathogen that forms part of the normal nasopharyngeal microbiota (1). This species has been classified into two different groups depending on the absence or presence of the polysaccharide capsule (serotypes a to f). Serotype b, as the most invasive serotype, was responsible for invasive diseases in children before the introduction of the successful type b polysaccharide-protein conjugate vaccine in developed countries (2). The second group, commonly known as nontypeable *H. influenzae* (NTHi), is formed by strains lacking the capsular structure. NTHi usually colonizes the nasopharynx asymptomatically in healthy individuals; nevertheless, this opportunistic pathogen is a frequent cause of otitis media (OM), sinusitis, conjunctivitis, community-acquired pneumonia (CAP), and exacerbations of chronic obstructive pulmonary disease (COPD) (3–5).

Chronic infections have been widely associated with the presence of biofilm-forming bacteria (6). Biofilm is defined as a community of microorganisms held together in a polymeric matrix and attached to an inert or living surface (7). This biofilm structure confers protection against the host immune system but also increases antimicrobial resistance (8–10). Despite controversial views with respect to the presence of a specific polymeric matrix (11) or biofilm formation as a controlled survival mechanism (12), NTHi biofilms are suggested to be present during colonization, OM, and exacerbations of COPD (13–15).

Various bacterial factors have been shown to affect NTHi biofilm formation (16), including the presence of sialic acid (NeuAc) (17) and phosphorylcholine (PCho) incorporation into the lipooligosaccharide (LOS) (18). Hong and coworkers presented convincing data where they correlated the presence of PCho in the LOS of three variants of NTHi strain 2019 with biofilm maturation in a continuous flow system *in vitro* as well as in a chinchilla model of OM *in vivo* (19). In that study, a *lic*D gene deletion mutant deficient for PCho showed decreased biofilm formation, whereas a phase-locked *lic*A gene variant showed increased PCho incorporation and increased biofilm maturation compared to wild-type (WT) strain 2019. These results corroborate recent findings by Morey et al., who showed decreased biofilm formation for a *lic1* mutant of NTHi strain 375 (20). Furthermore, NTHi *licD* mutants of strains 2019 and 86–028NP showed decreased biofilm density and increased clearance in a chinchilla model for OM compared to WT strains (18, 19).

The ability of NTHi to form biofilms *in vitro* is highly strain specific (21–24), but the mechanism that determines whether a particular strain is able to form a biofilm is not known. Based on...
those previous studies, our work aimed to investigate whether the presence of PCho was associated with the level of biofilm formation by clinical NTHi strains isolated from the oropharynx of healthy children, middle ear fluid of children with OM, and sputum of adult patients with COPD and CAP.

MATERIALS AND METHODS

Bacterial strains and culture conditions. One hundred eleven NTHi strains from different groups of patients were analyzed in this study: (i) 29 isolates from the oropharynx of healthy children in day care centers in Oviedo, Spain; (ii) 25 isolates from middle ear fluid of children with OM at the Radboud University Medical Centre, Nijmegen, The Netherlands; (iii) 27 isolates from sputum samples of patients with CAP from the Hospital Universitari de Bellvitge, Barcelona, Spain; and (iv) 30 isolates from sputum samples collected from COPD patients at the Hospital Universitari de Bellvitge, Barcelona, Spain; (ii) 25 isolates from middle ear fluid of children with OM at the Radboud University Medical Centre, Nijmegen, The Netherlands; (iii) 27 isolates from sputum samples collected from COPD patients at the Hospital Universitari de Bellvitge, Barcelona, Spain; and (iv) 30 isolates from sputum samples of patients with community-acquired pneumonia at the Hospital Universitari de Bellvitge, Barcelona, Spain. All the NTHi isolates were identified according to standard microbiological procedures (25). All strains used in this study are reported in Table 1. The strains were grown in brain heart infusion (BHI) medium (Becton, Dickinson) supplemented with 10 μg/ml hemin (Sigma-Aldrich) and 10 μg/ml NAD (Merck).

Static biofilm formation assay. Bacterial cultures grown overnight were diluted to a final optical density at 620 nm (OD_{600}) of 0.01 in 150 μl of fresh supplemented BHI (sBHI) broth in 96-well plates or 24-well plates with glass slides in triplicate and incubated at 37°C in 5% CO2 for 24 h. Before biofilm staining, the OD_{620} was determined to assess bacterial growth. Culture broth was removed, the wells were rinsed three times with distilled water, and glass slides were transferred to new 24-well plates. Biofilm was stained with 150 μl of 0.5% crystal violet for 20 min at room temperature and washed three times with distilled water to eliminate traces of unbound dye. Finally, the crystal violet was dissolved with 150 μl of 90% ethanol, and the A_{560} was measured. The experiments were performed on three independent replicates.

Generation of NTHi-directed licA mutants. Deletion of the licA gene was performed by allelic exchange of the target gene with an antibiotic resistance marker, as described previously (26), with the primers (Biolegio) listed in Table 1. Flanking regions (~1,000 bp) of the R2866 licA gene and the spectinomycin cassette with overlapping regions (indicated in Table 1) were used to target the licA gene and the spectinomycin cassette.
italic type in Table 1) were PCR amplified and purified with the Qiagen PCR purification kit (Qiagen). Equimolar concentrations of the flanking regions and spectinomycin cassette were linked together in a second PCR. The megaprimer PCR product amplified from strain R2866 was used to amplify regions and spectinomycin cassette were linked together in a second PCR.

2% SDS) and boiled for 5 min. Proteinase K (2.5 g/ml) was added to the samples and incubated for 16 to 24 h at 37°C. Samples were precipitated by adding 20 units of sodium acetate (pH 3.0) and 400 units of EDTA, incubated for 1 h at −20°C, and centrifuged at 15,000 × g. Pellets were washed twice with 500 μl of 70% ethanol and suspended in 180 μl H2O. LOS samples were separated on a Tris-Tricine SDS-PAGE gel with a Protein II xi cell electrophoresis system (Bio-Rad) and visualized by silver staining or transferred onto a polyvinylidene difluoride (PVDF) membrane for Western blotting with monoclonal antibody (MAB) TEPC-15 to detect PCho.

Silver staining. Tris-Tricine gel was fixed for 1 h (45% methanol, 10% acetic acid), washed 3 times with 20 min of 50% methanol, and incubated for 1 min in sensitizing solution (0.02% sodium thiosulfate). The gel was washed 3 times with distilled water and incubated for 20 min in impregnation solution (0.2% silver nitrate, 0.075% formaldehyde). After the gel was washed twice with distilled water, it was incubated for 1 min in sensitizing solution (0.02% sodium thiosulfate, 0.05% formaldehyde) until the bands were clearly visible. Development was stopped by incubation in 0.1% acetic acid followed by a 30-min wash with distilled water.

Western blot analysis. PVDF membranes were blocked with 5% bovine serum albumin (BSA) in PBS, incubated for 2 h with TEPC-15 (1:1,000), washed five times for 5 min with PBS plus 0.1% Tween, and subsequently incubated with rabbit anti-mouse Ig (1:5,000) in PBS. Membranes were washed five times for 5 min with PBS plus 0.1% Tween and detected with ECL Plus reagent (GE Healthcare).

Complement resistance. Experiments were conducted with pooled normal human serum (NHS) obtained from GTI Diagnostics (catalog number PBS-N100), as described previously (29). NTHi was grown in supplemented BHI medium to an OD620 of 0.5, washed once with PBS, and diluted to an OD620 of 0.1 in PBS-Hanks’ balanced salt solution (HBSS) without phenol red containing Ca2+/Mg2+ (60% PBS–40% HBSS). Samples were finally diluted 10,000-fold in PBS-HBSS to obtain a concentration of ~20,000 CFU/ml. Fifty microliters of the bacterial culture was mixed with 50 μl of 10% NHS or 10% heat-inactivated NHS (HI-NHS), diluted in PBS-HBSS, and incubated for 1 h at 37°C. Serial dilutions were plated onto sHBl plates and incubated overnight at 37°C in 5% CO2. Survival was determined by dividing the CFU counts in 5% NHS with the CFU counts in HI-NHS after 1 h of incubation.

Flow cytometry analysis. NTHi was grown in supplemented BHI medium to an OD620 of ~0.5, or NTHi cells present in biofilms after 24 h were scraped from the plate and suspended vigorously by pipetting up and down. TEPC-15 and C-reactive protein (CRP) binding was detected by flow cytometry, as previously described (30). HBSS without phenol red and Ca2+/Mg2+ containing 5% (vol/vol) heat-inactivated fetal calf serum, was used for all dilutions and washes. Surface opsonization was performed by incubating 100 μl of bacteria at an OD620 of ~0.5 (mid-log growth) with 10 μg/ml TEPC-15 (Sigma) or 5% NHS for 1 h at 37°C with 5% CO2. Bacteria were fixed for 20 min in 2% paraformaldehyde at room temperature, and surface-bound TEPC-15 or CRP was detected by using 1:200-diluted anti-mouse IgA fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma) or 1:100-diluted goat anti-human CRP (Sigma) and 1:500-diluted donkey anti-goose Alexa 488-conjugated antibody (Life Technology) by flow cytometry using a FACSCalibur II instrument (BD Biosciences, San Jose, CA, USA) shown as mean fluorescence intensity (MFI) in arbitrary units (AU). Data were analyzed by using FlowJo version 7.6.3.

Statistical analysis. Statistical analyses were performed with GraphPad Prism version 4, where a P value of <0.05 was considered significant. The specific statistical tests that were used for the various experiments are specified in the figure legends.

RESULTS AND DISCUSSION
Phosphorylcholine content and level of biofilm formation of clinical NTHi isolates. This study included a total of 111 NTHi clinical isolates collected from patients with different diseases, including OM, CAP, and COPD, as well as strains isolated from healthy colonized children. Growth of the isolates was consistent and not statistically different between the groups (Fig. 1A). As shown in Fig. 1B, isolates recovered from middle ear fluid of patients with OM showed statistically significant increased levels of biofilm formation compared to isolates from healthy children. In contrast, isolates from sputum samples of CAP and COPD patients presented statistically significant decreased levels of biofilm formation compared to OM isolates. The level of biofilm formation of CAP and COPD isolates was lower than that of isolates from OM patients.

FIG 1 Growth, phosphorylcholine levels, and levels of biofilm formation for 111 clinical NTHi strains. Overnight growth measured by the optical density at 620 nm (OD620) (A), the level of biofilm formation measured by crystal violet light absorbance at 560 nm (A560) (B), and phosphorylcholine (PCho) content of planktonically grown NTHi isolates from healthy children, children with otitis media (OM), adults with chronic obstructive pulmonary disease (COPD), and adults with community-acquired pneumonia (CAP) are shown. Means ± standard errors of the means of three independent replicates are depicted. One-way analysis of variance (P = 0.2458 [A], P = 0.0005 [B], and P = 0.0002 [C]) with the Newman-Keuls multiple-comparison test was used for statistical analysis (*, P < 0.05; **, P < 0.01; ***, P < 0.001). AU, arbitrary units.
from healthy children, although this did not meet statistical significance. Isolates collected from the middle ear fluid of patients showed a modest but significant increase in the level of biofilm formation. These results, to some extent, corroborate the results reported by Torretta and coworkers, who showed that biofilm-producing NTHi isolates were present in the nasopharynx of children with recurrent acute OM (22). More striking was the reduced level of biofilm formation by NTHi isolates collected from patients with CAP and COPD. Previously, Murphy and Kirkham showed high variability in the level of biofilm formation but overall showed no association between the source of the sample and biofilm formation (21). Our results imply that isolates from sputum samples from adult patients with COPD or CAP behave differently from isolates collected from the oropharynx and middle ear fluid of children. Whether this is dependent on the age of the patients, location of isolation, or type of inflammatory disease is thus far not known.

The presence of PCho on NTHi grown planktonically was measured by MAb TEPC-15 staining by flow cytometry. PCho is a molecule present on a large number of microorganisms (31). NTHi acquires choline from the environment, which is incorporated into its LOS in the form of PCho, which is regulated by the phase-variable lic operon (32). NTHi isolates showed variations in PCho integration into the LOS, being generally higher in strains isolated from healthy children and children with OM than in isolates obtained from COPD and CAP patients (Fig. 1C). This observation could be explained by PCho phase variation, as it has been shown that incorporation of PCho favors colonization and OM in an animal model and recently also in a human colonization model (33–35). A possible factor explaining decreased PCho levels in NTHi isolates collected from CAP and COPD patients is that PCho binds C-reactive protein (CRP), which initiates complement-mediated killing of NTHi (35). A detectable level of CRP was present in sputum samples of COPD patients (36), whereas it was not detected in 30 out of 31 middle ear fluid samples of patients with OM (37). Therefore, NTHi present in the lungs of COPD or CAP patients might decrease PCho incorporation into the LOS in response to increased levels of CRP, thereby preventing complement-mediated killing.

**Phosphorylcholine content is not related to the level of biofilm formation of clinical NTHi isolates in a static assay.** The evaluation of biofilm formation and PCho incorporation in the LOS of 111 NTHi isolates enabled us to test whether there was a positive relationship between these two conditions. Strains obtained from healthy children showed a very modest \( r^2 = 0.1917 \) and a significant \( P = 0.0175 \) negative correlation (Fig. 2A), whereas no significant correlation between the presence of PCho and biofilm formation was observed for strains isolated from children with OM and patients with CAP and COPD (Fig. 2B to D). The combination of all strains \( (n = 111) \) also showed no significant correlation between the presence of PCho and the level of biofilm formation (Fig. 2E). In addition, when strains were grouped into four quarters based on PCho expression (quarter 1 [Q1], TEPC-15 mean fluorescence intensity [MFI] in arbitrary units [AU] of 1,254 to 221 AU; Q2, TEPC-15 MFI of 214 to 105 AU; medium low (Q3, TEPC-15 MFI of 104 to 46 AU), or low (Q4, TEPC-15 MFI of 45 to 7 AU) PCho levels. Means ± standard errors of the means of three independent replicates are depicted. One-way analysis of variance \( (P = 0.4864 \) [F]) with the Newman-Keuls multiple-comparison post hoc test was used for statistical analysis.

**FIG 2** Correlation between phosphorylcholine levels and levels of biofilm formation for 111 clinical NTHi isolates. Shown is the correlation between the level of biofilm formation measured by the A_{560} and the PCho content of planktonically grown bacteria measured by flow cytometry shown as mean fluorescence intensity (MFI) in arbitrary units (AU). (A) NTHi strains from healthy children. (B) NTHi strains from patients with OM. (C) NTHi strains from patients with CAP. (D) NTHi strains from patients with COPD. (E) All NTHi strains combined. (F) Level of biofilm formation measured by crystal violet A_{560} of strains with high (Q1, TEPC-15 MFI of 1,254 to 221 AU), medium high (Q2, TEPC-15 MFI of 214 to 105 AU), medium low (Q3, TEPC-15 MFI of 104 to 46 AU), or low (Q4, TEPC-15 MFI of 45 to 7 AU) PCho levels. Means ± standard errors of the means of three independent replicates are depicted. One-way analysis of variance \( (P = 0.4864 \) [F]) with the Newman-Keuls multiple-comparison post hoc test was used for statistical analysis.
AU, Q3, TEPC-15 MFI of 104 to 46 AU; Q4, TEPC-15 MFI of 45 to 7 AU), no differences in the level of biofilm formation were observed.

Different effects of the presence of PCho on NTHi biofilm initiation, formation, and maturation have been observed in static and continuous flow systems previously. It is likely that differences in NTHi strains and biofilm techniques used influence the outcome of the effect of PCho in biofilm assays. For instance, Hong et al. showed a positive correlation between the presence of PCho in the LOS of three variants of NTHi strain 2019 and biofilm maturation in a continuous flow system (19). More representative for our experiments, static biofilm experiments performed with strains 86-028NP and 2019 showed no effects of PCho on biofilm initiation after 10 hours. Therefore, additional experiments were performed to evaluate the relationship between PCho incorporation and the level of biofilm formation in a static assay in more detail by modulating PCho incorporation into NTHi LOS.

The position of phosphorylcholine in LOS does not affect the level of biofilm formation. Incorporation of PCho can occur at multiple positions in NTHi LOS. For example, PCho can be incorporated as a terminal moiety on hexoses extending heptose I (HepI) or heptose III (HepIII), which differentially affects CRP and continuous flow systems previously. It is likely that differences in NTHi strains and biofilm techniques used influence the outcome of the effect of PCho in biofilm assays. For instance, Hong et al. showed a positive correlation between the presence of PCho in the LOS of three variants of NTHi strain 2019 and biofilm maturation in a continuous flow system (19). More representative for our experiments, static biofilm experiments performed with strains 86-028NP and 2019 showed no effects of PCho on biofilm initiation after 10 hours. Therefore, additional experiments were performed to evaluate the relationship between PCho incorporation and the level of biofilm formation in a static assay in more detail by modulating PCho incorporation into NTHi LOS.

Phosphorylcholine content in bacteria within a biofilm is not related to the level of biofilm formation of clinical NTHi isolates. Since other studies demonstrated that the PCho content was increased in bacteria growing within a biofilm structure (40), we determined the amount of PCho present in 32 NTHi strains in the planktonic or biofilm state of growth by flow cytometry and analyzed the relationship with the level of biofilm formation.

All the tested strains showed increased PCho content in a biofilm compared to planktonic growth (Fig. 4A), and the PCho contents of individual strains in either a planktonic culture or biofilm showed a significant correlation (Fig. 4B). However, we have to mention that it is very likely that we still had sufficient bacterial clumping in the biofilm preparations despite vigorous shearing by pipetting, which increases TEPC-15 binding per particle measured by flow cytometry. Although we attempted to loosen the bacteria in the biofilm samples by vigorous shearing, forward and scatter signals were increased for the biofilm samples (data not shown), which shows the presence of larger particles. Therefore, the PCho content for NTHi samples present in a biofilm might also be higher because of clumping. Nevertheless, as described for planktonic cells (Fig. 4C), no correlation was observed between the PCho content in NTHi strains present in a biofilm and the level of biofilm formation of the particular strain (Fig. 4D). These results show that although the PCho content might be increased within the biofilm, the level of PCho is not related to the level of biofilm formation.

Modulation of phosphorylcholine incorporation affects complement resistance and the level of biofilm formation. In order to address the effects of PCho in the LOS of NTHi on biofilm formation, we constructed five mutant strains by replacement of the licA gene with a spectinomycin cassette, which was confirmed by PCR analysis. Replacement of the licA gene by a spectinomycin cassette did not affect growth (data not shown). The PCho content of the WT and licA mutants was determined by flow cytometry and Western blotting. Flow cytometry analysis confirmed that all licA mutants (Fig. 5A, light gray) presented an absence of PCho compared to the WT strains (Fig. 5A, dark gray), which was sig-
significant for 4 out of 5 strains (Fig. 5B). Western blot analysis results corroborated the flow cytometry data, with clearly abrogated incorporation of PCho into the LOS of the licA gene deletion mutants (Fig. 5C).

PCho has been shown to affect the virulence of NTHi by multiple mechanisms. For instance, PCho was shown to prevent IgG binding, thereby preventing complement-mediated killing (39). However, PCho also binds CRP, which induces classical complement pathway activation (35). Therefore, we determined the effects of licA deletion on resistance to complement-mediated killing in pooled normal human serum (NHS) and observed strain-dependent changes. Strains C008, 14-1, 1/1, and 16/16 showed decreased resistance to complement-mediated killing, whereas strain 2215 showed increased resistance (Fig. 6A). The latter strain showed binding to CRP (Fig. 6B) that was significantly reduced upon licA deletion, which corresponds to its increased resistance to complement-mediated killing.

Finally, we analyzed the effect of decreased PCho incorporation into LOS on biofilm formation. Biofilm formation showed high variability among the five WT NTHi strains and their respective licA mutants (Fig. 7A). The 1/1 licA mutant strain formed slightly less biofilm than the WT, although it was not significant. In contrast, the C008 and 16/16 licA mutants formed more biofilm than the WT, whereas the 14-1 and 2215 licA mutants formed approximately the same amount of biofilm, which is in line with other static biofilm experiments with strains 86-028NP and 2019 (40).

The fact that the licA deletion affects the level biofilm formation either positively or negatively might be related to strain-dependent alterations in LOS size or charge. As seen for the 14-1 and 16/16 licA mutants, LOS appeared to be slightly altered compared to that of the WT, whereas this was not the case for the other strains. Additionally, alterations in the overall surface charge of NTHi might affect bacterial adhesion and biofilm formation, as was observed for a wide variety of other bacterial species, including Staphylococcus aureus (41), Enterococcus faecalis (42), and Campylobacter jejuni (43). PCho has a positively charged quaternary amine group, and depletion of PCho in the licA mutant might affect the overall surface charge depending on the amount of PCho and other charged molecules on the surface of the WT.

**FIG 5** Effect of licA deletion on phosphorylcholine incorporation into NTHi LOS. (A) PCho contents in 5 NTHi WT and licA mutant strains detected by TEPC-15 binding by flow cytometry in a representative experiment used for analysis in panel B. Solid and dotted black lines are the second antibody controls for the WT and the licA mutant, respectively. In dark gray and light gray are TEPC-15 binding with the second antibodies for the WT and licA mutant strains, respectively. The y axis depicts the relative number of events, and the x axis depicts the FITC mean fluorescence intensity (MFI) in arbitrary units (AU). (B) PCho content in 5 NTHi WT and licA mutant strains detected by TEPC-15 binding by flow cytometry. Means ± standard errors of the means of two independent replicates are depicted. A two-way analysis of variance with a Bonferroni post hoc test was used for statistical analysis (*, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, not significant). (C) Analysis of LOS purified from licA mutants separated by Tris-Tricine SDS-PAGE and visualized by silver staining or by Western blotting using TEPC-15 antibody.
strain. Therefore, we tested biofilm formation on glass, since it was shown previously that changes in charge affected adhesion to glass or plastic differently (44). Overall, the ability of the WT and \( \text{licA} \) mutants to form biofilm on glass (Fig. 7B) was similar to the results obtained on plastic (Fig. 7A). Altogether, we show that alterations in PCho affect the level of NTHi biofilm formation in a strain-dependent manner.

**Conclusion.** An understanding of which general factors are involved in NTHi biofilm formation is an important topic for future research because this knowledge will allow a better comprehension.
hension of the infection process, which is important for the evaluation and treatment of diseases caused by NTHi. Our work shows that incorporation of PCho into the LOS of clinical NTHi isolates does not predict the level of biofilm in vitro. We observed decreased biofilm formation in a static assay for the licA mutant of strain 1/1; however, strains C008 and 16/16 showed increased biofilm formation upon deletion of PCho incorporation. Therefore, we conclude that PCho in NTHi LOS affects the level biofilm formation in a strain-dependent manner and that its presence does not predict the ability to form biofilms in a static assay in vitro.

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We have no conflicts of interest to declare.

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Phosphorylcholine and NTHI Biofilm Formation

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