

Nontypeable *Haemophilus influenzae* releases DNA and DNABII proteins via a T4SS-like complex and ComE of the type IV pilus machinery

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Biofilms formed by nontypeable *Haemophilus influenzae* (NTHI) are central to the chronicity, recurrence, and resistance to treatment of multiple human respiratory tract diseases including otitis media, chronic rhinosinusitis, and exacerbations of both cystic fibrosis and chronic obstructive pulmonary disease. Extracellular DNA (eDNA) and associated DNABII proteins are essential to the overall architecture and structural integrity of biofilms formed by NTHI and all other bacterial pathogens tested to date. Although cell lysis and outer-membrane vesicle extrusion are possible means by which these canonically intracellular components might be released into the extracellular environment for incorporation into the biofilm matrix, we hypothesized that NTHI additionally used a mechanism of active DNA release. Herein, we describe a mechanism whereby DNA and associated DNABII proteins transit from the bacterial cytoplasm to the periplasm via an inner-membrane pore complex (TraC and TraG) with homology to type IV secretion-like systems. These components exit the bacterial cell through the ComE pore through which the NTHI type IV pilus is expressed. The described mechanism is independent of explosive cell lysis or cell death, and the release of DNA is confined to a discrete subpolar location, which suggests a novel form of DNA release from viable NTHI. Identification of the mechanisms and determination of the kinetics by which critical biofilm matrix-stabilizing components are released will aid in the design of novel biofilm-targeted therapeutic and preventative strategies for diseases caused by NTHI and many other human pathogens known to integrate eDNA and DNABII proteins into their biofilm matrix.

eDNA | Tra | secretin | IHF | HU

Biofilms contribute substantially to the chronic and recurrent nature of many bacterial diseases of humans and animals, including those of the airway, urogenital tract, skin, and oral cavity (1–3). Bacteria within a biofilm are protected from environmental stresses by a self-produced extracellular matrix, called the extrapolymeric substance (EPS), whose composition varies greatly depending upon the microbes that produce it and the environment in which it is formed. Despite vast compositional variability, the EPS is typically composed of a complex mixture of lipopolysaccharides, proteins, lipids, glycolipids, and nucleic acids (4). Extracellular DNA (eDNA) is a major constituent of the EPS formed by multiple human pathogens (5–7) and serves diverse roles within the bacterial biofilm. eDNA provides structural stability to the matrix, acts as a sink for antimicrobial peptides, protects resident bacteria from the host immune response, provides a source of “common goods” for the resident bacteria, acts as a universal structural material conducive to microbial community architecture, and facilitates the uptake of genetic material between bacterial species via a process known as horizontal gene transfer (5, 8–17). Members of the DNABII family of DNA-binding proteins, integration host factor (IHF) and histone-like protein (HU), are known for their strong structural influences on DNA intracellularly (18–24), and are also critical to the stability of the lattice-like 3D DNA structure found in biofilm matrices extracellularly (13, 25–31). To date, the presence of bacterial eDNA within biofilms has been attributed to release via

various mechanisms, including cell lysis (autolysis or phage-mediated) (12, 32, 33), or active secretion through type IV secretion systems (T4SSs) (34, 35). Recently, DNA has been shown to be released from *Pseudomonas aeruginosa* by explosive cell lysis that is predicated on the formation of giant rounded cells that ultimately rapidly lyse, thereby releasing cell contents into the environment (36). However, a mechanism for the specific release of DNABII proteins into the extracellular space has been heretofore unknown.

Our laboratory demonstrated that DNABII proteins bind at the vertices of crossed eDNA strands and act as lynchpin-like molecules to stabilize the structure of eDNA within the biofilm matrix formed by nontypeable *Haemophilus influenzae* (NTHI) and multiple other human pathogens in vitro (25–31, 37, 38). We also show that biofilms formed within the chinchilla middle ear during experimental otitis media, as well as those within clinical specimens recovered from children with chronic posttympanostomy tube otorrhea and pediatric cystic fibrosis patients infected with *Burkholderia cenocepacia*, are stabilized by these proteins (13, 26, 38, 39). Further, we show that targeting the DNABII proteins with IHF- and/or HU-specific antibodies induces catastrophic collapse of the biofilm structure and subsequent release of resident bacteria that are now significantly more susceptible to the action of traditional antibiotics (13, 28, 39). Moreover, in a rat model of periodontitis (oral osteolytic infection) due to the oral pathogen

Significance

Extracellular DNA and DNABII proteins are essential structural components of the extracellular polymeric substance, or matrix, of the nontypeable *Haemophilus influenzae* biofilm; however, the mechanisms by which these elements are released from the bacterial cell for incorporation into the biofilm matrix are not yet characterized. Here, we propose a mechanism for active DNA release during biofilm formation that involves an inner-membrane complex (TraCG) and the ComE pore through which the type IV pilus is typically expressed. Knowledge of how and when DNA and DNABII proteins are released into the extracellular milieu for integration into the biofilm matrix will further our understanding of biofilm formation and maturation and, in turn, guide development of directed therapies for diseases with a biofilm etiology.

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Aggregatibacter actinomycetemcomitans, therapeutic treatment with antiserum against the DNABII family of proteins induced significant resolution of experimental periimplantitis (29). This same antiserum also disrupted multispecies biofilms in sputum solids recovered from pediatric cystic fibrosis patients (25). However, to date, the exact mechanism(s) by which NTHI releases DNA and DNABII proteins into the extracellular milieu has not yet been fully characterized.

NTHI is a predominant causative agent of multiple upper and lower respiratory tract diseases, which include otitis media, sinusitis, bronchitis, chronic cough, and exacerbations of both cystic fibrosis and chronic obstructive pulmonary disease (40, 41). Biofilm formation by NTHI contributes greatly to the chronicity of these diseases. Given the predominance and essential structural role of both extracellular DNA and DNABII proteins within an NTHI biofilm, we used this pathogen as a model organism with which to determine the mechanism(s) and kinetics by which these critical components are released by this bacterium. DNA and DNABII proteins are typically found within the bacterial cytoplasm, and must pass across both the inner and outer membranes for release into the extracellular environment. Whereas no active DNA secretion systems are characterized for NTHI, *Neisseria*, a closely related species, actively secretes single-stranded DNA via a T4SS (34, 35, 42, 43). The T4SS of *Neisseria* is composed of an inner-membrane complex, formed by TraC, TraD, and TraG; TraB, which spans both membranes; an outer-membrane complex, formed by TraK and TraV; and several cytoplasmic chaperone proteins (34, 35, 42, 43). Herein we investigated the possibility that NTHI used a similar mechanism for transit of DNA and associated DNABII proteins from the cytoplasm into the periplasm and, further, that this microbe used the ComE pore, through which the type IV pilus (Tfp) is typically expressed, to export the DNA into the extracellular environment. This mechanism for release of DNA and DNABII proteins from NTHI was independent of cell lysis and not preceded by changes in bacterial cell size or shape. We hypothesize that this unique mechanism of controlled DNA and DNABII protein release from NTHI contributes significantly to the structural stability and robust architecture of biofilms formed by NTHI as well as other pathogens that use a similar strategy for integration of eDNA and DNABII proteins into the biofilm matrix.

Results

NTHI Released DNA and DNABII Proteins Soon After Attachment to the Substratum. Within 3 h of inoculation of NTHI onto a chambered coverglass, a lattice-like network of extracellular dsDNA was observed on the coverglass surface (Fig. 1A). In addition, DNABII proteins were detected bound with regular periodicity along these strands of eDNA and also positioned at the vertices of crossed strands of eDNA (Fig. 1B, red fluorescence). Because the inoculum consisted of NTHI in the mid-log phase of growth, we reasoned it unlikely that cell death was the primary mediator for the presence of the structured lattice of eDNA. For clarity, additional experiments were conducted with a viability stain to confirm this assertion. We next determined whether altruistic cell death by explosive cell lysis facilitated the observed deposition of DNA, as is described for *Pseudomonas* (36). Although a small number (<1 for every 1,000 cells) of NTHI did indeed form giant rounded cells similar in morphology to those described for *Pseudomonas*, these rounded cells did not form until much later in time [e.g., more than 3 h after inoculation compared with ≤1 h after incubation as reported for *Pseudomonas* (36)]. Moreover and importantly, these giant rounded cells were observed for up to 6 h of culture and never lysed. Conversely, once formed, 86% of large rounded *Pseudomonas* cells survive for <60 s before lysing (36). The limited (if any) bacterial cell death and absence of explosive cell lysis under the culture conditions we tested suggested that the observed release of eDNA and associated DNABII proteins from NTHI

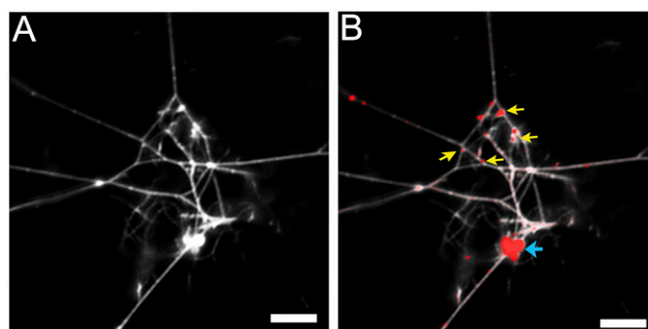


Fig. 1. Detection of eDNA and DNABII proteins early in biofilm formation in vitro. (A) Three hours after inoculation, eDNA released from NTHI was arranged in a lattice-like structure (white). (B) Overlay image depicts DNABII proteins (red) associated with eDNA (white). DNABII proteins appeared as punctate red fluorescent signals situated at vertices of crossed strands of eDNA (yellow arrows) and also positioned at regular intervals along the eDNA strands. Whole bacterial cells also appear red, as the antiserum against NTHI IHF also labels the bacterial cell (blue arrow). (Scale bars, 10 μ m.)

very early in biofilm formation likely occurred via an alternative nonlytic mechanism.

NTHI Released DNA and DNABII Proteins via a Non-Lysis-Dependent Mechanism. To visualize the release of DNA from NTHI during biofilm formation in vitro, we stained the NTHI outer membrane with FM1-43 membrane stain (Fig. 2, green fluorescence) and cultured the bacteria in the presence of the nucleic acid stain ethidium homodimer-2 (Fig. 2, red fluorescence), a membrane-impermeant stain that is only fluorescent when bound to DNA. As such, red fluorescence would indicate the presence of eDNA in the extracellular milieu or a bacterium with a compromised outer membrane. Via time-lapse fluorescence microscopy, we detected the release of DNA into the extracellular milieu by a subpopulation of NTHI within 10 to 120 min of inoculation, as evidenced by a “flare” of red fluorescence beyond the bacterial cell membrane (Fig. 2A and Movie S1). This release from a single site is more clearly viewed in Movie S2, which was captured by superresolution time-lapse microscopy. However, NTHI that released DNA by this mechanism did not first form giant rounded cells or exhibit explosive cell lysis before these events. This unique method of DNA release occurred on average from 17 of every 1,000 bacterial cells (Fig. 3, solid red bar) and within 2 h of inoculation.

To both confirm that the observed DNA staining represented release of DNA from NTHI and not uptake of DNA from the environment (as had been clearly indicated by time-lapse microscopy) as well as validate the labeling of internal versus external DNA, we also reversed the use of the fluorochromes and now labeled intracellular DNA with the membrane-permeable stain Syto 9 and the bacterial outer membrane with the membrane stain FM4-64. Under the same culture conditions, intracellular DNA now appeared as a green fluorescent signal encircled by a red fluorescent ring that represented the bacterial outer membrane (Fig. 2B). As before, DNA was released (now seen as a green flare) from a subpopulation of NTHI without formation of a giant rounded cell (Fig. 2B). Intriguingly, regardless of the fluorochrome used to detect intra- versus extracellular DNA, the release appeared to originate from a single location along the long axis of the cell and at a single subpolar location (Fig. 2).

To reveal the approximate location of the DNA release from the bacterial cell, we immunolabeled cultures of whole unfixed NTHI for the presence of both eDNA and DNABII proteins. By scanning transmission electron microscopy (STEM), eDNA and DNABII proteins were observed associated with the bacterial membrane at a single subpolar location similar to that observed via each fluorescence

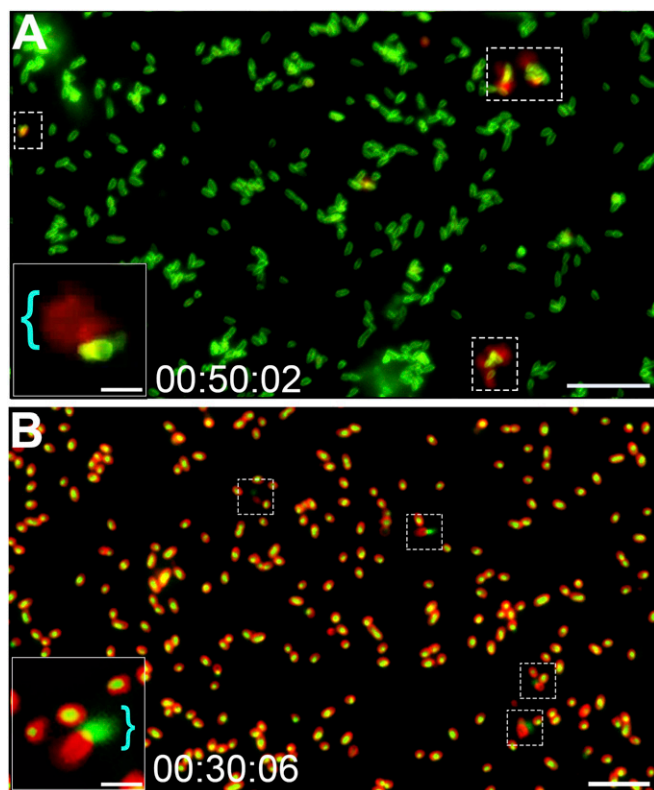


Fig. 2. Subpopulation of NTHI released DNA from a single subpolar location along one long axis of the cell. (A) Time-lapse fluorescence video microscopy demonstrated DNA release from NTHI via a mechanism that did not involve development of large round cells or explosive cell lysis. Representative images show NTHI (green) cultured in medium that contained the membrane-impermeable dsDNA stain ethidium homodimer-2 (red), and thus a fluorescent red flare indicated DNA release from the bacterial cell (dashed boxes). Representative still frame was taken after 50 min of incubation. (Scale bar, 10 μ m.) (A, Inset) Image of a cell with a red flare that indicated DNA release (blue bracket). (Scale bar, 2 μ m.) (B) To validate that DNA was released from the bacterial cell, intracellular DNA was first stained with the cell-permeable stain Syto 9, which fluoresces green when bound to DNA, and the bacterial outer membrane was stained with FM4-64 (a red fluorescent membrane stain). Fluorescence time-lapse microscopy images confirmed that DNA was released from a subpolar location of a subset of cells. The presence of eDNA (now green) adjacent to a bacterial cell (now red) supported the proposed release mechanism (dashed boxes). (Scale bar, 10 μ m.) (B, Inset) Magnified image of bacteria with a green flare that indicated DNA release (blue bracket). (Scale bar, 2 μ m.)

time-lapse microscopy strategy used (Fig. 4A). These corroborating data confirmed that NTHI released DNA and DNABII proteins from a discrete location of the bacterial outer membrane. The subpolar region of this DNA release appeared highly similar to the location from which we have shown the NTHI Tfp is expressed and that coincides with the location of the ComE pore (Fig. 4B). Further evidence of the release of DNA and DNABII proteins from a single subpolar site on the bacterial cell and their assembly into a lattice-like structure is provided in Fig. 4C. The NTHI Tfp is an adhesive protein that is extruded through the outer-membrane pore formed by ComE (10, 44, 45). In addition to its role in adherence and twitching motility, expression of the ComE pore and Tfp is also required for the uptake of DNA from the extracellular environment (44, 45).

To determine whether the Tfp machinery was perhaps also involved in the release of DNA, we examined an isogenic $\Delta comE$ mutant via fluorescence time-lapse microscopy to assess whether this mutant would release DNA into the extracellular environment.

In contrast to the parent, no extracellular fluorescence specific for DNA was observed with the $\Delta comE$ mutant, even after an extended incubation time of 6 h (compare Fig. 5A and Movie S3 with Fig. 2A and Movie S1). Interestingly, however, we did observe a subpopulation of cells that appeared to take up the ethidium homodimer-2 DNA stain, as indicated by red fluorescence within the borders of the green fluorescent bacterial outer membranes. The characteristic release of DNA seen with the parent as a red flare that extended beyond the cell membrane (as shown in Fig. 2A) was not observed. This intense red labeling of a subpopulation of $\Delta comE$ cells was observed at a later time point (>3 h) compared with the parent strain (~1 h until the appearance of a flare) and suggested an accumulation of ethidium homodimer-2 within this subset of cells, likely due to the presence of DNA within the periplasm. Complementation of the *comE* mutation restored the ability of NTHI to release DNA as a flare and from a subpopulation of cells comparable to that observed for the parental isolate (Figs. 3, green bar and 5B). To confirm that release of DNA and DNABII proteins in this manner was dependent upon expression of the ComE secretin and not another component of the Tfp machinery, we repeated this study with a mutant that could not express the majority subunit of the Tfp, PilA ($\Delta pilA$). As shown in Figs. 3, yellow bar and 5C, the $\Delta pilA$ mutant released DNA into the environment as did the parental isolate. These results indicated that the subpolar release of DNA and DNABII proteins by NTHI required expression of the ComE pore.

DNA Was Transported Across the NTHI Inner Membrane. To this point, we have described a mechanism by which DNA and DNABII proteins appeared to be released from the bacterial cell into the environment through the ComE outer-membrane pore. However, DNA and DNABII proteins in the cytoplasm must first cross the inner membrane to access the periplasmic space before they can exit the cell. No inner-membrane complex specific for DNA transport has been described in NTHI. However, *Neisseria* sp. possesses an active T4SS composed of an inner-membrane complex, an outer-membrane complex, and several cytoplasmic chaperone proteins to release ssDNA into the environment for horizontal gene transfer (35). *Neisseria* shares multiple similarities with NTHI, including noted mosaicism between genomes, despite each organism failing to recognize each other's uptake signal sequence (46–48). Bioinformatic analysis of the NTHI strain 86-028NP genome identified several genes that are homologous to those of the *Neisseria* T4SS. NTHI 86-028NP encodes orthologs of the *Neisseria* inner membrane-spanning proteins TraC and TraG, as well as the cytoplasmic chaperone proteins TraI, ParA, and ParB and the inner membrane-associated protein TraD. Surprisingly, no NTHI genes homologous to those that encode for the outer-membrane portion of the *Neisseria* T4SS were identified. Because NTHI has no known DNA secretion system and a relatively small conserved genome (1.9 Mb and 1,895 genes), we hypothesized that proteins similar to those of the inner-membrane complex in *Neisseria* were perhaps nonetheless sufficient to facilitate DNA translocation from the cytoplasm to the periplasm in NTHI. We also reasoned that once in the periplasm, DNA could then be released from the cell through the ComE pore situated within the outer membrane.

To test this hypothesis, we generated a $\Delta traCG$ mutant that lacked the predicted inner-membrane proteins TraC and TraG and assessed the ability of this mutant to release DNA into the extracellular environment. Similar to what we observed with the $\Delta comE$ mutant, the $\Delta traCG$ mutant did not release DNA via the described mechanism (Fig. 6A and Movie S4). Also similar to the $\Delta comE$ mutant, there was a minor subpopulation of cells that, over an extended time period, took up the ethidium homodimer-2 DNA stain but, unlike the $\Delta comE$ mutant, the stain was now localized to a central area of the cell with a clear demarcation (nonfluorescent,

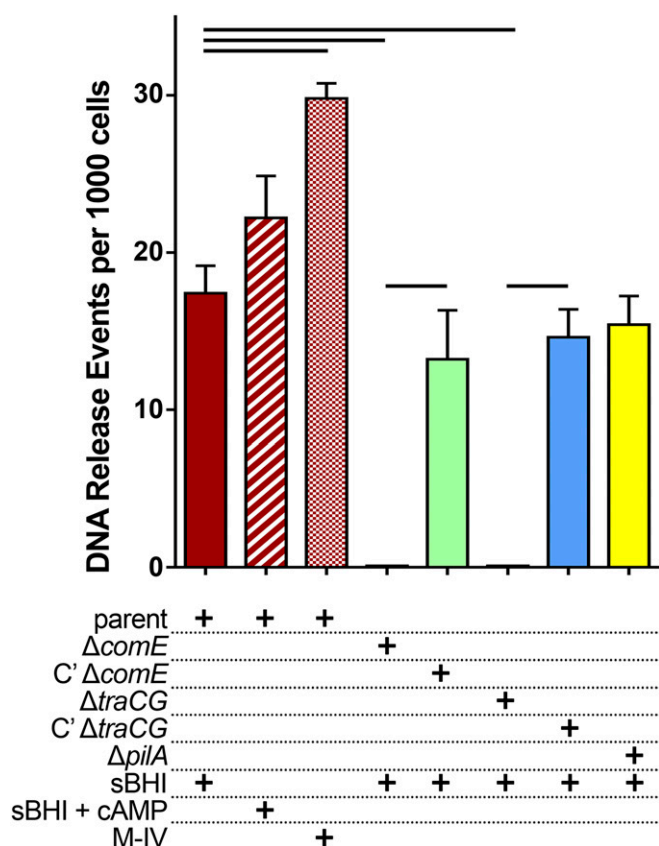


Fig. 3. Quantitation of DNA release events by NTHI. In rich medium (sBHI), we observed an average of 17 events per 1,000 parent cells after 2 h (solid red bar). There were no detectable events for either the $\Delta comE$ or $\Delta traCG$ mutant at this time point. Complementation of the mutations restored the average number of release events similar to that of the parent (compare solid red bar with green, blue, and yellow bars; no statistical differences). Induction of competence by incubation of NTHI in sBHI + cAMP (red striped bar) increased the number of DNA release events. This increase was statistically significant when NTHI was incubated in M-IV medium (red stippled bar). The number of DNA release events was calculated per 1,000 cells. Data are shown as the mean \pm SEM from five separate sets of 1,000 cells (5,000 total cells evaluated). Statistical significance was calculated using one-way ANOVA with significance shown at $P \leq 0.001$.

dark zone) between the green fluorescent outer membrane and the red fluorescence of the DNA, which suggested the presence of DNA within the cytoplasm but not within the periplasm. Complementation of the $\Delta traCG$ mutation restored the DNA release phenotype to similar to that of the parent (Figs. 3, blue bar and 6B). These data suggested that a Tra-like T4SS inner-membrane complex facilitated the translocation of cytoplasmic DNA across the inner membrane and into the periplasmic space of NTHI.

Release of DNA from the Cytoplasm into the Extracellular Milieu Required Expression of a Tra-Like Inner-Membrane Complex and the ComE Outer-Membrane Pore. Although no fluorescent flares indicative of a release of DNA to the extracellular milieu were observed by either the $\Delta comE$ or $\Delta traCG$ mutants, fluorescent staining specific for DNA was observed within a subpopulation of both mutants after extended incubation. As introduced above, examination of individual bacterial cells revealed a difference between the $\Delta comE$ and $\Delta traCG$ mutants in the pattern of fluorescent staining seen in the time-lapse videos. Whereas the $\Delta comE$ cells exhibited red fluorescence that was uniformly distributed throughout the bacterial cell, similar fluorescent DNA labeling in the $\Delta traCG$ cells was only present within the central

portion of the cell, presumably the cytoplasm (compare Fig. 7B and C). Interestingly, there was a distinct nonfluorescent area that separated the green fluorescent outer membrane and the red fluorescent DNA that was only observed in the $\Delta traCG$ mutant. This nonfluorescent ring suggested an absence of DNA within the periplasm. Collectively, these observations support the hypothesis that TraC and TraG are part of an inner-membrane complex through which DNA is translocated from the cytoplasm to the periplasm for subsequent release into the extracellular environment via the ComE pore.

To confirm the compartmentalization of DNA as we had observed within fluorescently labeled cells (Figs. 7A–C), we now immunolabeled ultrathin sections of parent, $\Delta comE$, and $\Delta traCG$ cells for dsDNA (using 6-nm colloidal gold) that were then imaged by STEM (Figs. 7D–F). Gold particles were identified by detection of secondary electrons and pseudocolored red in Fig. 7 for ease of interpretation; however, additional images that depict high-contrast secondary electron detection can be seen in Fig. S1. Immunostem of a 70-nm section of the parent strain showed a subpopulation of bacteria with positive labeling for DNA within the cytoplasm, periplasm, and extracellular milieu (Fig. 7D). Moreover, DNA release appeared to originate from a single location on the bacterial cell with both techniques (compare Fig. 7A and D). The $\Delta comE$ mutant exhibited positive labeling for DNA within the cytoplasm and periplasm; however, no labeling was noted beyond the bacterial outer membrane (Fig. 7E). This observation recapitulated the fluorescent localization observed in the time-lapse microscopy images (Fig. 7B). The distinct ring observed between the outer membrane and fluorescently stained DNA in the $\Delta traCG$ mutant (Fig. 7C) strongly suggested that DNA was confined to the cytoplasm of these cells. This observation was confirmed by immunostem, by which positive labeling for DNA was restricted to the cytoplasm of the $\Delta traCG$ mutant (Fig. 7F). Collectively, these data further supported our hypothesis that DNA was transferred across the NTHI inner membrane via a T4SS-like Tra complex and across the outer membrane and into the extracellular environment through the ComE pore.

The Frequency of DNA Release Increased with the Induction of Competence. Natural competence is defined as the state in which bacteria are capable of being transformed via the uptake of extracellular DNA and is typically induced when bacterial cell densities are high or when bacteria are nutrient-deprived (49–51), as they are when resident within a biofilm (52–57). Because uptake of DNA is increased within biofilms, we hypothesized that this growth phenotype might also be associated with an increased release of DNA by biofilm-resident bacterial cells. To determine whether induction of competence augmented the relative proportion of NTHI that released DNA via the TraCG inner-membrane complex and ComE pore, the parental isolate as well as the $\Delta comE$ and $\Delta traCG$ mutants were exposed to 10 mM cAMP or grown in nutrient-depleted M-IV medium, methods commonly used to induce competence in NTHI (49, 58, 59). We observed an increase in the relative number of bacteria that released DNA when grown in the presence of cAMP (24 events per 1,000 cells) (Fig. 3, red striped bar) and an even greater and statistically significant increase in the number of release events when bacteria were incubated in M-IV medium (27 events per 1,000 cells) (Fig. 3, red stippled bar; $P \leq 0.001$) compared with bacteria incubated in sBHI medium (brain heart infusion broth supplemented with β -NAD and heme, 2 μ g each per mL) alone (17 events per 1,000 cells) (Fig. 3, solid red bar; $P < 0.001$). DNA release was not observed from either the $\Delta comE$ or $\Delta traCG$ mutant when grown in the presence of 10 mM cAMP or in M-IV medium, whereas their corresponding complemented mutants were restored in terms of the relative number of release events to that reported for the parent (Fig. 3, green and blue bars, respectively).

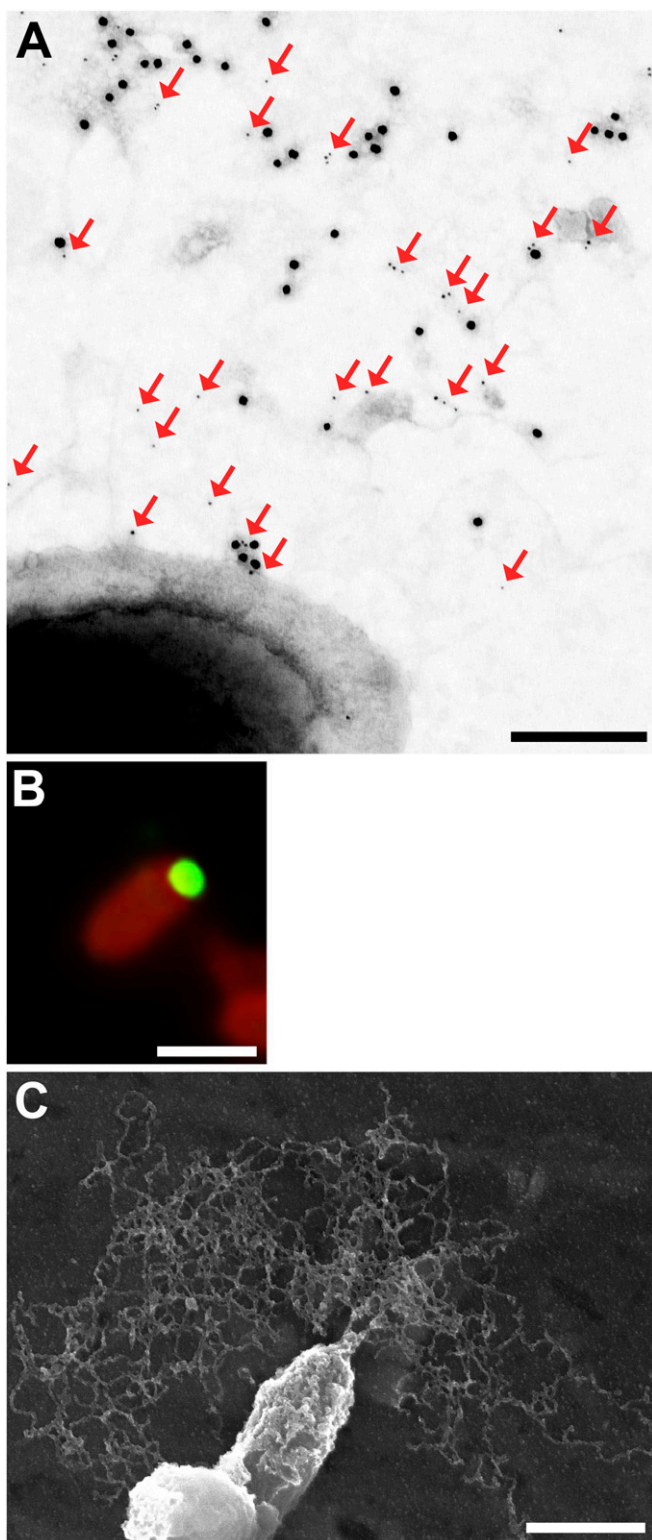


Fig. 4. Imaging release of DNA and DNABII proteins via the ComE secretion of NTHI. (A) To confirm the conserved localization of DNA and DNABII release, immunoSTEM of NTHI cells imaged 3 h after inoculation onto the surface of a formvar-coated grid demonstrated the presence of both eDNA (15-nm gold spheres; arrows) and DNABII proteins (6-nm gold spheres) in close proximity to the bacterial cell and at a location that corresponded to the DNA flare seen in fluorescence microscopy. (Scale bar, 250 nm.) (B) *E. coli* that expressed NTHI ComE was immunolabeled to determine the localization of the ComE pore. ComE (green) was localized to a single subpolar location (red), similar to that of DNA and DNABII protein release events shown in A.

To determine whether TraCG was necessary for competence/uptake of DNA from the environment, we conducted transformation efficiency assays with the parental isolate, the $\Delta traCG$ mutant, as well as the complemented $\Delta traCG$ mutant. All isolates demonstrated equivalent competence to the parental isolate (the negative control, $\Delta comE$, was not competent, as expected) (Fig. S2). These data demonstrated that whereas TraCG facilitated export of DNA and DNABII proteins into the periplasm, it did not appear to be involved in transit of DNA across the inner membrane and into the cytoplasm. To rule out a specific nutritional limitation of the M-IV medium as a cause for the increase in the observed DNA release, given that *H. influenzae* has a strict growth requirement for iron, we assessed NTHI grown in M-IV medium supplemented with heme at the concentration typically used for culture in rich medium, sBHI (2 μ g heme per mL). There was no difference in the relative number of bacteria that released DNA when NTHI was grown in M-IV medium supplemented with heme compared with unsupplemented M-IV medium. Therefore this avenue of investigation was not pursued further. These data thus confirmed that the observed increase in DNA release events under competence-inducing conditions was not due to increased cell death as a result of an iron deficiency nor was it likely due to increased biogenesis of outer-membrane vesicles known to occur in *H. influenzae* in response to iron starvation (60). Although competence has long been associated with the uptake of DNA, here we provide evidence that, in NTHI, induction of competence is also associated with the increased release of DNA and likely also the associated DNABII proteins.

eDNA and DNABII Proteins Released by TraCG and ComE Were Required for the Structure of NTHI Biofilms. To determine the role of eDNA and DNABII proteins released via TraCG and ComE in the overall architecture of an NTHI biofilm, we assessed biofilms formed by the parent strain, the $\Delta comE$ mutant, and the $\Delta traCG$ mutant for relative biofilm structure as well as for the presence of eDNA and DNABII proteins in the biofilm matrix. Biofilms were grown for 24 h and then labeled for the presence of eDNA and DNABII proteins. The parent strain formed a robust, viable biofilm that contained eDNA and associated DNABII proteins, as expected for these culture conditions (Fig. 8 A, E, and I). In contrast, biofilms formed by both the $\Delta traCG$ and $\Delta comE$ mutants were of significantly reduced height (see each panel for mean biofilm height) and lacked the characteristic shape and architecture of the biofilm formed by the parent strain (Fig. 8 B and C, respectively). In addition, biofilms formed by either mutant had considerably less or no detectable eDNA or DNABII proteins within the biofilm matrix compared with what was observed with the parent strain (compare among Fig. 8 E–G and among Fig. 8 I–K). We attributed the apparent slight increase in labeling for DNA by the $\Delta traCG$ mutant (Fig. 8F) over that by the $\Delta comE$ mutant (Fig. 8G) to the increase in number of adherent cells along the glass surface. Fluorescent staining of the adherent cells resulted in the faint green haze seen in Fig. 8B and can cause some background fluorescence in the light path used to detect DNA. Because the $\Delta comE$ mutant does not express Tfp, a major adhesin, there are fewer individual bacteria adherent to the glass surface to generate similar background labeling. The growth rates of both mutants were similar to that of the parent strain when grown in sBHI and, as such, a growth defect would not account for the observed reduction in biofilm biomass (Fig. S3). The absence of both eDNA and DNABII proteins within the biofilm matrix, both essential for

and C. (Scale bar, 2 μm .) (C) Low-magnification SEM image of an NTHI cell releasing DNA and DNABII proteins from a single subpolar site and the assembly of these components into a lattice-like matrix. (Scale bar, 500 nm.) These data indicated that the release of DNA and DNABII proteins occurred at a similar location as does expression of the ComE pore.

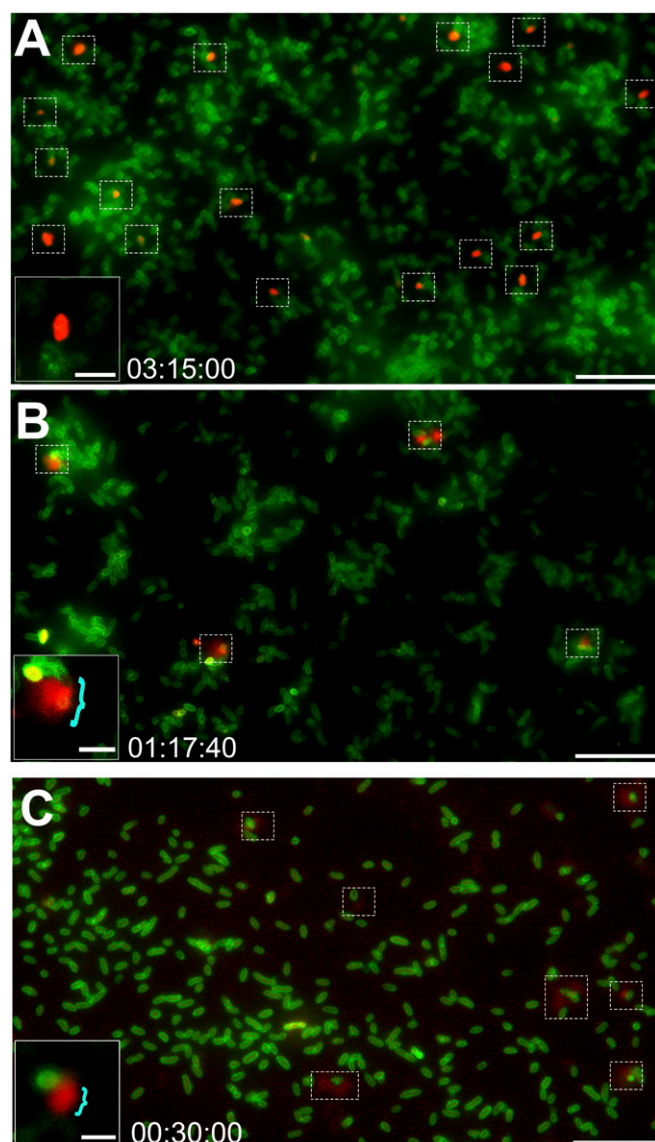


Fig. 5. Expression of ComE, but not PilA, is required for the release of DNA by NTHI. Time-lapse fluorescence microscopy images of the $\Delta comE$ mutant and the complemented $\Delta comE$ mutant are shown. Time stamps indicate elapsed incubation time. (A) No DNA was released from the $\Delta comE$ mutant at any time point; however, a subset of cells (dashed boxes) was uniformly red due to dye uptake. (Scale bar, 10 μm .) (A, Inset) Magnified image of a uniformly red fluorescent cell. (Scale bar, 2 μm .) (B) Complementation of the $\Delta comE$ mutant restored the DNA flare release phenotype (dashed boxes). (Scale bar, 10 μm .) (B, Inset) Magnified image of a complemented $\Delta comE$ mutant cell with the characteristic red flare (blue bracket). (Scale bar, 2 μm .) (C) DNA was released from $\Delta pilA$ cells via the characteristic red flare phenotype (dashed boxes), which indicated that deletion of this component of the Tfp machinery did not interfere with the described release mechanism. (Scale bar, 10 μm .) (C, Inset) Magnified image of a $\Delta pilA$ mutant cell with the characteristic red flare (blue bracket). (Scale bar, 2 μm .)

biofilm structural stability, was reasoned to be responsible for the altered architecture of biofilms formed by the $\Delta traCG$ and $\Delta comE$ mutants.

To further address the importance of DNA and DNABII proteins in the biofilm matrix and provide substantiating evidence that a lack thereof was responsible for the altered architecture and reduced biomass formed by the $\Delta comE$ mutant, we added back both exogenous NTHI DNA and DNABII proteins to the culture

system at the time of seeding. This supplementation restored the biofilm height, biomass, and overall architecture to ~50% of that observed with the parental isolate when grown in sBHI (compare Fig. 8D with Fig. 8A). Moreover, both DNA and DNABII proteins were observed within these reconstituted biofilms, as demonstrated by immunolabeling similar to what was observed in parent strain biofilms (compare Fig. 8H and L with Fig. 8E and I, respectively).

Discussion

Bacteria within a biofilm are significantly more protected from environmental stresses compared with their planktonic counterparts. The extracellular polymeric substance serves as a semipermeable barrier that protects resident bacteria from both immune effectors and therapeutics such as antibiotics (61, 62). The EPS is composed of a variety of cell-associated and secreted molecules, which include polysaccharides, proteins, lipids, and nucleic acids (63). Extracellular DNA protects bacteria within the biofilm from host defenses via the chelation of antimicrobial peptides and divalent cations (14, 64). In addition, we have shown that eDNA is an important structural component of the biofilm matrix and that the DNABII proteins IHF and HU are required to stabilize the eDNA scaffold that provides structural support to biofilms formed by NTHI and many additional human pathogens (7, 13, 25, 27, 28, 30, 37–39).

There are a number of possible mechanisms that may explain the presence of bacterial DNA in the extracellular environment.

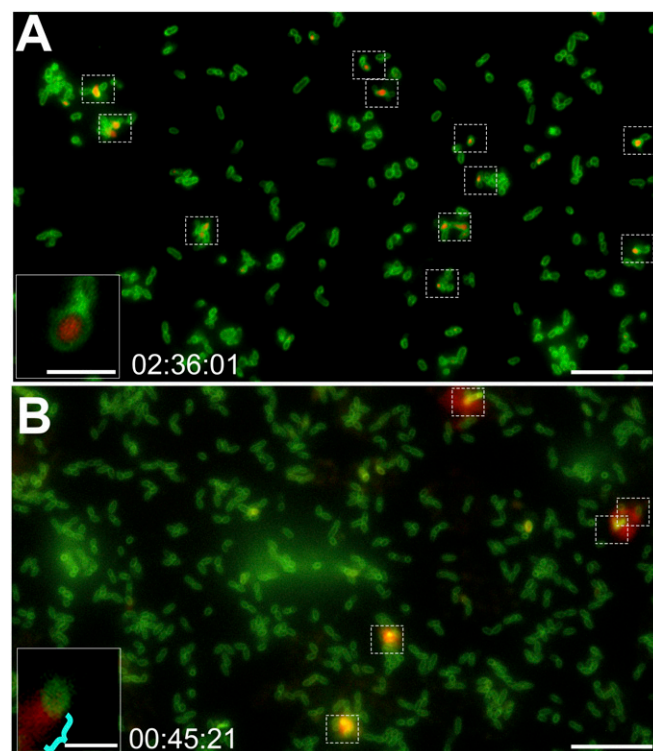


Fig. 6. T4SS-like inner-membrane complex is required for the release of DNA from NTHI. Time-lapse fluorescence microscopy images of the $\Delta traCG$ mutant and the complemented $\Delta traCG$ mutant are shown. Time stamps indicate elapsed incubation time. (A) No DNA was released from the $\Delta traCG$ mutant at any time point. However, a subset of cells (dashed boxes) had taken up the ethidium homodimer-2 DNA stain. (Scale bar, 10 μm .) (A, Inset) Magnified image of a $\Delta traCG$ mutant cell showed a clear demarcation between red fluorescent DNA and green fluorescent outer membrane. (Scale bar, 2 μm .) (B) Complementation of the $\Delta traCG$ mutant restored the characteristic DNA flare release phenotype (dashed boxes). (Scale bar, 10 μm .) (B, Inset) Magnified image of a complemented $\Delta traCG$ mutant cell showed the characteristic red flare release phenotype (blue bracket). (Scale bar, 2 μm .)

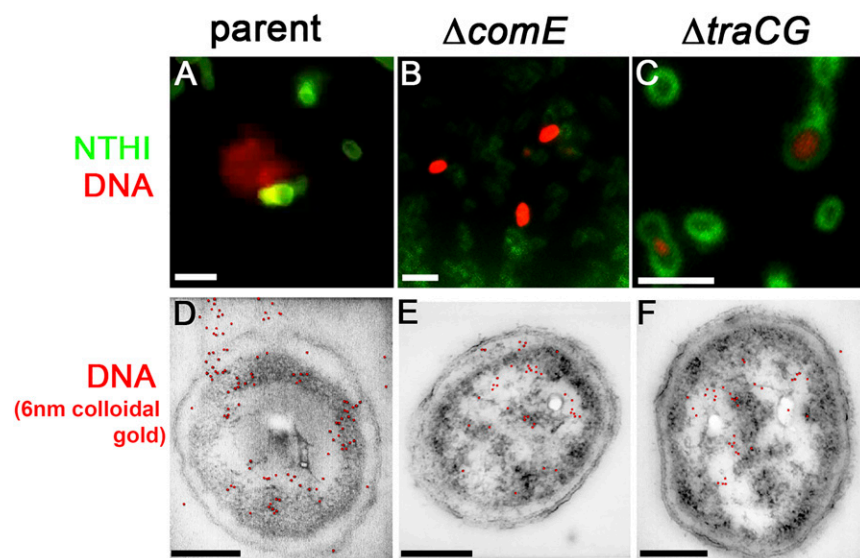


Fig. 7. DNA was released from the parent but not from the $\Delta comE$ or $\Delta traCG$ mutants. (A–C) Duplicates of insets already presented in Figs. 2A, 5A, and 6A are repeated here for comparison (please see respective Fig. 2 legends for a detailed description). (D–F) ImmunoSTEM for the presence of DNA-confirmed fluorescent labeling seen in A–C. (D) A subset of NTHI parent cells positively labeled for DNA within the cytoplasm, periplasm, and extracellular environment as seen by 6-nm gold particles (pseudocolored red based on detection of secondary electrons; see Fig. S1 for additional images). (E) Subset of $\Delta comE$ mutant cells positively labeled for DNA within the cytoplasm and in the periplasm, but no extracellular labeling was detected. (F) DNA was detected within the cytoplasm of the $\Delta traCG$ mutant cells with no labeling within either the periplasm or extracellular space. (Scale bars, 200 nm.)

Some bacteria have a mechanism by which a subpopulation of cells will undergo programmed cell death and lysis to release all of their cellular contents into the environment (9). This type of cell lysis provides common goods for neighboring bacteria and releases DNA for horizontal gene transfer (4, 65). *Staphylococcus aureus* has been shown to undergo such an altruistic cell death (9). Recently, it was reported that a small proportion of *Pseudomonas* cells form very large rounded cells that then rapidly undergo explosive cell lysis and release their cellular contents, including DNA, into the extracellular environment (36). In addition, some species such as *Neisseria* have an active type IV secretion system that transports DNA from the cytoplasm to the extracellular space, presumably for gene transfer (35).

Herein, we identified a mechanism of release of both DNA and DNABII proteins into the environment by a subpopulation of NTHI. To first determine how DNA and DNABII proteins were able to get from within the bounds of the inner membrane and into the periplasm, we analyzed the NTHI genome for po-

tential genes that encode for proteins known to be involved in DNA transport across the inner membrane. Although the genome of NTHI strain 86-028NP lacked orthologs of any of the periplasmic or outer-membrane components found in *Neisseria*, we did find orthologs of the *Neisseria* proteins that constitute the T4SS inner-membrane complex. Indeed, work by Juhas and colleagues (66–68) has shown that a novel T4SS, acquired through gene transfer of genomic islands of the ICE_{Hin} 1056 subfamily, is likely the origin of the *traC*- and *traG*-like genes found in strain 86-028NP, as well as other NTHI strains. In addition to *traC* and *traG*, the genomic island contains genes that encode homologs of the T4SS cytoplasmic chaperone proteins TraI, ParA, and ParB as well as the inner membrane-associated protein TraD. The genomic island of NTHI strain 86-028NP also includes genes involved in excision, integration, replication, and stabilization of integrative and conjugative elements (ICEs) (68).

In the current study, we found that the NTHI TraC and TraG proteins, predicted to form an inner-membrane complex of a

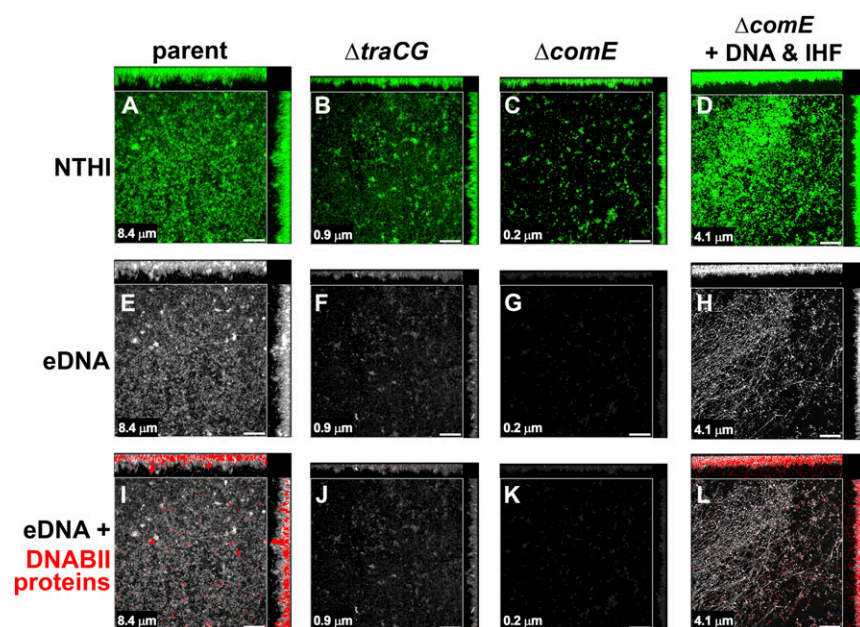


Fig. 8. eDNA and DNABII proteins were present in biofilms formed by the parental isolate but not the $\Delta comE$ or $\Delta traCG$ mutants. Biofilms were immunolabeled for DNA and DNABII proteins. (A–C) Bacteria within the biofilms were stained with FM1-43 outer-membrane stain (green fluorescence) with both top-down and orthogonal views of representative biofilms shown. (E–G) eDNA (white) is visible throughout the biofilm formed by the parental isolate with minimal labeling in biofilms formed by either mutant. (I–K) DNABII proteins (red) were visualized in addition to eDNA (white) throughout biofilms formed by the parental isolate. In comparison, minimal to no labeling for DNABII proteins (red) was observed in biofilms formed by either of the mutants. Addition of exogenous NTHI DNA and a DNABII protein (IHF) restored $\Delta comE$ biofilm characteristics comparable to the parent (compare D, H, and L with A, E, and I). (Scale bars, 20 μ m.) Note: Mean biofilm height as determined by COMSTAT2 analysis is indicated in the lower left-hand corner of each panel.

T4SS, were required for the release of DNA during early biofilm formation. Fluorescence time-lapse microscopy provided evidence to suggest that this complex, similar to that of the *Neisseria* T4SS inner-membrane complex, actively translocated DNA across the NTHI inner membrane. ImmunoSTEM of cells that could not express either TraC or TraG further confirmed this finding, as no labeling for DNA was observed in the periplasm of any of the cells or in the extracellular space. Although a system similar to the *Neisseria* T4SS is likely involved in translocation of DNA across the inner membrane, NTHI lacks the components necessary to form a complete T4SS outer-membrane complex. As such, DNA or DNABII proteins transported into the periplasm via the Tra inner-membrane complex would require a heretofore uncharacterized additional mechanism to cross the outer membrane and be released into the extracellular environment.

In data presented here, we show how quickly NTHI releases DNA, with associated DNABII proteins into the extracellular environment, in a lattice-like array of DNA strands. This array is similar to what was observed by Barnes et al. (69) with *Enterococcus faecalis*, wherein they observed a network of strands of DNA in 4-h cultures by both immunofluorescence and scanning electron microscopy by an as-yet uncharacterized DNA export system. Here, by fluorescence imaging and electron microscopy, we revealed that DNA and DNABII proteins were released from NTHI at a single subpolar location on the cell. The location of this release is consistent with the location of Tfp expression through the ComE pore (44). Unlike the less well-understood mechanism(s) by which NTHI releases DNA into the extracellular milieu, uptake of DNA from the environment is well-studied. Many bacteria, including NTHI and *Neisseria*, take up DNA from the environment via a process that involves the machinery for expression of type IV pili. NTHI expresses Tfp from a single subpolar location along the long axis of the bacterial cell. These Tfp are essential for twitching motility, adherence to respiratory tract epithelial cells, colonization of the mammalian respiratory tract, and competence (10, 44, 45, 50, 51). The Tfp machinery in NTHI is encoded by two operons, *pilABCD* and *comABCDEF*. *pilA*, *pilB*, and *pilD* encode the Tfp major subunit, an ATPase, and the prepilin peptidase, respectively (44, 45). *comE* encodes a protein (ComE) that is homologous to the PilQ secretin of *Pseudomonas*, which complexes to form the outer-

membrane pore through which the Tfp is expressed and foreign DNA is taken up during competence (44).

Collectively, these observations suggested to us that the ComE pore might be involved in the release of DNA and DNABII proteins from the periplasmic space into the extracellular environment, given that the secretin pore is just wide enough to accommodate a pair of DNA double helices (51). Therefore, we constructed an isogenic *comE* mutant and found that NTHI that could not express the ComE pore did not release DNA via the mechanism observed with the parent strain. These results suggested that, in addition to the Tra complex that was required for transport of DNA across the inner membrane, the ComE pore was required for the transfer of DNA across the outer membrane. In analysis of the completed genomes of 46 strains of *H. influenzae* available in the National Center for Biotechnology Information, we found that 25/46 (55%) possessed *tra* genes whereas 46/46 (100%) had the *comE* gene. This observation suggests that the majority of NTHI isolates are capable of using this mechanism to release DNA and DNABII proteins into the EPS of their biofilms. Other bacterial secretins are similarly known to function in dual processes (70–72).

A model of the predicted transfer of DNA from the cytoplasm into the periplasm via the Tra inner-membrane complex and then out of the cell via the ComE pore is presented in Fig. 9 (Left). Based on this model, we would predict that a $\Delta comE$ mutant would be able to translocate DNA across the inner membrane via the Tra complex but would be unable to release DNA from the cell due to the absence of the ComE pore (Fig. 9, Center). In a $\Delta traCG$ mutant, DNA would be unable to cross the inner membrane and, as such, no DNA would be present within the periplasmic space or be available to be released by the cell despite the presence of the ComE pore (Fig. 9, Right). This model is supported by our observations of both DNA release by fluorescently labeled whole cells and immunolabeled ultrathin sections of parent, $\Delta comE$ and $\Delta traCG$ cells. Whereas we cannot exclude the possibility that these inner-membrane (TraCG) and outer-membrane (ComE) components form a complex, given the canonical arrangement of the type IV pilus machinery, which does indeed require linkage of its own inner- and outer-membrane components both spatially and temporally, we do not currently favor this possibility. Further, we do not yet know whether the described method of release of both DNA and

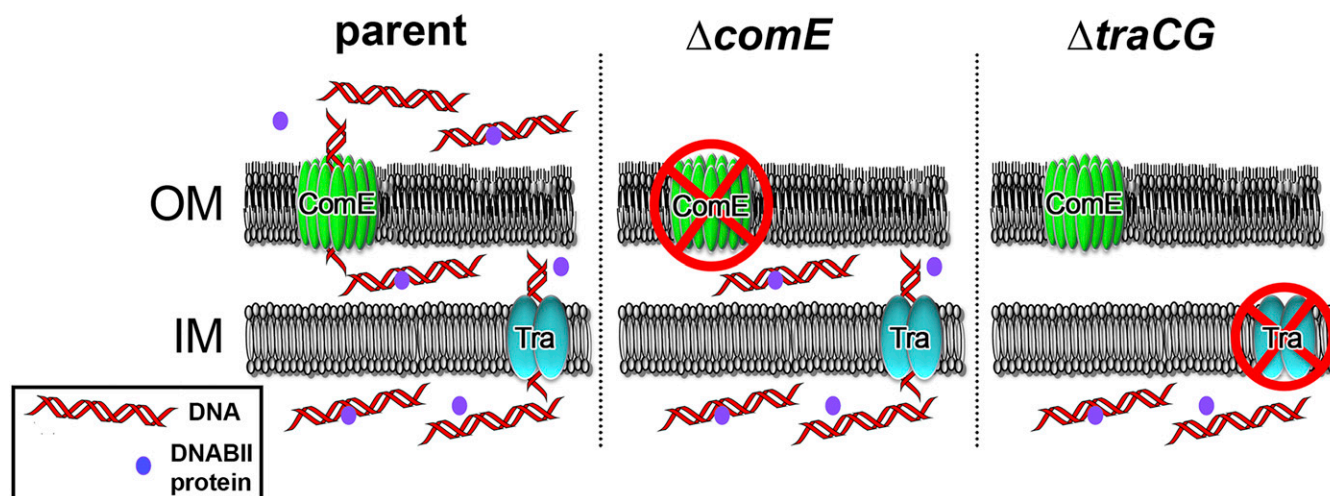


Fig. 9. Graphic representation of the proposed mechanism for DNA and DNABII protein release from NTHI. In the parental isolate, DNA and DNABII proteins cross the inner membrane (IM) via the Tra complex, and are released from the cell through the ComE pore. In the $\Delta comE$ mutant, DNA and DNABII proteins can cross the IM via the Tra complex but are unable to cross the outer membrane (OM) in the absence of the ComE outer-membrane pore. Therefore, no DNA or DNABII proteins would be released outside of the cell via this mechanism. In the $\Delta traCG$ mutant, wherein cells lack the T4SS-like inner-membrane Tra complex, DNA and DNABII proteins would be confined to the cytoplasm and unable to cross the IM.

DNABII proteins into the extracellular milieu requires adherence of NTHI to a substratum or not; however, this is the subject of ongoing investigation.

Diseases caused by NTHI are often chronic and recurrent in nature due to the formation of highly recalcitrant biofilms. Extracellular DNA and DNABII proteins are required for biofilm structure and stability. The targeted removal of eDNA and/or DNABII proteins has been shown to cause catastrophic biofilm collapse in vitro and disruption of biofilms in three distinct animal models of disease induced by three important human pathogens of the middle ear, oral cavity, and lung (13, 26, 29). Here we show that release of DNA and DNABII proteins via a mechanism that involves both the Tra inner-membrane complex and the ComE pore is critical for biofilm formation in vitro. Biofilms formed in vitro by bacteria unable to release DNA and DNABII proteins via this mechanism lacked extracellular DNA and DNABII proteins in the biofilm matrix, which resulted in a compromised and atypical biofilm architecture. This mechanism of release of both DNA and DNABII proteins is therefore likely critical to biofilm stability in vivo as well.

Naturally competent bacteria can take up foreign DNA from the environment via a two-step process that involves both transfer of DNA from the bacterial surface to the cytoplasmic membrane and translocation of DNA across the cytoplasmic membrane that may or may not be spatially and temporally coupled (73, 74). There is a relationship between cell growth and development of competence in NTHI (58), wherein a greater cell density with slowed growth, as in a biofilm, increases competence. We have shown that induction of competence also modestly increased the number of NTHI within a population that released DNA and DNABII proteins. We surmise that this increase was limited due to the state of competence, which induced *comE* expression but not concomitant expression of *traCG*. This outcome resulted in the rate-limiting step being the transit of DNA through the inner membrane into the periplasm. Importantly, other genera demonstrate a similar DNA release phenotype induced by competence. *Streptococcus pneumoniae* can trigger cell lysis of neighboring bacteria of the same species (fratricide) and DNA release when competence is induced (75). A relationship between competence and eDNA has also been shown in *Neisseria gonorrhoeae* (76), *Pseudomonas stutzeri* (77), and *Bacillus subtilis* (78). However, to the best of our knowledge, the induction of competence has heretofore not been associated with the release of DNA by NTHI.

Herein, we described a mechanism by which NTHI releases DNA and DNABII proteins into the environment that is independent of explosive cell lysis. We have shown that DNA appeared to transit from the cytoplasmic space to the periplasm via a T4SS-like inner-membrane complex and was then released,

along with DNABII proteins, into the environment through the ComE outer-membrane pore. Taken together, these data indicated that this TraCG- and ComE-dependent mechanism of release is critical for NTHI biofilm formation. These findings describe a mechanism by which a subset of NTHI released DNA and DNABII proteins into the extracellular milieu, where they are known to provide structural support to the biofilm, which protects resident bacteria. Knowledge of the mechanism and kinetics of release of DNA and DNABII proteins can be integrated into our development of biofilm-targeted disruption and prevention strategies.

Materials and Methods

NTHI strain 86-028NP is a low-passage clinical isolate. NTHI was maintained as frozen stocks and cultured on chocolate agar (Remel) at 37 °C with 5% CO₂ for 18 to 20 h before use. A ComE deletion mutant ($\Delta comE$) and its complement (*C'* $\Delta comE$) were described previously (44). A TraCG mutant ($\Delta traCG$) was generated by replacement of the *traCG* region of the chromosome with a kanamycin resistance cassette. Briefly, regions ~1 kb upstream and downstream of the *traCG* region were amplified by PCR and digested with SphI and EcoRI, respectively. The flanking regions were then ligated to a kanamycin resistance cassette that had been amplified by PCR from pKMLN-1 (79) and digested with SphI and EcoRI. NTHI strain 86-028NP was transformed with the resultant DNA fragment using a modified M-IV transformation protocol (80, 81). Transformants were selected on chocolate agar supplemented with kanamycin (20 μ g/mL) and sequenced to verify allelic exchange.

The $\Delta traCG$ mutant was complemented via use of a derivative of pSPEC1 (44) that contained the *traCG* genes. Briefly, the *traCG* region of NTHI strain 86-028NP was amplified by PCR, digested with NcoI and PvuI, and ligated into pSPEC1 that had been digested with NcoI and PvuI. The resultant plasmid, pJAJ1, was confirmed by sequencing and used to transform the $\Delta traCG$ mutant via electroporation. All PCR assays were performed with Q5 High-Fidelity DNA polymerase (New England Biolabs). All restriction enzymes were purchased from New England Biolabs.

To demonstrate the singular subpolar location of expression of ComE, the *com* locus from NTHI strain 86-028NP was amplified from genomic DNA by PCR using a forward primer containing a BglII site and a reverse primer that encodes a FLAG epitope tag and an EcoRI site. The product was digested with BglII and EcoRI and ligated with pCOLADuet-1 (EMD Millipore) previously digested with BamHI and EcoRI. The construct was transformed into ElectroMAX *Escherichia coli* DH5 α (Thermo Fisher Scientific), and transformants were selected on Luria agar containing 20 μ g kanamycin per mL. Isolated plasmids were confirmed by restriction analysis and sequencing and then transformed into *E. coli* BL21 Star (DE3) (Thermo Fisher Scientific). Transformants were again selected on Luria agar containing 20 μ g kanamycin per mL.

Detailed materials and methods can be found in [SI Materials and Methods](#).

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