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Identification and characterization of three novel LytM proteins of non-typeable *Haemophilus influenzae* involved in cell division, Outer Membrane Vesicles production and pathogenesis

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1 - Abstract

Metalloproteases are a class of proteins very important for bacterial physiology, they are involved in many different aspects of microbial life and in the recent years they are target of many relevant studies.

Through a genomic approach, we identified three hypothetical metalloprotease in Non-typeable *Haemophilus influenzae* (NTHi): NT013, NT017 and NT022. These proteins belong to LytM family, which is composed by factors mainly involved in cell division and in pathogenesis.

The analysis of knockout mutant strains phenotypes confirms the role of protein NT013 and NT022 in cell splitting process; in particular we observed defective phenotypes in terms of cell morphology, formation of chains and bacterial aggregates. Moreover, we demonstrated a direct activity of protein NT013 in peptidoglycan cleavage, meanwhile NT022 seems to have a regulatory function.

Furthermore, the alteration in the cell division in the KO strains resulted in an increase in the release of Outer Membrane Vesicles (OMVs), probably due to a decrease in membrane stability.

NT017 does not seem to be involved in cell division process, but has a possible role in host colonization, since NT017 deletion reduces the capacity of NTHi to adhere to epithelial cells, to form biofilm and shows a susceptibility to human serum mediated killing.

The results obtained so far clearly highlight the importance of LytM factors in NTHi physiology and pathogenesis.

2 - Introduction

2.1 - Metalloproteases are essential enzymes for bacterial physiology

Proteolytic enzymes play many physiological roles and are essential factors for homeostatic control in both eukaryotes and prokaryotes. Bacterial metalloproteases are mainly involved in the hydrolysis of large polypeptide substrates into smaller molecules for the recruitment of peptide nutrients for the microorganism [1], the enzymes produced by pathogenic microorganisms, especially by opportunistic pathogens, occasionally act as toxic factors to the host.

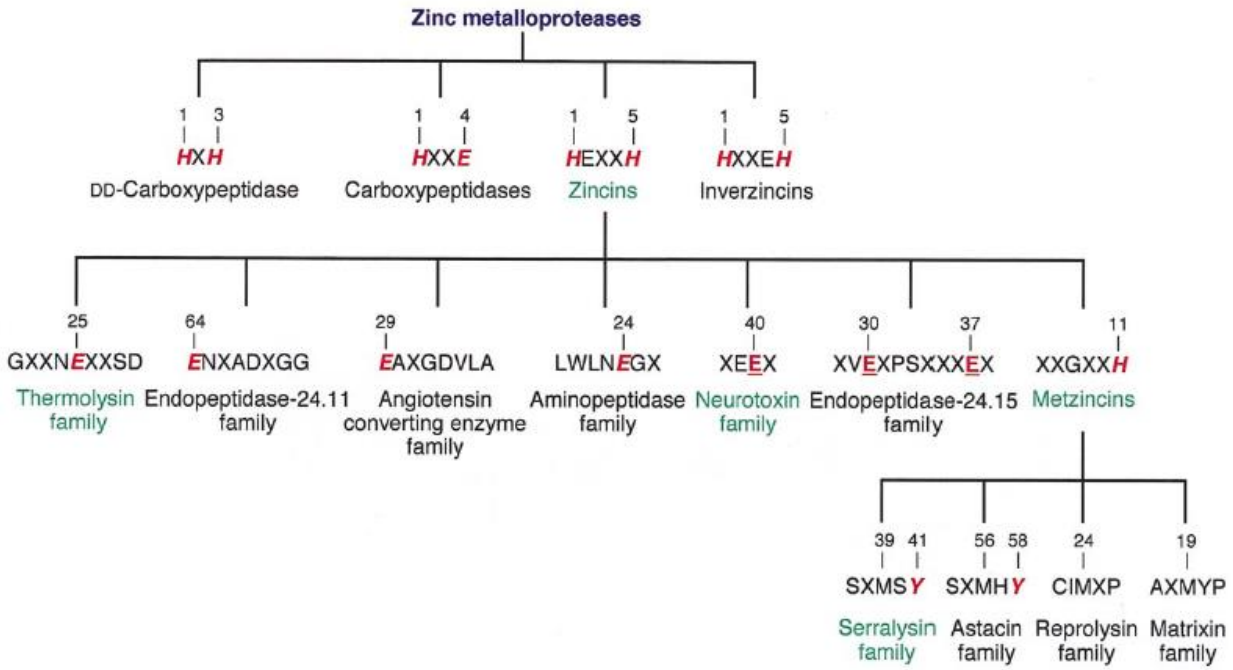


Figure 1: **classes of zinc metalloproteases**
Families of zinc metalloproteases based on the sequence around the zinc-binding residues. Italicized red letters represent identified zinc ligands; underlined red letters represent putative zinc ligands; and X stands for any amino acid. Residues in the first line correspond to the first and second ligands; residues in second line to the third ligand; and residues in the third line to the putative fifth ligand **[3]**.

Many of the toxic proteases are metalloproteases having a zinc (II) ion in the catalytic site. Zinc is an integral component of many proteins which are involved in virtually all aspects of metabolism of the different species of all phyla. X-ray crystallographic

analyses of several zinc containing proteins have defined the features of the catalytic and structural zinc-binding sites [2]. In all zinc enzymes whose crystal structures are known, a catalytic zinc atom is coordinated to three amino acid residues of the protein and an active water molecule, whereas structural zinc atoms are coordinated to four Cys residues [2]. A combination of His, Glu, Asp, or Cys residues creates a tridentate active zinc site, and an activated water molecule fills and completes the coordination sphere.

Zinc-containing metalloproteases are widely distributed from prokaryotes to eukaryotes and are classified into four groups: zincins, inverzincins, carboxypeptidases and DD-carboxypeptidases [3].

Secreted bacterial metalloproteases have been identified in both gram-positive and gram-negative pathogens, but they are certainly not unique to pathogenic species. Many bacterial proteases from pathogenic organisms that have been studied in detail have either been demonstrated or suggested to play important roles in virulence: they are used by the bacteria as direct defense mechanisms, cleaving proteins involved in the host immune defense, as is the case of ZapA from *Proteus mirabilis* [4]. Or they can have other functions as maturation factors for other virulence factors, such as the secreted metalloprotease PlcB from *Listeria monocytogenes* which is responsible for the activation of two phospholipases important in the pathological process [5].

For instance, a metalloprotease produced by *Vibrio vulnificus*, which is an opportunistic human pathogen causing serious septicemia accompanied by edematous skin lesions, has been documented to enhance the vascular permeability by stimulating the generation of inflammatory mediators, histamine and bradykinin [6]. *Pseudomonas aeruginosa*, another opportunistic human pathogen, also produces two metalloproteases, these can digest a wide variety of host proteins, such as structural components of the cornea or basement membrane, and plasma proteins involved in coagulation or complement action [7]. In addition, some microbial proteases may play indirect roles in

pathogenicity. A metalloprotease from *Vibrio cholerae* serovar O1, which is responsible for epidemic cholera characterized by a voluminous amount of rice water stool, is known to accelerate the bacterial attachment to intestinal epithelial cells through digestion of the small intestinal mucosa [8].

Proteins like metalloproteases are so important for bacterial physiology and they could be a target as potential vaccine antigens. Development of protective immunity against bacterial metalloproteases was observed and it could have practical implications in the design of future vaccines or therapies [9-10-11].

2.2 - Bacterial cell division in gram negative bacteria

The study of bacterial cell division, especially for gram negative, was focused on organisms as *Escherichia coli* and *Caulobacter crescentus*.

Escherichia coli and other Gram-negative bacteria divide by coordinately constricting all three of their envelope layers, the inner and outer membranes along with the peptidoglycan (PG) layer sandwiched between them [12-13]. Envelope constriction is driven by a ring-shaped, multiprotein complex called the septal ring or divisome [12]. The assembly of this machine is initiated by polymerization of the tubulin-like FtsZ protein into a ring-like structure, the Z-ring, just underneath the cytoplasmic membrane at the prospective site of fission [14].

Several Z-ring associated proteins (FtsA, ZipA, ZapA, ZapB, and ZapC) play important roles in Z-ring formation and are thought to decorate and stabilize the structure as it forms [15, 16, 17, 18, 19, 20].

Placement of the division site at its correct location in *Escherichia coli* requires a division inhibitor (MinC), that is responsible for preventing septation at unwanted sites near the cell poles, and a topological specificity protein (MinE), that forms a ring at midcell and

protects the midcell site from the division inhibitor [21]. However, the mechanism responsible for identifying the position of the midcell site or the polar sites used for spore septum formation is still unclear.

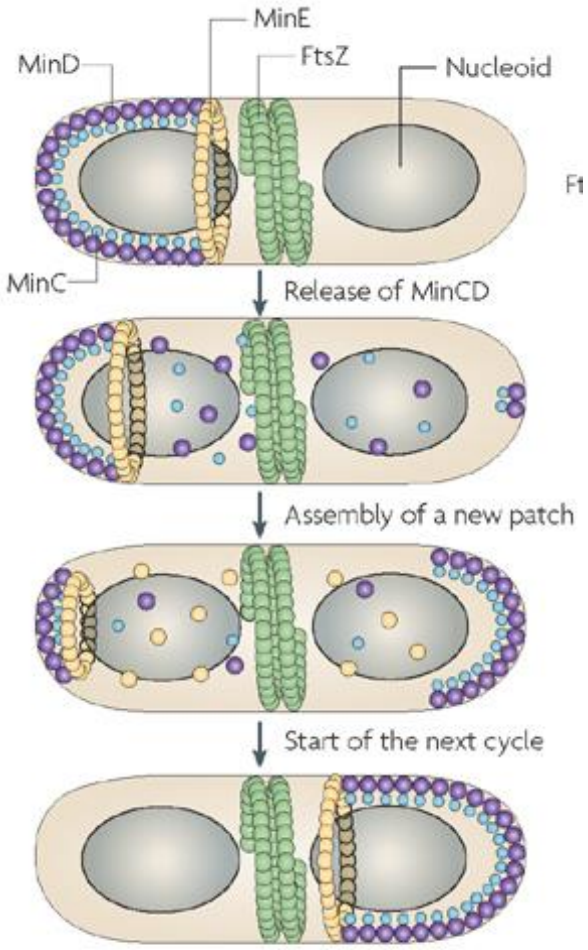


Figure 2: In Escherichia coli, assembly of the FtsZ ring is restricted to the mid-cell by nucleoid occlusion and the MinE-driven pole-to-pole oscillation of the cell-division inhibitor MinCD [4].

Regulation of the division process and its coordination with other cell cycle events, such as chromosome replication, are poorly understood. However, a protein has been identified in *Caulobacter* (CtrA) that regulates both the initiation of chromosome regulation and the transcription of *ftsZ*, and that may play an important role in the coordination process.

Once assembled, the Z-ring is thought to serve as a scaffold for the recruitment of a large set of essential and auxiliary division proteins to the division site, together forming the *trans*-envelope septal ring machine. Studies in which the subcellular localization of

one essential divisome component is observed in the absence of another have revealed a mostly linear dependency pathway for divisome assembly that starts with FtsZ and ends with FtsN (FtsZ [FtsA, ZipA], FtsK [FtsQLB], FtsW, FtsI, FtsN) [22, 23, 24, 25, 26, 27, 28, 29, 30, 31]. The dependency pathway does not appear to reflect the temporal order of divisome assembly. Rather, analysis of septal ring assembly during the cell cycle suggests that maturation takes place in just two steps, with stable Z-rings forming and persisting for about 20% of the cell cycle before most of the remaining divisome components (from FtsQ to FtsN) are simultaneously recruited (32). This second "maturation" step is then closely followed by the initiation of cell constriction (32).

The contraction of the septal ring is associated with the highly localized production of new PG that is thought to be initially shared by the developing daughter cells [33, 34, 35, 36]. The periplasmic PG amidases, AmiA, AmiB, and AmiC, are required to split this shared septal PG to shape the new poles and allow constriction of the outer membrane to closely follow that of the inner membrane [13, 37, 38, 39]. Amidases are PG hydrolases that break peptide cross-links in the PG meshwork by cleaving bonds that link stem peptides to the *N*-acetylmuramic acid component of the glycan strands. Mutants lacking amidase activity complete inner membrane constriction and fusion but fail to split septal PG. Consequently, they form long chains of cells connected by shared layers of PG and a partially constricted outer membrane layer [38, 39].

2.3 - LytM metalloproteases are involved in cell division process

Metallopeptidases can often be recognised by the presence of a short conserved signature sequence containing histidine and glutamate residues. The most common motif is HExxH ("zincins"), but other motifs such as HxxEH ("inverzincins"), HxxE ("carboxypeptidase family") and HxH (e.g. lysostaphin-like) have also been described [3].

HxH metalloproteases of the lysostaphin-type (LytM) of peptidases occur in bacteriophages, in Gram-positive and in Gram-negative bacteria. Metalloproteases containing the catalytic LytM domain belong to the M23 peptidase family [43], this domain was identified for the first time in a secreted autolysin from *Staphilococcus aureus* [44].

Lysostaphin-like peptidases from bacteriophages and Gram-positive bacteria cleave polyglycine crossbridges in the peptidoglycan of Gram-positive bacterial cells. Their role in Gram-negative bacteria is less clear: some peptidases, like b-lytic protease from *Achromobacter lyticus*, target cell walls of Gram-positive bacteria, possibly providing a competitive advantage to the producer organism, others seem to have additional roles, like LasA from *Pseudomonas aeruginosa* that is believed to participate in host elastin degradation [45].

In *E.coli* three LytM proteins (EnvC, NlpD and YebA) were characterized and they are involved in the cell division process [13].

Strains lacking the divisome-associated LytM factors, EnvC and NlpD show defects in cell splitting [13]. It was reported that these LytM proteins are potent and specific activators of PG hydrolysis by the amidases [46]. In a purified system, EnvC was found to specifically activate AmiA and AmiB, while NlpD was found to specifically activate AmiC [13]. The LytM factors are therefore key regulators controlling the activation of PG hydrolysis at the cytokinetic ring.

To activate the amidases, EnvC and NlpD most likely stabilize the open conformation of their cognate amidases, thus biasing the equilibrium to favour the active state. An attractive mechanism by which the open form of the amidase might be stabilized is shown in Fig.3, it acts through the direct binding of the autoinhibitory helix by the LytM activator, it [47].

YebA maintains an active role in peptidoglycan cleavage [48] but appear to play minor, yet observable, roles in cell separation is likely to participate in other aspects of PG biogenesis and only have weak cell separation activity [13].

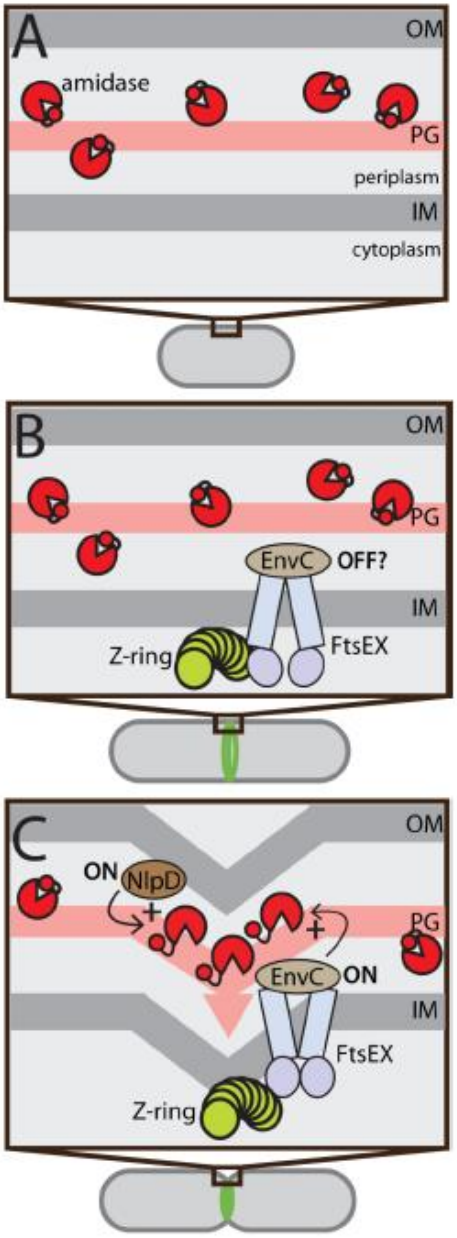


Figure 3: Conformational control of amidase activity during the cell cycle
Shown is a schematic diagram illustrating the activation status of cell separation amidases through the cell cycle. (**A**) At early stages in the cell cycle prior to the formation of the Z-ring, periplasmic amidases (red pac-men) are likely to be largely inhibited by their regulatory helices (red circles). (**B**) FtsEX and EnvC are early recruits to the Z-ring, arriving well before the initiation of constriction. It is not known if the FtsEX EnvC system is capable of amidase activation immediately following its recruitment, or if it requires further septal ring maturation. Even if it is active at this stage, it is unlikely to stimulate a high level of amidase activity because the amidase are not concentrated at mid-cell before the onset of cell constriction. (**C**) Once constriction is initiated NlpD, AmiB, and AmiC are recruited to the septal ring and both amidase activation systems are presumably activated to stimulate amidase activity. Our results indicate that amidase activation proceeds via the release of the regulatory helix from their active site. For simplicity, the three different amidases are not individually identified in the figure **[47]**

Consistent with this idea, both EnvC and NlpD are specifically recruited to the division site, whereas YebA shows a more dispersed peripheral localization pattern.

2.4 - LytM Metalloproteases in pathogenesis

LytM factors are also found in other bacteria, they are involved in cell division, but also in pathogenesis, in particular HdpA from *H. pylori* has a role in the regulation of morphology and in the colonization process [49], the inactivation of the HdpA gene led to a stocky and branched phenotype, affecting *H pylori* colonization capacity despite a normal motility phenotype in vitro. In contrast, the overexpression of the HdpA gene induced the transformation of *H. pylori* from rod to dividing cocci shaped bacteria. Altogether, the morphological abnormalities of the mutant were likely to be directly involved in its colonization

defect. These could impact on several important features of *H. pylori* required for efficient colonization of the gastric mucosa: motility in a viscous environment, poor adhesion to target cells, or reduced growth. Individually or collectively, these altered phenotypes can contribute significantly to explain the reduced fitness of the HdpA mutant *in vivo.*

NG1686 from *N. gonorrhoeae* has roles in resistance to hydrogen peroxide and PMN-mediated killing [50]. The deletion of Δ*ng1686* affects probably the overall permeability of the cell wall. If this were the case, the *ng1686* strain should be more susceptible to killing by a variety of chemicals. However it was only observed increased susceptibility to killing by H_2O_2 and the inorganic peroxide cumene hydroperoxide and not to other oxidants or antibiotics, suggesting that small defects in PG structure in the ng1686 mutant result in discernable phenotypes only for peroxide and PMN-mediated killing.

NlpD is essential for the development of bubonic and pneumonic plague in *Y. pestis*, a chromosomal deletion of the nlpD gene sequence resulted in a drastic reduction in virulence to an LD50 of at least 10^7 cfu for subcutaneous and airway routes of infection. Interestingly, the highly attenuated phenotype of the nlpD mutant and its inability to colonize host organs did not seem to prevent the development of immunity against plague following s.c. infection. Rather, this strain seemed to effectively stimulate a long-

term adaptive immune response as demonstrated by the generation of high antibody titers [11].

Although LytM metalloproteases are well characterized in *E. coli* and in other bacteria, not much information is available in *Haemophilus influenzae.*

2.5 – Non-typeable *Haemophilus influenzae* (NTHi) and pathogenesis

Haemophilus influenzae is a gram negative bacterium that colonizes human nasopharynx. *H. influenzae* strains that lack capsular polysaccharides are referred to as non-typeable (NTHi).

Although it is most commonly associated with asymptomatic colonization, it could be also pathogenic causing serious infections [51].

The questions must surely arise how and why this organism changes from a relatively harmless commensal into an agent of infection. Survival of the bacterial species depends on the ability of the NTHi to parasitize the mucous membranes of the human host [52]. Infection and disease represent an imbalance of colonization.

From this perspective, NTHi should coexist in a balanced relationship with its human host. Of course, the spread of different subtypes to allow exchange of genetic information and subsequent evolution of adaptive diversity for NTHi is essential, and it may be due to this requirement that the balanced state is disturbed.

The success of this organism as a colonizer and pathogen is due to its lack of reliance on any single mechanism of attachment and its ability to respond rapidly to host defense mechanisms by antigenic variation of proteins and enzymes.

As an opportunistic pathogen, NTHI colonization depends on a variety of host and bacterial factors, most of them already characterized, however, the complete scenario of NTHi pathogenesis is still not clear. Adhesion to and invasion of epithelial cells,

macrophages and other components and cells from the human nasopharynx by NTHi have been studied in details and represented in figure 6: NTHI adheres to respiratory mucus and to an unidentified non-ciliated cell type(s) of the multiple cell types of the respiratory epithelium [53], then it is able to persist in the nasopharynx mucosa evading the host defense and forming biofilm until it is internalized in the cells.

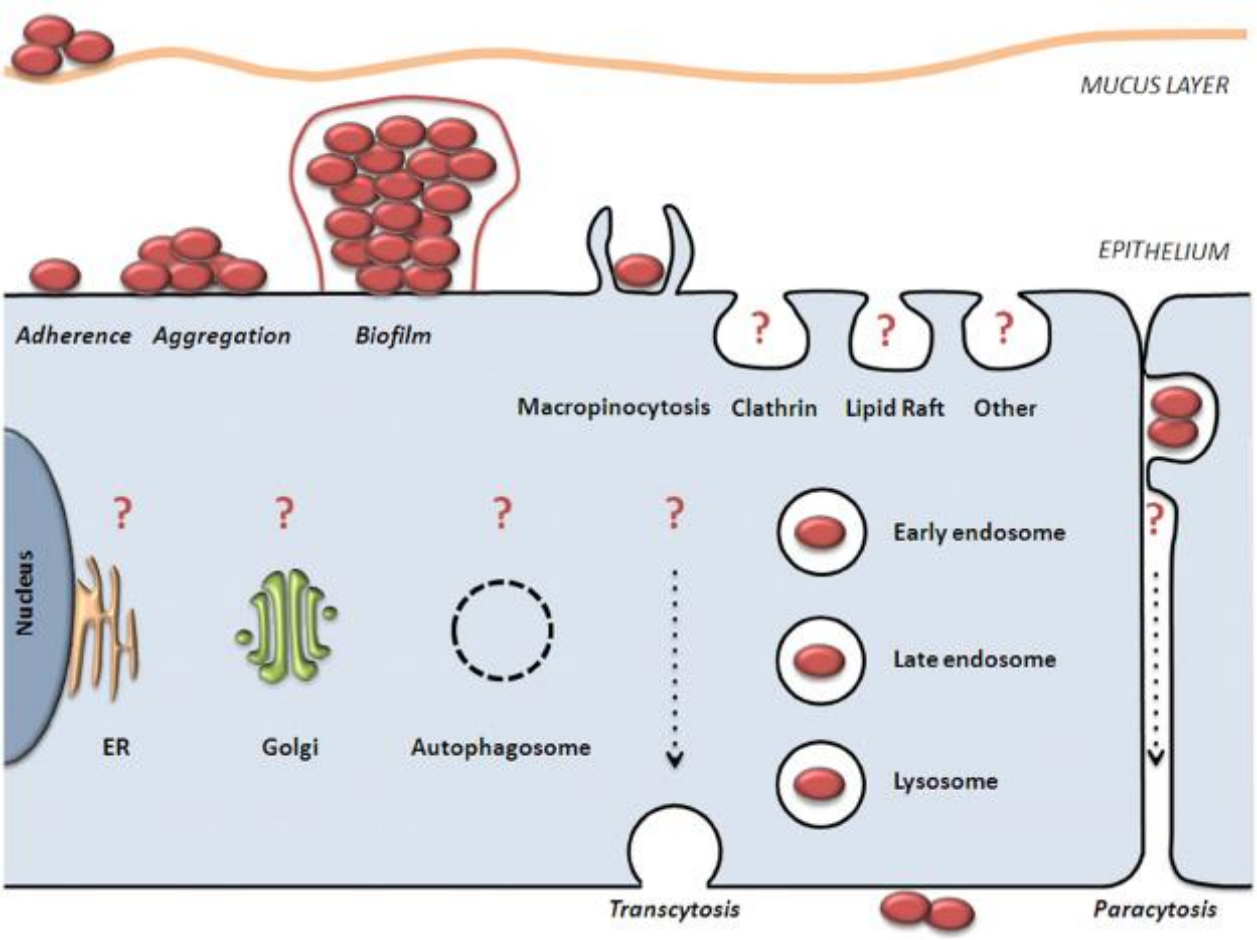


Figure 6: Model of NTHI colonization and invasion of epithelial cells.
NTHI adheres to mucus and non-ciliated epithelial cells. NTHI aggregates mature into a biofilm composed of bacterial and host components. NTHI has been observed within, between, and beneath epithelial cells in vitro and ex vivo. It is clear that NTHI are internalized by macropinocytosis and are trafficked to vesicles that are positive for endolysosomal markers. It is unclear what role(s) are played by other host internalization and trafficking pathways (noted by question marks), or how these pathways affect NTHI viability. Further examination of these pathways in relation to NTHI is needed to fully characterize the journey and fate of intraepithelial NTHI. [42].

The complexity of bacterial-host interactions at mucosal surfaces is an area of intensive research and new insights into bacterial-host cell cross-talk and bilateral consequences have been gained [54, 55].

NTHi has the capacity to express adhesins and invasins. The long-range pili:fimbriae adhesion system has been characterized [56,57]; aside from the structural HifA, candidates for tip proteins exist, e.g. HifE. Surprisingly, however many NTHi otitis and nasopharynx isolates appear to lack the pilus gene cluster [58]. The existence of alternative long-range adhesion mechanisms is unclear. NTHi strains were found to express proteins of the family of high molecular weight adhesins such as Hmw1, Hmw2 and Hia [58].

All NTHi strains also appear to express an adhesin: invasin Hap associated with the capacity to enter eukaryotic cells [58]. It has also been demonstrated that regions of the integral OMP P5 are clearly associated with NTHi adhesive properties [59,60]. The association between P5 and fimbrial structures merit further clarification [59]. Invasion can also be observed by in vitro NTHi-cell culture interactions [61]. Such invasive states could imply the need for T-cell mediated protection mechanisms. As well as adhesion and invasion, resistance to complement, C-reactive protein and other innate immune mechanisms will play a role at the epithelial and subepithelial level, NTHi LPS most likely being a key bacterial factor in such processes.

NTHI encodes specific defense mechanisms: ProteinD enhanced damage to cilia in a nasopharyngeal tissue culture model [62], the Sap transporter and lipooligosaccharide (LOS) phosphorylcholine (PCho) appear to protect NTHI against human antimicrobial peptides β-defensin and cathelicidin LL-37, respectively, which are important respiratory defense molecules [63,64]. NTHI LOS glycosyltransferase Lic2B activity and the ability to bind host complement inhibitors C4 binding protein, factorH, and vitronectin promote NTHI evasion of complement-mediated killing [65,66,67,68,69]. The exact mechanism of

how *H. influenzae* evades the host complement attack is unclear. In a previous study is shown that NTHi binds C4BP and that this interaction significantly contributes to bacterial serum resistance [70]. NTHi binds surface-associated vitronectin and it has been suggested that adhesins are involved in the binding [71]. In addition, both NTHi and encapsulated H. influenzae bind the alternative pathway inhibitor factor H, which is able to inhibit lysis of H. influenzae [66]. NTHI also encodes IgA1 proteases to inactivate IgA1, likely facilitating colonization, although this is difficult to confirm in animal models since IgA1 is a human-exclusive antibody. However, human lactoferrin cleaves IgA1 proteases and the homologous NTHI adhesin, Hap, suggesting that the healthy host might neutralize at least some of these potential colonization factors [73,74].

Adherent aggregates of bacteria may mature into biofilm, an important and intensely studied form of NTHI persistence in vitro and in vivo [75-76-77-78-79-80]. NTHi biofilms can exist in at least two clinically relevant phenotypes: (1) a ‘classical’ biofilm, wherein bacteria are attached to a surface such as the mucosa of the middle ear, in adenoid tissue, or tympanic tube implants; (2) large biofilm aggregates consisting of host material, especially neutrophils, proteins, and lipooligosaccharide that are not attached to the mucosa or host surfaces *per se* [81]. Many reports are aimed to establish a link between NTHi biofilm formation and its ability to cause disease, such as otitis media (OM). NTHi presenting at inflammatory sites, such as the middle ears of children with OM, is usually terminal for the bacteria as they are normally cleared from the site by host defense mechanisms and therefore are not transmitted to another host. In this regard, the ability of NTHi to form biofilms would not be expected to evolve or be retained within the bacterial population. Therefore, NTHi biofilm formation should have other advantages besides the ability to cause disease. Possibly, biofilm formation is already needed for colonization. Much less is known about the potential for NTHI persistence by invasion of host tissue.

NTHi strains have not traditionally been thought of as invasive. More recent work, however, has demonstrated that the bacterium may enter the host cell, presumably to evade the local immune response. This response has been demonstrated with several *Haemophilus* species. Capsule-deficient *H. influenzae* strains invade endothelial cells and remain within membrane-bound vacuoles over an extended period with no apparent effect on the host cell [82]. Wild-type NTHi strains have been found, in vitro, to adhere to, invade, and persist in Chang epithelial cells [83]. The relationship between the bacterium and he mammalian cell appeared to be a dynamic interaction, since both adherence and invasion of the NTHi increased over time. Intracellular entry was also accompanied by penetration of the mucosal surface at points of necrosis and cell junction. Following on from this, Van Schilfgaarde et al. [84] coined the phrase “paracytosis” when describing NTHi adherence to and subsequent passage through lung epithelial cell lines. In these experiments, highly adherent strains demonstrated greater paracytosis. The passage time was independent of inoculum size, fimbriae, or capsule but, was dependent on the rate of bacterial multiplication, with rapidly growing strains taking 10 to 18 h and slower growing strains taking 30 h to pass between the apical and basolateral chambers. Chloramphenicol addition prevented paracytosis, indicating that de novo protein synthesis was required for the process to occur. Paracytosis is both an *in vitro* and *in vivo* phenomenon. Adenoids removed from children with persistent otitis media or adenoidal hypertrophy were shown to have up to 105 viable intracellular *H. influenzae* cells in the reticular crypt epithelium and macrophage-like cells in the subepithelial layer of tissue [85]. The mechanism for uptake into the cell is unknown; however, under strategies outlined by Falkow [86] and Isberg and Tran Van Nhieu [87], it may be associated with the Hap protein or indeed the gag-dependent binding of the high-molecular-weight proteins on the bacterial surface [88,89]. Other proteins that contribute to invasion are ChoP, ProteinD, and ProteinE [89; 90; 91; 92].

Studies on the significance of NTHI–host cell interactions, and indeed all NTHI studies, are complicated by the aclonal nature of this bacterium. Enormous strain-to-strain heterogeneity exists in the possession, expression, and composition of many NTHI outer membrane molecules.

Aside from the genetic population diversity NTHi has been found to demonstrate extensive sequence and antigenic variation amongst gene products interacting with the human immune system such as outer membrane proteins and secreted virulence factors [93].

Amongst well studied examples of antigens demonstrating antigenic variation by way of mutations and:or phase variation are the outer membrane proteins P1, P2, P5, TbpB; pili or fimbriae; lipopolysaccharide; IgA-protease [94-100].

Antigenic drift, i.e. intrastrain variability has been demonstrated to occur with respect to surface regions of P2 [95]. OMP characteristics are described in van Alphen et al. [101]. As well as phase variation, LPS can be further modified by substitutions with phosphorylcholine and sialic acid which impact on serum sensitivity [99,102]. The crucial requirement for protoporphyrin IX has been met by NTHi by way of the potential expression of various hemoglobin, haptoglobin, hemopexin surface bound receptors and excreted products [103,104]. A redundancy of hemoglobin-haptoglobin-binding NTHi proteins exists allowing possible adaptation in the face of immunity [103].

Added complexity comes from variable expression and availability of certain host cell receptors and matrix proteins. NTHI cells for example express, on their outer surfaces, a number of LOS core oligosaccharide epitopes, and the expression of these epitopes is subject to frequent, reversible phase variation. Four chromosomal loci, *lic-1* to *lic-3* and *lgtC*, which contain long stretches of 4-bp tandem repeats within their 5′ coding regions, have been reported to generate phase-variable LOS structures [105,106,107]. *lic-1* functions to add phosphorylcholine (PCho) to the LOS molecule

[108], *lic-2* and *lgtC* are necessary for the expression of Galα1-4Gal [109], and the effect of variation in *lic-3* is unknown. Phase variation may represent a mechanism whereby NTHI evades the host immune response or concurrently modulates its surface in order to colonize different anatomical sites, each with a unique complement of host cell receptors [110-111]. Elucidating the role of LOS phase variation is important both for understanding OM pathogenesis and for designing a candidate vaccine [93].

Analysing the different aspects of NTHi pathogenesis there is one key factor that is important for all the steps: phosphorylcoline (PCho). The addition of PCho to bacterial surfaces is a recurring theme among bacteria that inhabit mucosal surfaces. Contributions of PCho to the persistence of NTHi *in vivo* include increased adherence to and invasion of airway epithelial cells [91], influence on biofilm formation [77,112], resistance to host defense [111], and diminished potency of LOS as an inflammatory agonist [113]. The contribute of this factors will be evaluated also in this thesis and its importance for NTHi virulence will be underlined.

2.6 - Eradication of NTHi infections

Respiratory tract infections associated with nontypeable *Haemophilus influenzae* (NTHi) are a major cause of morbidity and mortality in both developed and nonindustrialized nations. NTHI strains are the leading cause of bacterial otitis media infections (both acute and recurrent) in young children and are also responsible for chronic obstructive pulmonary disease (COPD) exacerbations in current and former smokers [51].

These pathologies are multifactorial diseases caused by other nasopharyngeal commensal too. For example the predominant bacterial agent causing Acute otitis Media (AOM) are *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis*.

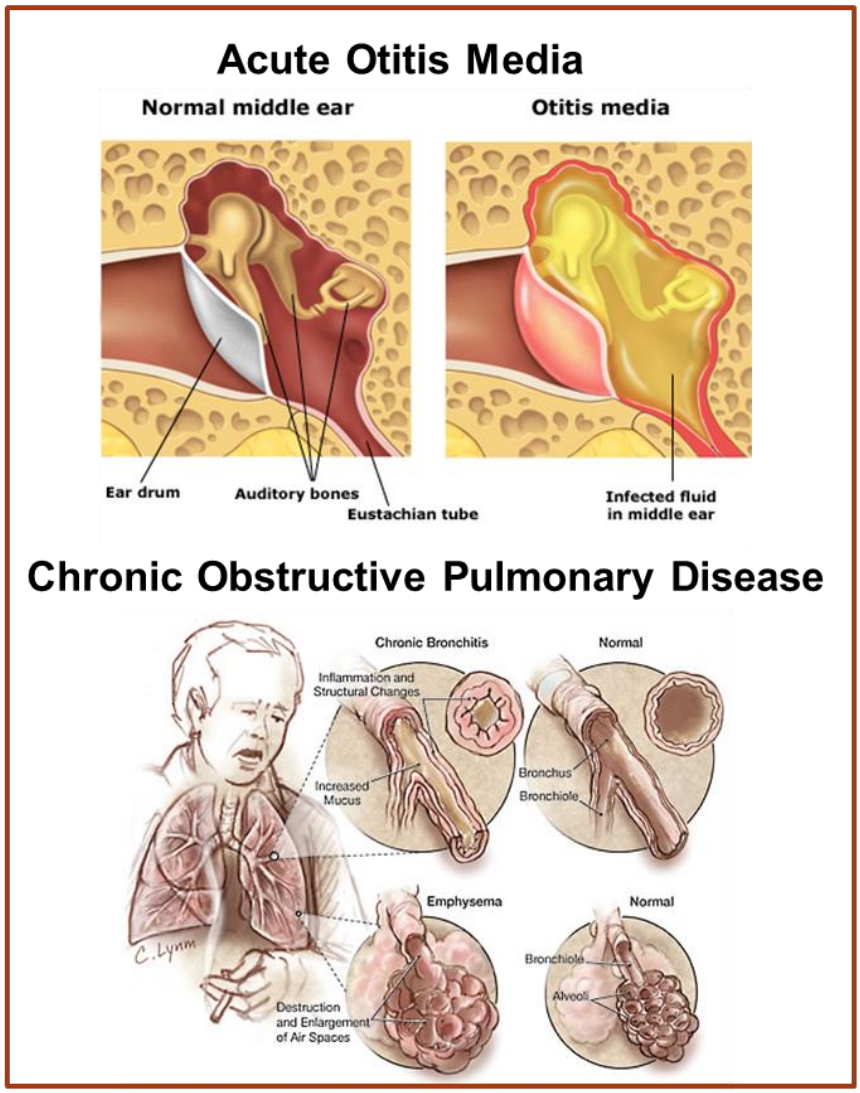


Figure 4: Main pathologies associated to NTHi infections (http://doctorrennie.wordpress.com/tag/otitis-media/ and http://nursingcrib.com/case-study/chronic-obstructive-pulmonary-disorder-copd-case-study)

Following introduction of anti-pnumococcal vaccine some studies have demonstrated a shift in the proportions of causative pathogens of acute otitis media (AOM), there was an increase in the proportion of AOM episodes due to NTHi. Other studies have seen similar changes in the proportions of bacteria isolated from the middle ear and the nasopharynx of children with AOM, with NTHi becoming a more frequently isolated pathogen and replacement with non-conjugate vaccine *S.pneumoniae* serotypes [114-115].

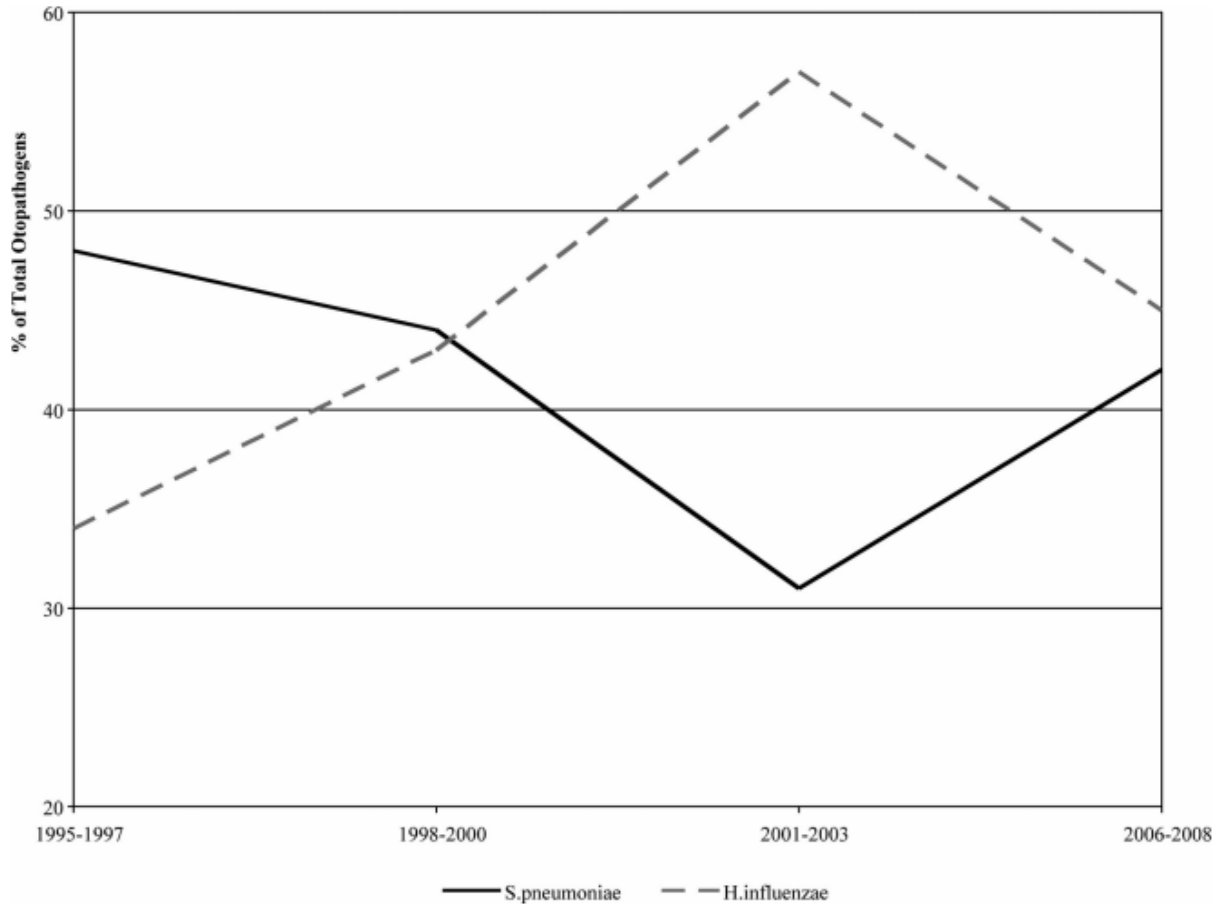


Figure 5: Percentage of S. pneumoniae and NTHi causing recurrent AOM in Rochester, NY 1995–2008 **[114]**

Strategies to prevent host tissue damage due to infection by NTHi must take into account the differences between prevention of acute respiratory infection and prevention of chronic respiratory infection. The occurrence of an acute exacerbation, indicates a breakdown in the innate immune response, while chronic infection indicates that the immune response mounted

to infection is either acting inappropriately or being countered by NTHi. Therefore, while prevention of acute infections may focus on prevention of attachment, invasion, and multiplication of the bacteria, dealing with an established chronic infection requires a strategy of modulating the immune response for successful treatment.

Development of the ideal vaccine to prevent infection from this member of the normal microbiota is a great challenge. Vaccine strategies can be targeted toward prevention of

primary infection or immunomodulation of the host response that has resulted in either repeated acute exacerbations or chronic infection. However, the final goal is complicated by the fact that NTHi uses several strategies to evade immune response as: huge genomic variability, IgA protease production, paracytosis and antigenic variation [83-84-85-116-117-118]. Moreover AOM and COPD are multifactorial pathologies, often caused by polimicrobial infections.

3 - Aim of the study

The aim of this work is to elucidate the role of the new identified LytM proteins in NTHi, analysing their contribution both in cell division and in pathogenesis.

4 - Experimental procedures

4.1 - Computer analysis

LytM proteins were analyzed using several online applications. Putative signal peptides were identified using SignalP (http://www.cbs.dtu.dk/services/SignalP/), Pfam (http://pfam.sanger.ac.uk/) was used to detect the presence of domains of known function and SMART software (http://smart.embl-heidelberg.de/) helped us to reconstruct architectural structure of each protein. Homologues of NTHi LytM factors were found using BLASTP (http://blast.ncbi.nlm.nih.gov/). Identity percentages between different proteins were obtained comparing amino acid sequences with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and BLASTP.

4.2 - Bacterial strains and growth conditions

NTHi Strain 176 was used for this study. It was part of a Finnish otitis media cohort study, as isolate obtained from the middle ear. NTHi was cultivated on chocolate agar polivitex (BioMerieux) incubated at 37°C with 5% CO_2. Brain-heart infusion (BHI) broth (Difco Laboratories) supplemented with 10 μg/mL each of haemin (Fluka Biochemika) and nicotinamide adenine dinucleotide (NAD, Sigma) was used as fluid growth medium. *Escherichia coli* strains DH5α, HK100 and BL21 (DE3) (Invitrogen) were used for cloning and expression of LytM proteins. They were cultured at 37°C in Luria Bertani (LB) medium and, when required, supplemented with 100 μg/mL ampicillin.

4.3 - Cell cultures

Tissue culture cells used in this study are Chang epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4, human conjunctiva, ATCC® CCL-20.2™) and HEK293 (human kidney, ATCC® CRL1573™). Chang cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM; Gibco) supplemented with 25 mM Hepes, 15 mM L-glutamine, antibiotics and 10% (vol/vol) heat-inactivated fetal calf serum (FCS, Invitrogen Corporation). They were grown at 37°C with 5 % CO_2.

HEK293 cells stably expressing TLR2 or TLR4/MD2/CD14 and the NF-κB–luciferase reporter cassette, were cultured in DMEM containing 4.5 g/ml glucose, supplemented with 10% heat inactivated FBS, 100 U/ml pelicillin, 100 µg/ml streptomycin, 2 mM glutamine, 5 µg/ml puromycin 250 µg/ml hygromycin (and plus 10 µg/ml Blasticidin for HEK293-TLR4 cells).

4.4 - Cloning of genes coding for LytM proteins

LytM genes were cloned into the pET15b+ vector (Novagen) by the polymerase incomplete primer extension (PIPE) method (119). In brief, sequences coding for each protein were amplified by PCR from the *HI176* genomic DNA, removing the signal peptide (primers are listed in Table S1 in the supplemental material). PCRs generated mixtures of incomplete extension products; by primer design, short overlapping sequences were introduced at the ends of these incomplete extension mixtures, which allowed complementary strands to anneal and produce hybrid vector-insert combinations. *Escherichia coli* HK100 cells (120) were then transformed with vector-insert hybrids. Single ampicillin-resistant colonies were selected and checked for the presence of the recombinant plasmid by PCR. Plasmids from positive clones were isolated and subcloned into competent *E. coli* BL21(DE3) cells.

4.5 - Expression and purification of recombinant proteins

For protein purification, one single colony of *E. coli* BL21(DE3) strain expressing NTHI0532, NTHI0915 and NTHI0830 were inoculated in LB + ampicillin and grown overnight at 37°C, diluted in fresh LB medium and grown at 30°C to an OD of 0.6-0.8. The protein over-expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG; Sigma) for 4 hours. Recombinant 6 x His-fusion proteins was purified by affinity chromatography on Ni^{2+}-conjugated chelating fast-flow Sepharose 4B resin (Pharmacia). The purity was checked by SDS-PAGE electrophoresis staining with Coomassie blue. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo Scientific).

4.6 - Construction of the Knockout mutants

Deleted mutants of NTHI0532, NTHI0915 and NTHI0830 were constructed by allelic replacement of each whole gene with an erythromycin resistance cassette. Upstream and downstream regions of the three genes were amplified by PCR using the primers listed in table 1 and cloned in Stratagene pSC-A TOPO vector.

Table1: Oligos for Knockouts generation

NT013	NT013 5'FOR	TTGCACGCGCCAATAATACC
	NT013 5'REV	**TGCATGCAT**TTACGTGTTGCACTGGCATC
	NT013 3'FOR	**TGCATGCAT**TGTTCGTGTTCGTGAAGCAG
	NT013 3'REV	AACGCGATTGCGTAATGCAG
NT017	NT017 5'FOR	TGCTGGTGCAATTTGATCTTC
	NT017 5'REV	**TGCATGCAT**TGATTAACGCCAAAACGCAAC
	NT017 3'FOR	**TGCATGCAT**ATTAGCCGTAAAGGAACGCC
	NT017 3'REV	TGGCGATCTAATGAACGCAC
NT022	NT022 5'FOR	AAACATTGTGCAACAATGGGG
	NT022 5'REV	**TGCATGCAT**ACAAGACTCAAAGGGAGTAAG
	NT022 3'FOR	**TGCATGCAT**GGATCCAGTACGTTACCTAC
	NT022 3'REV	GTTTCTTTGTCCGCAGGTTC

Erythromycin resistance cassette was purified from pIM13 plasmid. The constructs containing upstream regions, resistance cassette and downstream regions were

assembled. Plasmids obtained were linearized and used to transform 176 NTHi strain using MIV protocol [40]. Knockout strains obtained were confirmed by PCR, western blot and locus sequencing.

4.7 - Preparation of polyclonal antisera

Groups of four CD1 mice were immunized to produce polyclonal antisera; 10 μg of purified protein was used for each mouse. The recombinant protein was given intraperitoneally in the presence of aluminum. A second (day 21) and a third (day 35) booster doses were administered. Blood sample was taken on day 49.

The treatments were performed in accordance with internal animal ethical committee and institutional guidelines.

4.8 - Cell fractionation and western blot analysis

Haemophilus strains were grown in BHI until mid-log phase at 37°C with 5 % CO_2.

Whole cell lysates and periplasmic fractions were purified using PeriPreps Periplasting kit from Epicentre. Outer membrane proteins (OMPs) were recovered on the basis of Sarkosyl-insolubility following the rapid procedure as described by Carlone *et al*. [129].

To prepare culture supernatants, bacteria were harvested at 13000 g for 10 min at 4°C. 1 ml of culture supernatant was filtered through a 0.22 mm filter and precipitated with vol of 50% TCA for 1 h at 4∞C.

After centrifugation at 13000 g for 30 min, the achieved pellet was washed once with 70% ethanol and resuspended in 1X sample loading buffer.

Proteins of each cell fraction were separated by SDS-PAGE electrophoresis using NuPAGE Gel System, according to the manufacturer's instructions (Invitrogen), and

revealed by Coomassie-blue staining or transferred onto nitrocellulose membranes for Western blot analysis.

Western blots were performed according to standard procedures. The different LytM proteins were identified with a polyclonal mouse antiserum raised against recombinant NTHI0532, NTHI0915 and NTHI0830 (diluted 1:1000) and an anti-mouse antiserum conjugated to horseradish peroxidase (DAKO), as secondary antibody. Bands were visualized with Super Signal Chemiluminescent Substrate (Pierce) and with Opti 4CN Substrate Kit (Bio-Rad) following the manufacturer's instructions.

4.9 - Confocal Microscopy

The presence of LytM proteins on NTHi surface was checked using confocal imaging. Knockout mutants were used as negative controls. Bacteria were grown until exponential phase, and fixed in 4% paraformaldehyde (Sigma). After multiple washings, bacteria were spread on polylisine-coated slides and blocked with PBS + 3% bovine serum albumin (BSA) (Sigma) for 30 min at room temperature. Samples were washed and incubated with specific antisera (1:1000) for 15 min at room temperature. LytM antisera were preadsorbed with intact KO bacteria to minimize cross-reactivity. Bacteria were washed several time with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:400) (Molecular Probes). Labelled samples were mounted with ProLong®Gold antifade reagent with DAPI (Molecular Probes) and analysed with ZeissLSM710 confocal microscope.

4.10 - Scanning and transmission electron microscopy

Electron microscopy was performed on 176wt and ko strains to observe defects in bacterial morphology. Bacteria were grown until exponential phase, washed with PBS

and fixed overnight in cacodylate sucrose buffer containing 2.5% glutaraldehyde and 2.5% paraformaldehyde. Samples were then postfixed in 1% OsO4 and 0.15% ruthenium red in cacodylate buffer, blocked with 1% uranyl acetate and dehydrated with serial dilution of acetone.

For SEM, samples were then dried by the critical point method using CO2 in a Balzers Union CPD 020, sputter-coated with gold in a Balzers MED 010 unit, and observed with a JEOL JSM 5200 electron microscope. For TEM, samples were fixed and dehydrated as described above then embedded in Epon-based resin. Thin sections were cut with a Reichert Ultracut ultramicrotome by use of a diamond knife, collected on collodion copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX II electron microscope.

4.11 - Peptidoglycan extraction and analysis

Peptidoglycan was purified from NTHi WT and mutants following the method of Uhehara [13]. 1 l of exponential phase culture was centrifuged and final pellet was resuspended in 20 ml of PBS. Resuspended pellet was boiled with 80 ml of 5% SDS for 30 minutes and let overnight at room temperature. Samples were ultracentrifuged for 1 h at 25000 rpm at room temperature and then washed with water several times to remove SDS. Peptidoglycan were resuspended in 1 ml of PBS and incubated with 200 µg/ml Amilase (Sigma, A6380) overnight at 37°C. Samples were pelleted by ultra-centrifugation using TLA 100.3, 80000 rpm, 15 min, washed with water three times and resuspended in 1 ml water.

Dried peptidoglycan samples purified from selected strains (Hi176wt, 176ΔNT013 and 176ΔNT 22) of non typeable *Haemophilus influenzae* were suspended in 0.7 ml of 90% H2O + 10% D2O. Enzymatic digestions of the peptidoglycan samples were performed to solubilize it adding 125 µg of dried mutanolysin (Sigma Aldrich) to the suspended

samples followed by an overnight incubation at 37°C. 1H NMR spectra of the digested peptidoglycans were recorded on a Bruker AVANCE III 400 MHz, equipped with a precision temperature controller, using a 5 mm broadband probe (Bruker). For data acquisition and processing, the TopSpin 2.1 software (Bruker) was used. 1H NMR spectra were collected at 4°C ± 0.1°C, with 4k data points over a 10ppm spectral width, using the a diffusion filter pulse sequence. The transmitter was set at the water frequency which was used as the reference signal (4.79 ppm).

Dye release assay was performed staining purified peptidoglycan with remazol brilliant blue (Sigma, R8001) [47] and then incubating it with 4 µM of purified LytM proteins or mutanolysin (positive control) for different incubation times at 37°C.

4.12 - Preparation of outer membrane vesicles

Native Outer membrane vesicles (OMVs) were isolated from WT and mutant strains, growing the bacteria until exponential phase in 200 ml BHI cultures. Bacteria were then centrifuged and supernatant were filtered and let at 4°C overnight adding proteases inhibitor and EDTA. Supernatant were ultracentrifuged for 3 hours at maximum 200000 X g and final pellet containing OMVs was resuspended in PBS.

4.13 - Mass spectrometry

SDS-PAGE Coomassie stained bands were excised and destained in 50 mM NH4HCO3 50% acetonitrile. After a drying step, bands were in-gel digested with 12.5 ng/ml Trypsin in 5 mM NH4HCO3 overnight at 37°C. The reaction was stopped by the addition of 0.1% final concentration Trifluoroacetic acid (TFA) and the samples were subjected to MALDI-TOF Mass Spectrometry analysis. 1 µl of digestion solution was spotted on a PAC target (Prespotted AnchorChip 96, set for Proteomics, Bruker Daltonics) and air-dried at room

temperature. Spots were washed with 0.6 µl a solution of 70% (vol/vol) ethanol, 0.1% (vol/vol) TFA. Peptide mass spectra were externally calibrated using the standards pre-spotted on the target. Peptide molecular masses determination was performed using a MALDI-TOF/TOF mass spectrometer UltraFlex (Bruker Daltonics, Bremen, GmbH). Ions generated by laser desorption at 337 nm (N2 laser) were recorded at an acceleration voltage of 25 kV in the reflector mode. In general, approximately 200 single spectra were accumulated for improving the signal/noise ration and analyzed by FlexAnalysis (version 2.4, Bruker Daltonics) Peptide mass fingerprints were performed using MASCOT searches against Haemophilus influenzae 86-028NP database using the following parameters: (i) 1 as number of allowed missed cleavages, (ii) methionine oxidation as variable modification, (iii) 75 ppm as peptide tolerance. Only significant hits were considered, as defined by the MASCOT scoring and probability system.

4.14 - Reactogenicity assays

For luciferase assay HEK293-TLR2 and HEK293-TLR4 cells were seeded into microclear 96-well bottom plates in 90 µl of complete medium in absence of selection antibiotics. After overnight incubation, cells were stimulated in duplicates with different concentration of OMVs (10 µl/well) starting from 1 mg/ml diluted 1:2 in PBS, for 6 h. Then the medium were discarded and cells were lysed with 20 µl of Passive Lysis Buffer (Promega) for 20 min at room temperature. Luciferase levels were measured by addition of 100 µl/well Luciferase Assay Substrate (Promega) using LMax II^{384} microplate reader (Molecular Devices). Raw light units (RLU) from each sample were divided by the RLU of the control sample (PBS) and expressed as Fold Induction (FI).

PBMCs (Pheripheral Blood Mononuclear Cells) were isolated from buffy coats of healthy donors using Ficoll (Amersham Biosciences) density gradient centrifugation. Cells were seeded into microclear 96-well bottom plates in 180 µl of RPMI (GIBCO) supplemented

with 10% of heat-inactivated FBS, 100 U/ml pelicillin, 100 µg/ml streptomycin, 2 mM glutamine. Cells were stimulated with different concentration of OMVs (20µl/well) starting from 1 mg/ml diluted 1:2 in PBS, for overnight. Mesoscale Assay Human-Proinflammatory 7-spot (MSD Technology) is used for detection of inflammatory cytokines following manufacturer's instructions.

4.15 - Infection of epithelial cells: adhesion assays with NTHi strains

Chang conjunctiva epithelial cell suspensions obtained from confluent monolayers were seeded at 1,5 x 10^5 cells per well in 12 well tissue culture plates (NUNC) and incubated for 24 hours in an antibiotics–free medium.

Overnight culture of bacteria were washed once and resuspended in DMEM + 1 % FCSi to a concentration of 3 x 10^7 bacteria ml^{-1} at a multiplicity of infection (MOI) of approximately 1:100, aliquots of 1 mL of each strain were added to monolayer cultures of Chang cells and incubated for 3 hours at 37°C in 5 % CO_2.

Non-adherent bacteria were removed by washing three times with DMEM + 1 % FCSi and twice with PBS. The remaining bacteria were released by addition of 1% Saponin (Sigma) and incubation at 37°C for 15 min: serial dilutions of the associated bacteria suspension were plated onto agar chocolate plates. Adhesion capability was quantified by counting CFU.

4.16 - Biomass assay on plastic

Bacteria from overnight culture were diluted 1:100 and incubated statically in BHI on plastic multiwell plates at 37°C. After 24 hours, wells were gently washed once with sterile 1 ml PBS and then allowed to dry for 10 minutes. The biofilm was stained with 1 ml of filter-sterilized 0.2% crystal violet and incubated for 30 minutes at room

temperature (RT). Crystal violet was removed from the wells, followed by two washes with 1 ml PBS. The dye was extracted by adding 1 ml 96% ethanol to each well and incubation for 30 minutes at RT. The absorbance was measured by Tecan plate reader at 540 nm.

4.17 - Serum resistance assay

Bacteria were grown until early exponential phase and then diluted in DPBS and splitted in a multiwell plate to have 10^4 bacteria/ml. Normal human serum from healthy individuals were added to each sample at 2% concentration. Heat inactivated serum was also added as negative control. 10 µl of each well were spotted at different time points on agar chocolate plates to evaluate bacterial survival.

4.18 – Facs analysis

PCho: Briefly, Wild type and 176*ΔNT017* bacteria were grown until midlog phase (~1 × 10^8/ml bacteria), suspended in PBS plus 1% (wt/vol) BSA, and incubated with antibody against pCho (1:200) for 1 h at room temperature (RT). Primary antibody binding was detected using an anti-mouse (whole molecule) FITC-conjugated antibody (Sigma) at a 1:500 dilution. The bacterial cells were fixed with 0.5% formaldehyde in PBS buffer and after 1 h at RT incubation, were analyzed by flow cytometry. Assay controls included secondary antibody alone as a negative control.

Factor H: Wild type and 176*ΔNT017* bacteria were were grown until midlog phase (~1 × 10^8/ml bacteria), suspended in PBS plus 1% (wt/vol) BSA, and incubated with Human Factor H (10 µg/ml) for 30' at room temperature. fH binding was detected with a goat polyclonal antiserum to fH (Calbiochem) diluted 1:200 and incubated for 30 min at RT

followed by an additional 30 min incubation with a donkey anti-goat IgG–FITC conjugate (Jackson Immunoresearch) diluted 1:100 in PBS–1% BSA buffer.

5 – Results

5.1 - NTHi genome contains LytM genes showing significant homology to bacterial metalloproteases

The emerging evidence that LytM metalloproteases play an important role in both bacteria physiology and pathogenesis lead us to investigate by *in silico* genomic analysis whether genes containing LytM signatures could be also identified in Non-Typeable *Haemophilus influenzae* (NTHi). By this analysis, we discovered three interesting genes, named NT013, NT017 and NT022, which codify for three proteins belonging to the M23 family of metalloproteases and that are well conserved among a panel of public available *H. influenzae* genomes (Fig. 7).

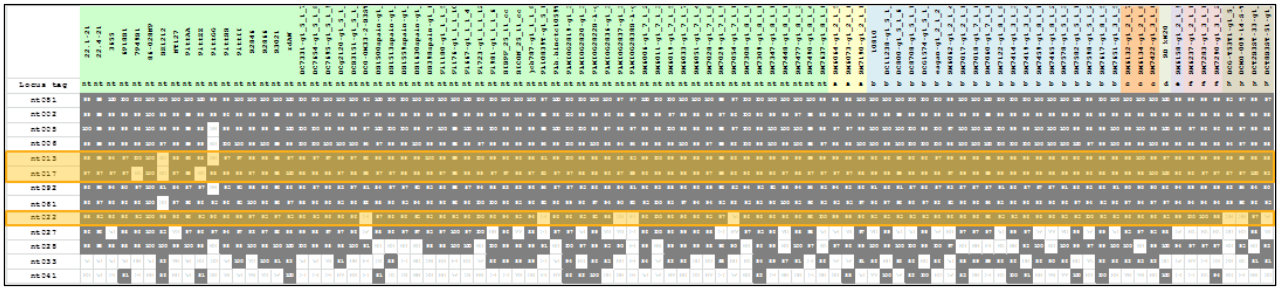
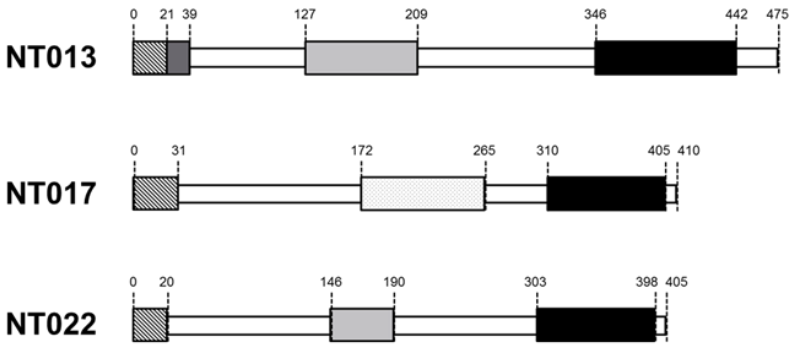


Fig. 7: Metalloprotease in NTHi
Conservation level of 13 hypothetical metalloprotease identified in NTHi among a panel of strains. Grey boxes indicate a percentage of amino acid identity over 80%. LytM factors are highlighted in orange.

All the three proteins contain a catalytic LytM domain that is localized at the C-terminus, while the presence of a canonical N-terminal signal sequence suggests a hypothetical extra-cytoplasmic localization. Moreover NT013 and NT022 have in common a central LysM domain that is usually responsible for the binding to the peptidoglycan [130].

A)



B)

LytM	NAME	PATHOGEN	AA IDENTITY	PROTEIN FUNCTION
NT013	YebA	*E. coli*	49%	Involved in peptidoglycan cleavage during cell division
	HdpA	*H. pylori*	50%	Required for cell shape. Pole formation and virulence
	NG1686	*N. gonorrhoeae*	32%	Influences resistance to hydrogen peroxide and colony morphology
NT017	EnvC	*E. coli*	40%	Activator of Amidase A and B during cell division
NT022	NlpD	*E. coli*	43%	Activator of Amidase C during cell division
	NlpD	*Y. pestis*	47%	Required for cell shape and plague development
	LppB	*H. somni*	58%	Immunogenic antigen

C)

NT013-YebA

```
NT013  PHKGVDFSVSQGTPVIAPADGTVEKVAYQAGGAGRYVMLRHGREYQTV
YebA   PHRGVDFAMPQGTPVLSVGDGEVV-VAKRSGAAGYYVAIRHGRSYTTR
       **:****::.*****:: .** *  ** ::*.** ** :****.* *

NT013  YMHLSKSLVKAGQTVKKGERIALSGNTGISTGPHLHYEFRINGRAVNP
YebA   YMHLRKILVKPGQKVKRGDRIALSGNTGRSTGPHLHYEVWINQQAVNP
       **** * ***.**.**:*:********* *********. ** :****
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NT017-EnvC

```
NT017  VRWKGMVIGASAGTPVKAIAAGRVILAGYLNGYGYMVIVKHGETDLSL
EnvC   LRWKGMVIGASEGTEVKAIADGRVILADWLQGYGLVVVVEHGKGDMSL
       :********** ** ***** ******.:*:*** :*:*:**: *:**

NT017  YGFNQAVSVKVGQLVSAGQVIAQVGNTGEISRSALYFGISRKGTPVNP
EnvC   YGYNQSALVSVGSQVRAGQPIALVGSSGGQGRPSLYFEIRRQGQAVNP
          **:**:. *.**. * *** ** **.:*  .*.:*** *
*:* .***
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NT022-NlpD

```
NT022  GGNKGIDISGSRGQAVKAAAAGRIVYAGNALRGYGNLIIIKHNDDFLS
NlpD   GGNKGIDIAGSKGQAIIATADGRVVYAGNALRGYGNLIIIKHNDDYLS
       ********:**:***: *:* **:**********************:**

NT022  AYAHNDKILVADQQEVKAGQDIAKMGSSGTNTVKLHFEIRYKGKSVDP
NlpD   AYAHNDTMLVREQQEVKAGQKIATMGSTGTSSTRLHFEIRYKGKSVNP
       ******.:** :********.**.***:**.:.:************:*
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Fig. 8: *In silico* analysis of LytM proteins.
(A) Architectural domain structures of NT*Hi* LytM proteins. LytM domains are indicated in black, LysM domains in light grey, trans-membrane regions in dark grey, signal sequences in dashed boxes and coil coiled regions in dotted boxes.
(B) The table summarizes the percentage of identity of NTHi LytM factors with homologues in other bacteria.
(C) Alignments of LytM domains from NTHi and *E. coli* are shown. Metal binding sites motifs are underlined and critical residues are highlighted in grey.

NT013, NT017 and NT022 show a significant homology with a number of previously characterized LytM-proteins expressed by other Gram-negative bacteria (Fig. 8 panel B). In particular, *E. coli* proteins known to be involved in the cell division process such as

YebA, EnvC and NlpD show an amino acid identity of 49% with NT013, 40% with NT017 and 43% with NT022, respectively. LytM catalytic domains are the most conserved regions between NTHi and *E. coli* proteins, in fact, the homology percentage grows up until 79% for this specific domain (alignments are shown in Fig. 8C). We observed that the typical M23 metalloproteases metal binding sites (HxxxD and HxH) are present only in NT013-YebA, whereas in NT022-NlpD only few critical residues are conserved and in NT017-EnvC these motifs are completely absent.

5.2 - LytM proteins are differently distributed on NTHi compartments

The presence of a typical signal peptide in NT013, NT017 and NT022 proteins suggests that they could be exported from the cytoplasm to the membrane. In particular NT022 is characterized by a signal peptide with a lipo-box motif of the type –Leu-X-X-Cys. Bacterial lipoproteins are components of the cell envelope of Gram-negative bacteria and are usually localized at the periplasmic space anchored to either the outer or the inner membrane [38]. NT022 has a serine residue in position +2 after the fatty-acylated cysteine and is therefore predicted to reside in the outer membrane similarly to the *E. coli* NlpD [38-39].

In order to verify their expression and the subcellular localization, single deletion mutants of the genes codifying for the three proteins were generated in Hi176 strain. Immunobotting with specific antisera raised against each of the LytM recombinant proteins was performed to determine the level of expression in periplasmic, outer membrane and supernatant fractions.

As shown in Fig. 9A, NT013 was detected in the outer membrane protein extracts, NT017 in the periplasmic fraction, while NT022 was found in all fractions. As a control, none of the antisera recognized specific bands at 53 kDa, 46 kDa and 42.5 kDa

corresponding to NT013, NT017 and NT022 in cell preparations from the respective knockout mutant strains (Fig. 2A).

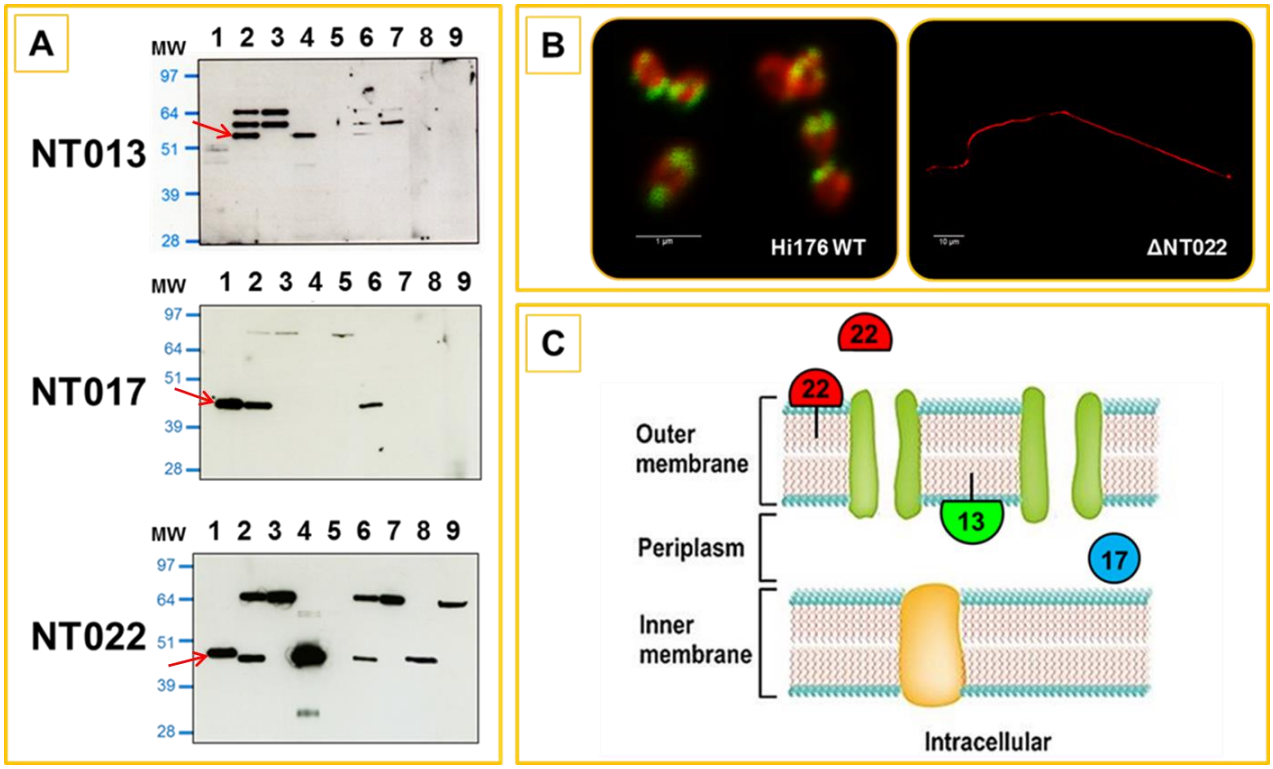


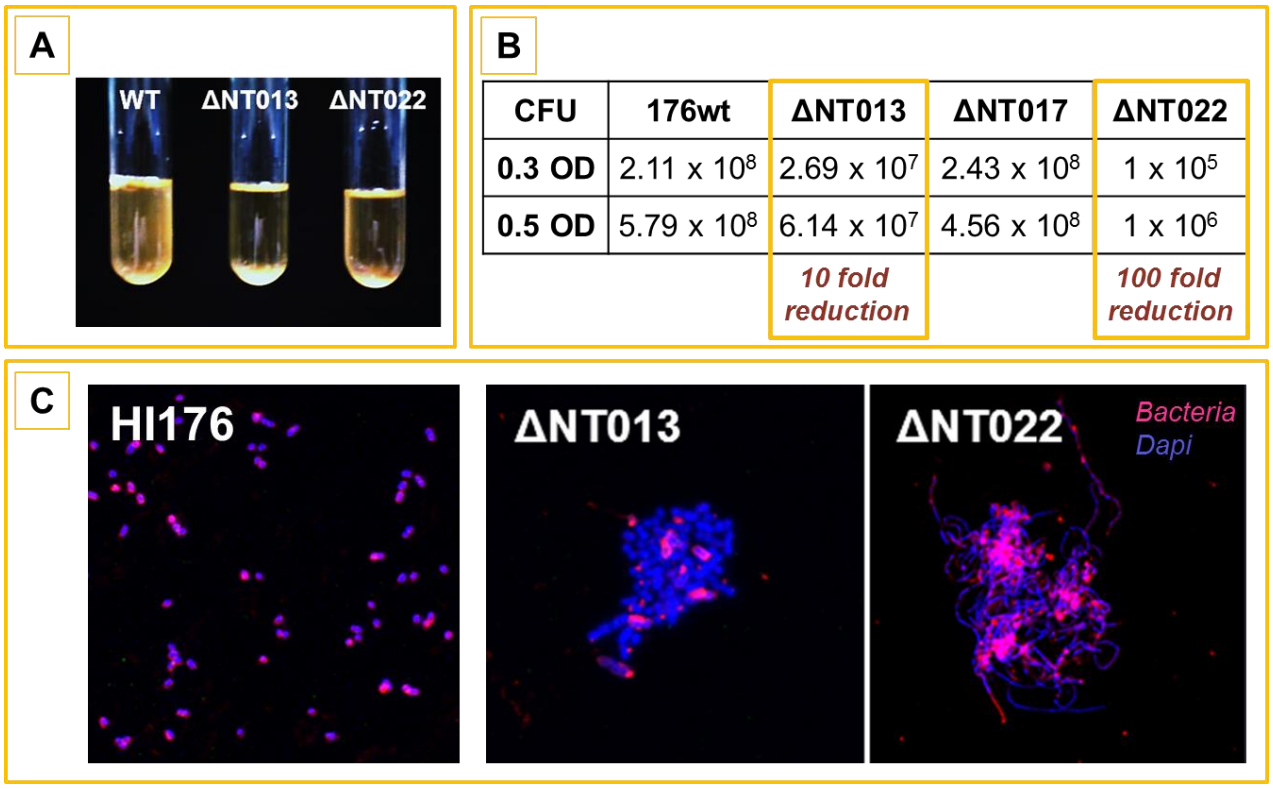
Fig. 9: Expression and subcellular localization of NTHi LytM factors.
(A) Western blot analysis on different cell compartments extracts were performed using specific antisera raised against NT013, NT017 and NT022. 1 - Recombinant protein, 2 - total extract WT, 3 total extract KO, 4 - outer membrane proteins WT, 5 - outer membrane proteins KO, 6 - periplasmic fraction WT, 7 - periplasmic fraction KO, 8 – supernatant, WT 9 – supernatant KO. Red arrows indicate the specific signals. As expected, no specific reactivity is observed with the mutant strains; however the antisera cross-react with other not specific bands present also in the knockout strains which were not characterized.
(B) Immunofluorescence microscopy analysis on Hi176 wild type strain and 176ΔNT022 mutant confirming the surface localization of protein NT022. Bacteria are red and LytM factors in green.
(C) Model of LytM proteins localization in NTHi.

Surprisingly, confocal immunofluorescence (IF) microscopy of bacteria stained for NT013 revealed no specific signal of the protein on the bacterial surface, indicating that NT013 could be associated to the inner layer of the outer membrane as it was found to be present in the outer membrane fraction by western blot analysis (data not shown). As expected, NT017, which was found in the periplasmatic fraction, was negative by confocal microscopy analysis (data not shown) and NT022 was confirmed to be exposed

on the bacterial surface (Fig. 8B). Of interest, it appears that the antigen is translocated on the bacterial surface at specific foci close to the division septum (Fig. 8B).

5.3 - 176ΔNT013 and 176ΔNT022 exhibit aberrant cell morphology and severe cell separation defects

Since EnvC and Nlpd from *E. coli* are known to have a crucial role in cell separation, we investigated whether also NTHi LytM factors could have a similar contribute. Single isogenic mutants cultured on solid or liquid medium and compared to the wild type strain showed no differences in colony morphology as visualized by light microscopy (data not shown).



CFU	176wt	ΔNT013	ΔNT017	ΔNT022
0.3 OD	2.11 x 10^8	2.69 x 10^7	2.43 x 10^8	1 x 10^5
0.5 OD	5.79 x 10^8	6.14 x 10^7	4.56 x 10^8	1 x 10^6
		10 fold reduction		*100 fold reduction*

Fig. 10: Phenotypic characterization of NTHi Hi176 wild type and LytM mutants.
Aggregation phenotype in liquid static cultures growth for 16h at 37°C **(A)** and CFU per milliliter **(B)** CFU counts was performed at two different OD. The 176ΔNT017 strain has a growth rate and a CFU similar to the parent strain, while the 176ΔNT013 and 176ΔNT022 mutants showed a reduced growth rate growth and a lower CFU. **(C)** Confocal imaging showing bacterial aggregation of 176ΔNT013 and 176ΔNT022 strains, bacteria are stained in red and Dapi in blue.

Moreover, to evaluate the effect of the mutations, knockout strains were grown in liquid BHI at 37°C. Interestingly, there are no significant differences in the growth rate of WT and mutants (data not shown), but a phenotype of aggregation was observed in liquid cultures for 176Δ*NT013* and 176*ΔNT022* (Fig 10A), and was confirmed by confocal imaging (Fig. 10C). The number of bacterial colonies was also measured at two different OD by plating cultures serial dilution on agar chocolate plates. Colony forming units (CFU) per milliliter of 176Δ*NT013* and 176Δ*NT022* was much lower than the wild type Hi176 and of 176Δ*NT017*, indeed CFU derived from 176Δ*NT013* and 176Δ*NT022* are respectively only about 10% and 1% with respect to the parent strain (Fig. 10B). This result could be related to bacterial aggregation observed in 176Δ*NT013* and 176Δ*NT022*.

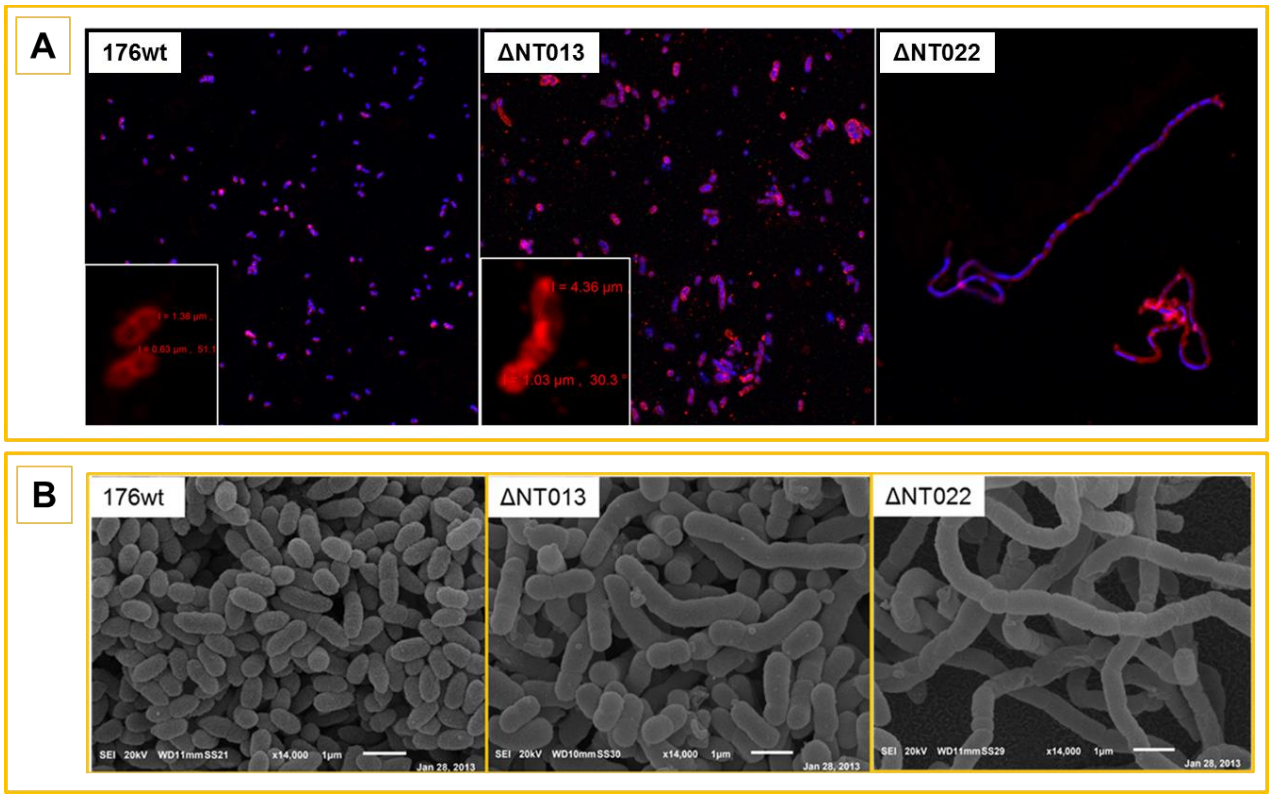


Fig. 11: Confocal and electron microscopy on LytM mutants.
(A) Confocal imaging of Hi176 wt, 176ΔNT013 and 176ΔNT022, bacteria are stained in red (anti total bacterium) and blue (DAPI).
(B) Scanning electron microscopy of 176 wt, 176ΔNT013 and 176ΔNT022. The mutant 176ΔNT017 does not show any difference compared to the wild type strain (data not shown).

To verify whether the bacterial aggregation phenotype was due to a failure in cell separation, we used confocal and scanning electron microscopy which clearly showed that 176*ΔNT013* and 176*ΔNT022* mutants differ from the wild type in dimension and morphology (Fig. 11A and 11B).

In particular, 176*ΔNT013* cells appeared roughly four times longer than the wild type strain and are bended in the central portion. On the other hand, 176*ΔNT022* mutant forms longer chains (up to 0.1 mm), while no evident morphological differences were observed for 176*ΔNT017* (data not shown). The same phenotype was observed when LytM mutants were generated in a different strain (Hi162), indicating the ubiquitous functional properties of such determinants (data not shown).

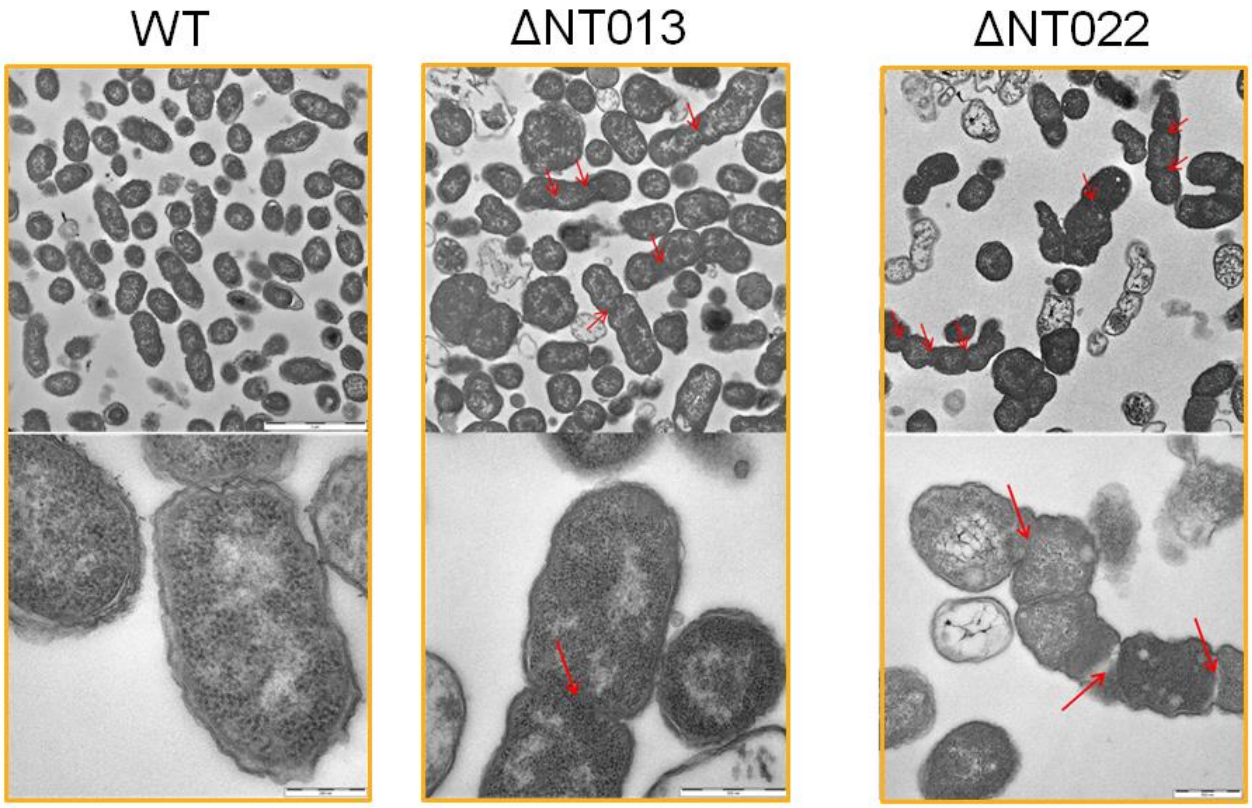


Fig.12: Septum formation in LytM mutants.
Transmission electron microscopy on Hi176 wt and LytM mutants. Red arrows indicate impaired septum formation in mutants 176*ΔNT013* and 176*ΔNT022.*

Transmission electron microscopy analysis confirmed the abnormal filamented cell morphology observed *in vitro* for 176*ΔNT013* and 176*ΔNT022.* Moreover in these mutants there is an aberrant septum formation that could be responsible for the defects in cell splitting (figure 12).

The results described in this paragraph indicate that NT013 and NT022 are involved in bacterial separation, although they are not essential for NTHi cell growth, at least under laboratory conditions.

5.4 - Peptidoglycan cleavage activity of NTHi LytM proteins

LytM proteins in gram-negative bacteria are very well characterized and they are known to be involved in peptidoglycan cleavage during cell division. It is demonstrated that homologues of NT013 are directly involved in peptidoglycan cleavage, for example NG1686 from *N. gonorrhoeae* have two different target sites that are indicated by the red crosses (fig13 A). Homologues of proteins NT017 and NT022 instead activate the amidases that are responsible for peptidoglycan cleavage during cell splitting in *E.coli.*

In order to verify if these functions are conserved in NTHi, several experiments were performed with purified peptidoglycan.

First of all, purified peptidoglycans from 176*ΔNT013* and 176*ΔNT022* knockouts were compared to the WT by NMR analysis. The results (Fig 13B) clearly show that there are no major differences in the overall structure, in the region of aminic protons in fact the spectra are very similar and there are no remarkable shifts as was also reported for NT013 homologues [49-50].

Further investigation on peptidoglycan degradation was carried out setting up a peptidoglycan cleavage assay using recombinant LytM proteins, basically the catalytic activity of protein NT013, NT017 and NT022 was tested on purified peptidoglycan

stained with remazol brilliant blue. Peptidoglycan cleavage is confirmed by the release of blue dye after centrifugation of reaction mixture. Mutanolysin (green crosses) and Proteinase K (yellow crosses) were used as positive controls and their target sites are indicated in fig 13B.

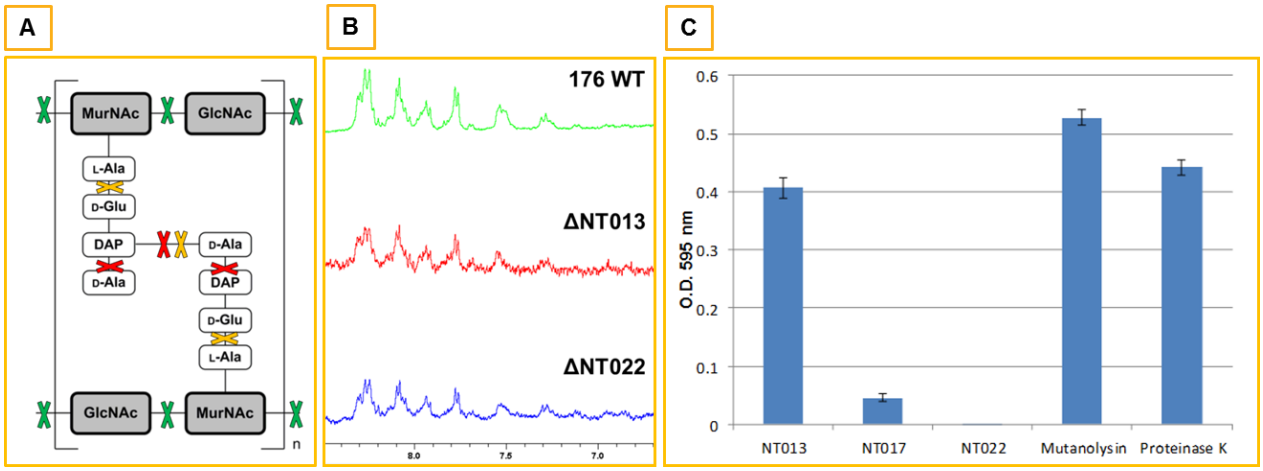


Fig.13: LytM proteins contribution in peptidoglycan cleavage.
(A) Architectural structure of gram negative peptidoglycan. Crosses indicates target sites for proteases and enzymes: Mutanolysin (green), Proteinase K (yellow) and NG1686 (red). **(B)** NMR Spectra of WT and 176*ΔNT013* and 176*ΔNT022*. **(C)** Peptidoglycan Dye release assay. OD of reactions supernatants are measured after O.N. incubation at 37°C.

The assay was performed with different incubation times and concentrations, in fig 13C are reported the results after O.N. incubation at 37°C of 4 μM proteins. It's clearly visible an activity of protein NT013 that is comparable to the positive controls. Not significant results were obtained for the other proteins.

As described in the introduction, in *E.coli,* EnvC and NlpD (respectively NT017 and NT022 homologues) are the specific activators of the amidases [46]. In particular, EnvC was found to specifically activate AmiA and AmiB, while NlpD was found to specifically activate AmiC [46]. Since analyzing the whole NTHi genome we found only one Amidase (AmiB), the capacity of NT017 and NT022 to activate AmiB and to cleave peptidoglycan was tested in the remazol assay using recombinant proteins. To test this directly, we

purified the amidase and assayed its PG hydrolase activity in the presence and absence of NT017 and NT022, but no significant activity was observed (result not shown) suggesting a possible different mechanism of amidase activation in NTHi.

5.5 - 176ΔNT013 and 176ΔNT022 mutants release outer membrane vesicles

Outer Membrane Vesicle (OMVs) formation was shown to be abundant at the site of cell division in species such as Vibrio, Escherichia coli and Brucella melitensis [121,122,123]. Hoekstra et al. reported that the peptigoglycan binding lipoproteins play a considerable role in *E. coli* OMVs production [123] (Fig. 14, Model. 1).

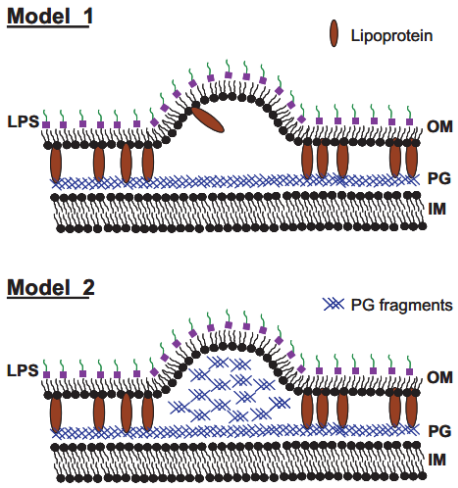


Fig.14: Proposed model for OMVs formation (Mashburn-Warren and Whiteley, 2006).
Model 1: MVs originate from regions of the cell without peptidoglycan-associated lipoproteins resulting from the outer membrane expanding faster than the underlying peptidoglycan layer.
Model 2: Peptidoglycan fragments generated during normal turnover are not efficiently transported back into the bacterial cytoplasm. Turgor pressure resulting from a build-up of peptidoglycan in the periplasm causes blebbing of the outer membrane [124].

They showed that OMVs formation starts with an outward bulging event towards the outer membrane, occuring mostly where there are less peptigoglycan binding lipoproteins, which can cause a weak linkage between the peptigoglycan and the outer membrane of the bacterium.

Therefore, mutants impaired in cell separation could exhibit defects in membrane protein assembly or stability. In order to investigate whether this paradigm is associated also to

NT013 and NT022 proteins, we performed a detailed examination of knockouts bacterial surface by transmission electron microscopy (TEM) and we observed the exclusive formation of blebs on the surface of both 176*ΔNT013* and 176*ΔNT022* bacteria (Fig.15).

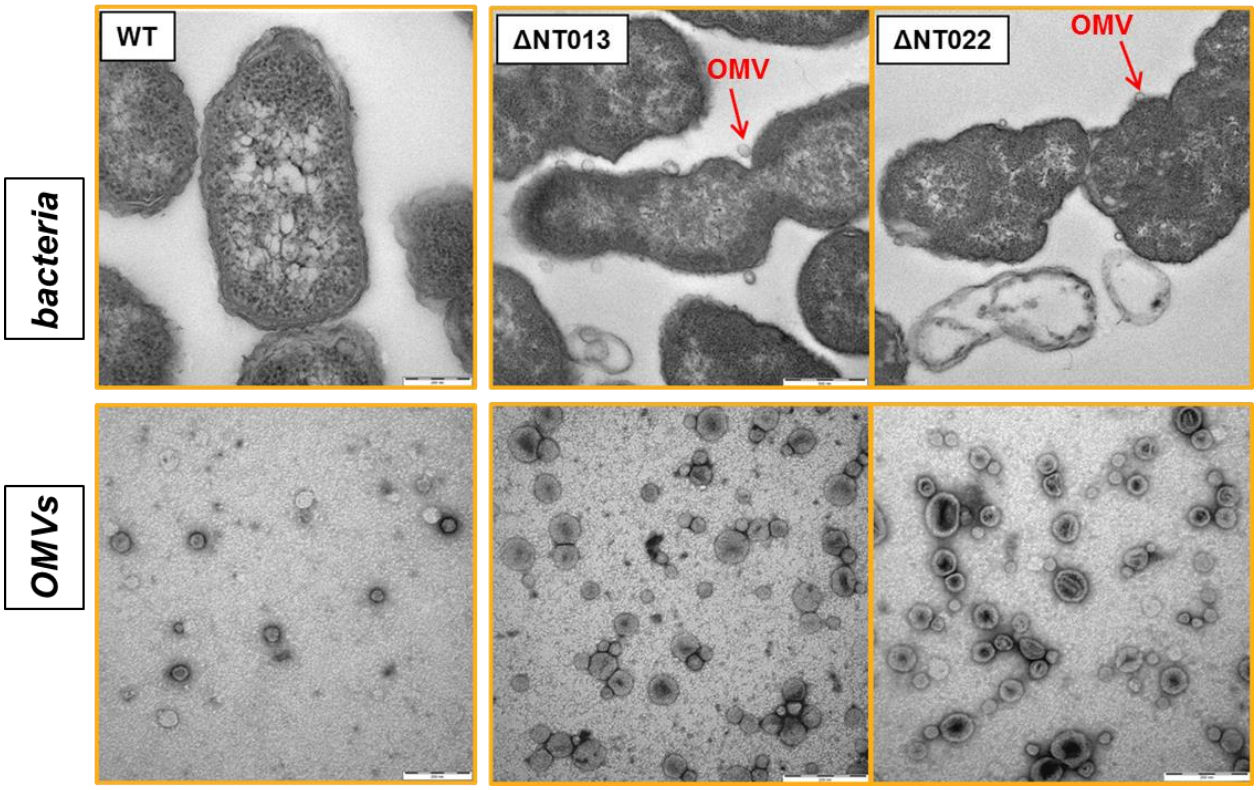
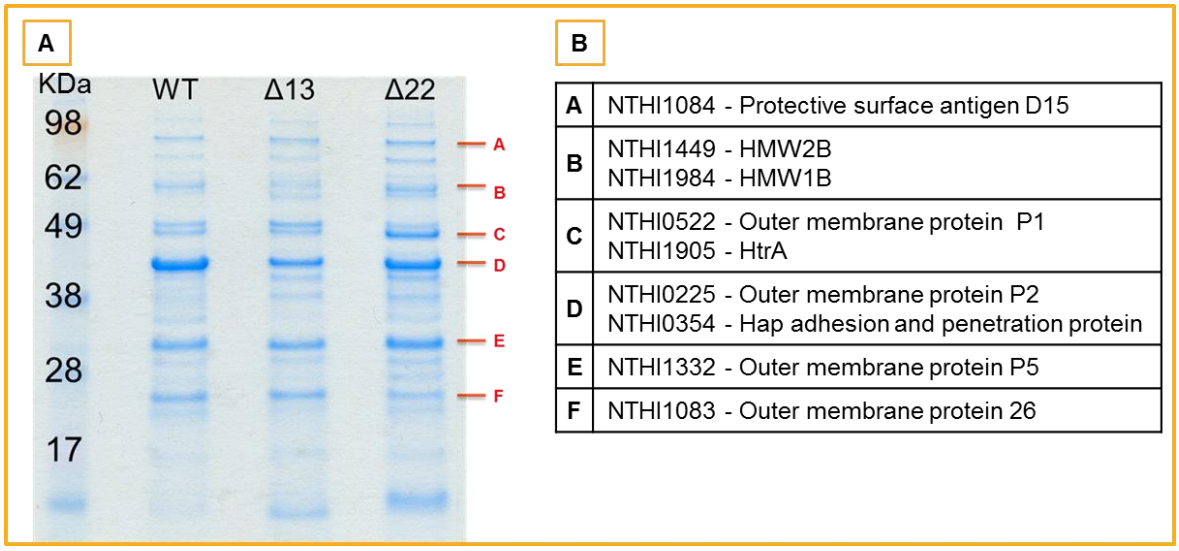


Fig. 15: The mutants 176ΔNT013 and 176ΔNT022 release more OMVs than the wild type strain. Transmission electron microscopy of Hi176WT, 176ΔNT013 and 176ΔNT022 mutants and of their respective OMVs preparations. Red arrows indicate OMVs that are released from bacterial surface.

Membrane blebbing could be due to an overproduction of Outer Membrane Vesicles (OMVs), On the basis of this observation, native OMVs were purified from these two mutants and from the Hi176 strain to verify the quality and to quantify OMVs release. Isolation of OMVs revealed that both mutant strains produce more vesicles with respect to the wild type strain. TEM analysis of OMVs preparations confirmed the presence of vesicles with an apparent diameter of 20 to 100 nm (Fig. 15) and LytM mutants OMVs overproduction was quantified in four fold increase respect than WT (Lowry Method for protein quantitation).

To compare the protein composition of the OMVs extracted from each strain, samples were run on a SDS-page gel and a Coomassie blue staining was performed (Fig.16A). Protein patterns were similar between wild type and mutant strains, although a few bands showed a different intensity. As expected, mass spectrometry analysis associated these bands to a number of known surface determinants, including HMW1 and 2, HtrA, P2, P5 and OMP26 (Fig. 16B).



A	NTHI1084 - Protective surface antigen D15
B	NTHI1449 - HMW2B NTHI1984 - HMW1B
C	NTHI0522 - Outer membrane protein P1 NTHI1905 - HtrA
D	NTHI0225 - Outer membrane protein P2 NTHI0354 - Hap adhesion and penetration protein
E	NTHI1332 - Outer membrane protein P5
F	NTHI1083 - Outer membrane protein 26

Fig. 16: Analysis of OMVs.
Coomassie stained SDS page gel of OMVs prepared from the wild type, 176ΔNT013 and 176ΔNT022 strains **(A).** Mass spectrometry identification was performed on selected bands **(B).**

Immunological studies were performed on OMVs from wild types and mutants to determine if the differences observed in OMVs protein patterns could influence TLRs activation by LPS or lipoprotein components. HEK293-hTLR2 and HEK293-hTLR4/CD14-MD2 cells were stimulated with different dilutions of OMVs from the wild type and knockout strains, but, no significant differences were detected (Fig.17A-B).

Moreover the same stimulation was extended to human Peripheral Blood Mononuclear Cells (hPBMCs) to measure proinflammatory cytokines production (IL-6 and TNFα are shown in figure 17 C and D), but also in this case not significant differences were

observed. Further experiments using quantitative mass spectrometry are still ongoing to understand the biological significance of outer membrane proteins differences.

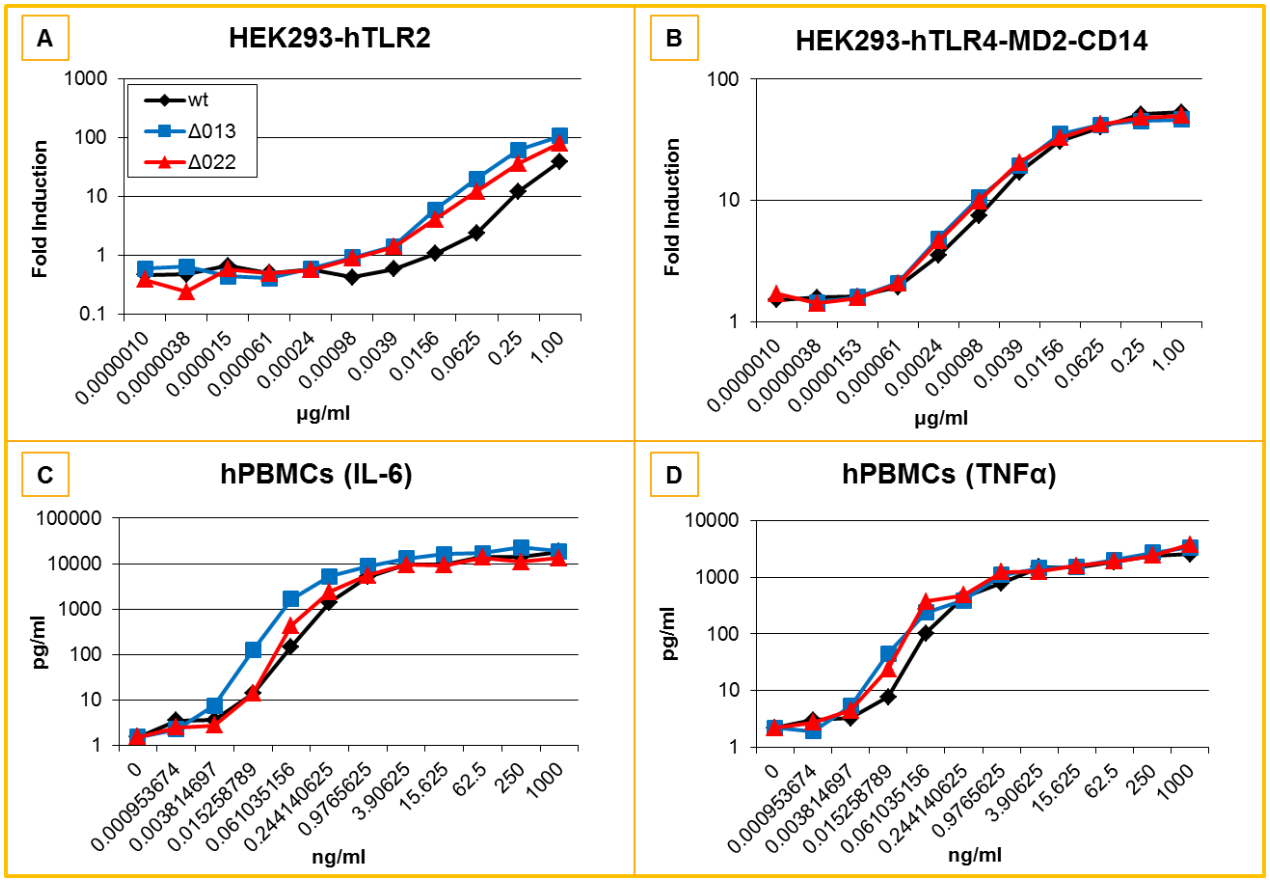


Fig. 17: Reactogenicity assays of OMVs.
Luciferase assay using HEK293 cells stably expressing NF-κB–luciferase reporter cassette and TLR2 **(A)** or TLR4/MD2/CD14 **(B).** The stimulation of TLR receptors is assessed by measuring the NF-κB-induced luciferase activity after 6 hours incubation with serially diluted OMVs.
IL-6 and TNFα levels were measured in hPBMCs stimulated (O.N.) with different dilutions of OMVs purified from wt and mutant strains **(C-D)**.

5.6 - NT017 plays a role in NTHi adherence to epithelial cells

Adhesion to the epithelium is one of the most studied and characterized events associated to NTHi pathogenesis [53, 54, 55, 58, 59, 60]. In order to investigate whether the expression of LytM-containing proteins may affect the interaction of NTHi with host cells, we compared the adhesive phenotype of Hi176 strain to LytM knockout strains.

Briefly, Chang cells monolayers were infected at MOI 100 with the different strains and incubated for 2 h at 37°C. Bacterial adhesion was evaluated using confocal imaging, because of the impracticality to perform accurate CFU counting on plate for 176*ΔNT013* and 176*ΔNT022* strains due to the aggregative phenotype.

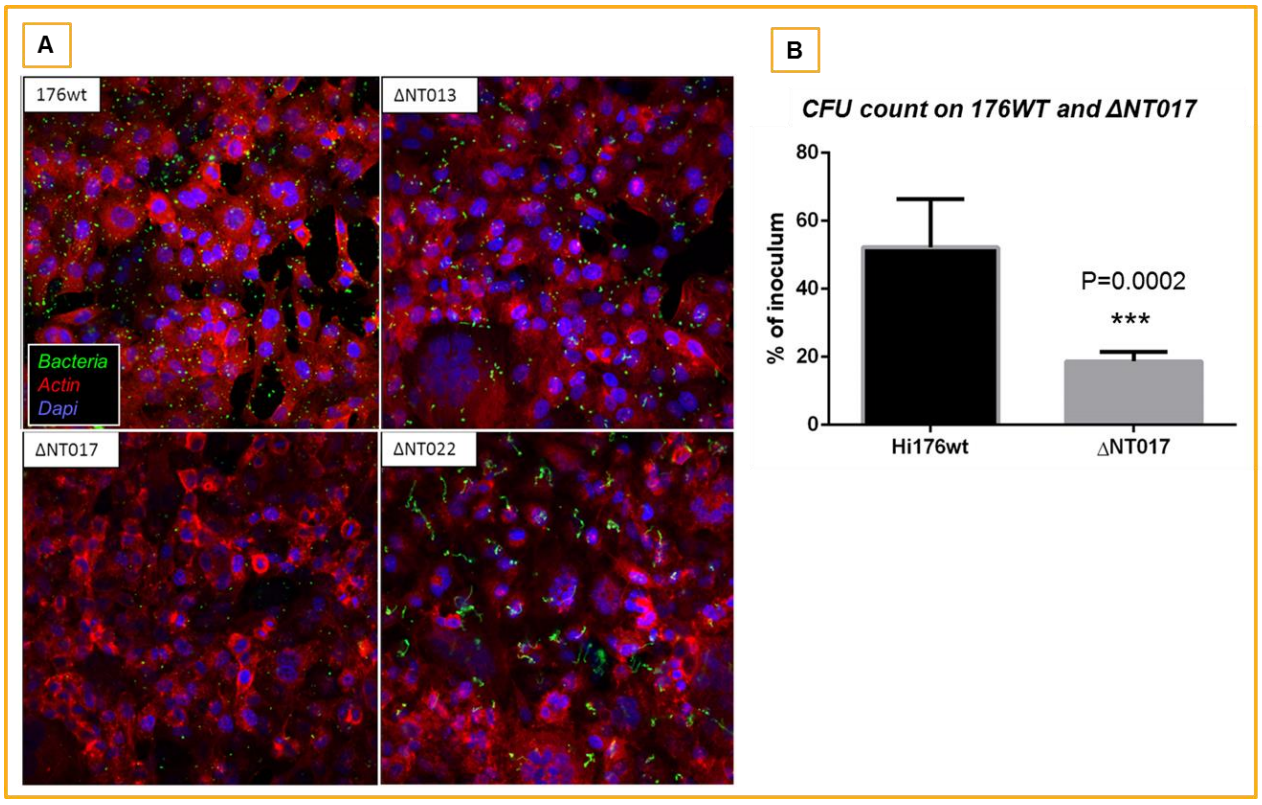


Fig. 18: Adhesion to epithelial cells of NTHi LytM mutants.
(A) Immunofluorescence microscopy showing adhesion to Chang epithelial cells of Hi176 wild type strain and LytM mutants. Chang monolayer was infected with the different NTHi strains for 2 h. Cells are stained in red (actin), blue (DAPI) and green (bacteria). (**B)** Quantification of the level of adhesion of 176ΔNT017 with respect to the wild type strain was calculated using cell-associated colony-forming units (CFU) counting. Adherence was expressed as percentage of adherent bacteria respect to the starting inoculum. Data represent the means and standard deviations of several experiments, each performed in triplicate. ***P < 0.001.

As shown in Fig. 18A, although 176*ΔNT013* and 176*ΔNT022* mutants exhibit a compromised division phenotype they retain similar adhesive capacity with respect to the wild type strain. In contrast, 176*ΔNT017* strain showed a severe reduction in bacterial binding to cells (Fig. 18A). Quantification of 176*ΔNT017* adhesion to Chang cells by CFU

counting confirmed a 3 fold decrease in the number of cell-associated bacteria compared to the Hi176 wild type strain (Fig. 18B). These results suggest a contribution of NT017 in NTHi adhesion to epithelial cells, although whether this is a direct or an indirect effect needs to be further evaluated.

5.7 - Biofilm formation on plastic is reduced in 176ΔNT017 and abolished in 176ΔNT022

Ability of NTHi to form biofilm *in vitro* and *in vivo* has been already reported [75-76-77-78-79-80] and associated to the capacity of bacteria to successfully reside in the middle year. Stemming from the observation that *NT013* and *NT022* LytM mutants showed a defective division phenotype resulting in a propensity to aggregate, we decided to investigate whether their expression may affect the formation of large biomass *in vitro*.

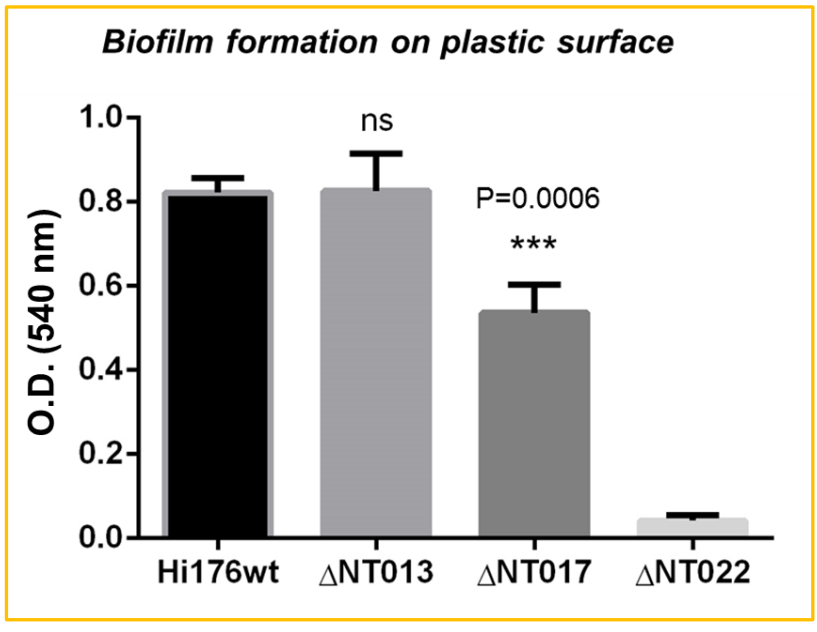


Fig. 19: Biomass formation assay.
Quantification of formed biomass after 24h of static growth by crystal violet staining for NTHi176 wild type strain and LytM mutant strains. Data represent the means and standard deviations of several experiments, each performed in triplicate; ***P < 0.001.

Bacterial biomass was measured by the crystal violet assay in which wild type and single LytM factors knockout mutants were grown on plastic for 24 hours of static incubation. The results reported in Fig. 19 indicate that, while 176ΔNT013 did not show any difference with respect to wild type strain, for 176*ΔNT022* strain no biomass was detected. However, 176ΔNT017 that is phenotypically similar to the wild type strain but has a reduced capacity to adhere to cells, showed a reduction in biomass formation. These results postulate that the capacity of NTHi to form biomass is independent from the bacterial aggregative phenotype, corroborating the ability of NT017 to influence bacterial binding.

5.8 - 176ΔNT017 is not able to survive in presence of Normal Human Serum

Defects in adhesion and biofilm formation suggest an indirect contribution of protein NT017 in NTHi virulence. 176ΔNT017 seems to have defect in colonization, but other steps of pathogenesis could be impaired too. We tested the ability to escape from host defense comparing the survival of WT and mutant in presence of human sera.

Complement mediated bactericidal activity could be evaluated using Normal Human Serum (NHS). Human sera from healthy patients in fact contain antibodies against NTHi (it is usually a commensal) and are able to mediate its killing.

Susceptibility of WT and 176ΔNT017 to human sera was tested in a serum resistance assay and the results are shown in figure 20. 176ΔNT017 is clearly more susceptible to complement mediated killing than WT and this result highlights the contribution of protein NT017 in NTHi pathogenesis.

The mechanism by which there is an increase in the sensitivity of 176ΔNT017 is still under investigation, but it could be related to the way of activation of complement

pathway.

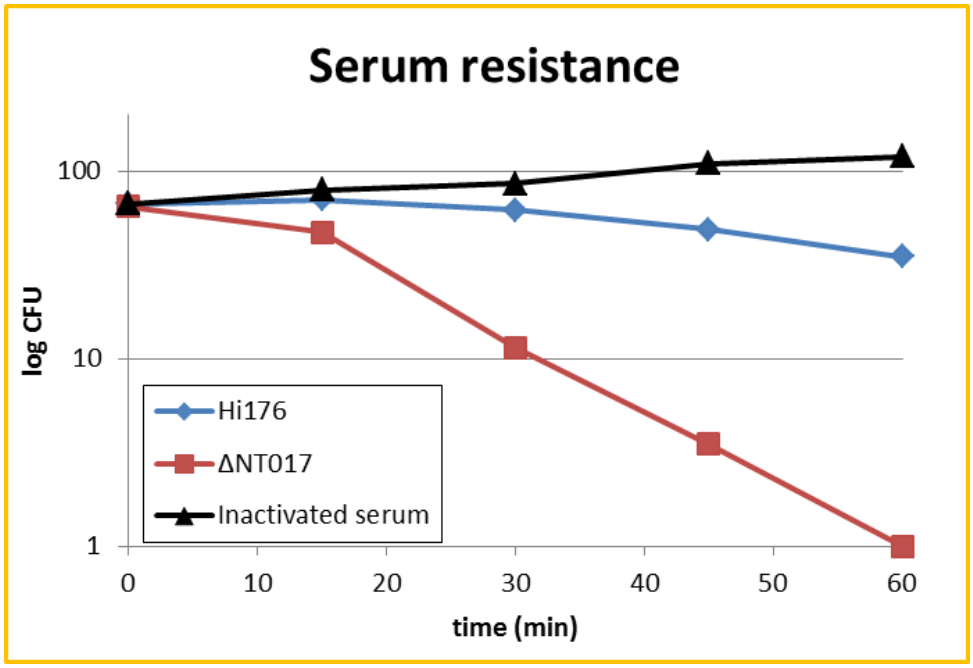


Fig. 20: Serum resistance assay.
Bacteria are incubated with 2% of NHS from healthy patients and number of CFU was evaluated at different time points plating aliquots of the mixture on agar chocolate plates. Heat inactivated serum was used as negative control.

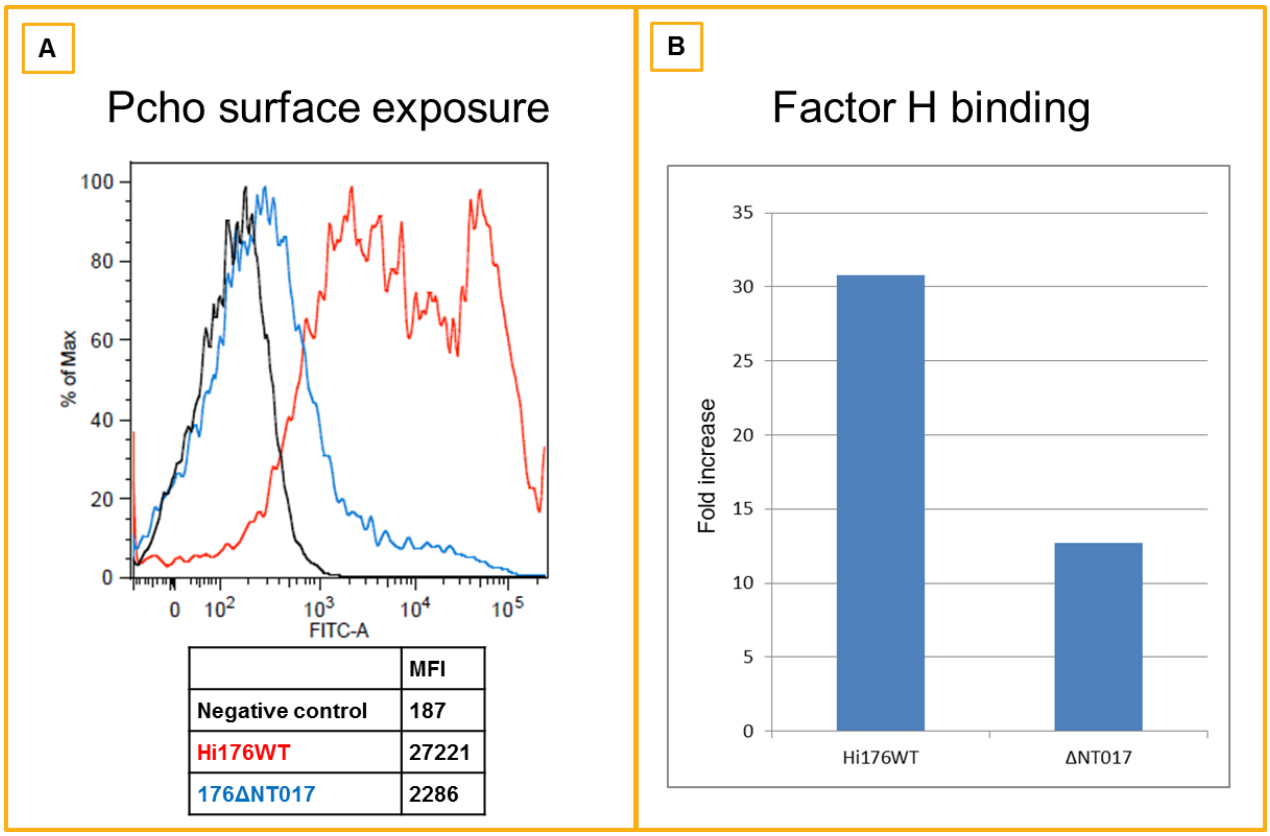
It was already reported that expression of PCho increases the sensitivity of *H. influenzae* isolates to NHS, since human serum contains C-reactive protein, which binds to PCho, activating complement classical pathway and bacterial killing [19].

We tested by FACS analysis Hi176 WT and 176ΔNT017 to verify the presence of PCho on bacterial surface and in contrast of what we expected, we found that 176ΔNT017 does not express phosphorylcoline (Fig. 21A).

This result indicates that there is another way of complement-mediated killing activation in 176ΔNT017 which probably involves factor H (fH) binding.

Factor H is a member of the regulators of the complement activation family and is a complement control protein. Its principal function is to regulate the alternative pathway of the complement system, ensuring that the complement system is directed towards

pathogens or other dangerous material and does not damage host tissue. A common strategy used by some pathogens is the binding of host complement inhibitors such as fH to promote survival in the host.



	MFI
Negative control	187
Hi176WT	27221
176ΔNT017	2286

Fig.21: Complement mediated killing activation.
FACS analysis on Hi176WT and 176ΔNT017 to verify the presence of PCho on bacterial surface **(A)**. Bacteria (WT and KO) were previously incubated with purified FH and analysed by FACS to evaluate factor H binding capability. Untreated bacteria were used as negative control. Results are presented as the fold increase of the Factor H bound bacteria mean fluorescence intensity relative to untreated control samples **(B).**

We tested factor H binding capability of WT and ko strains and the results are shown in Fig. 21 B. As we can observe, in 176ΔNT017 there is a decrease in the capability of the bacteria to bind fH, which could be one of the explanation of its increased susceptibility to human sera.

In conclusion, the lack of protein NT017 could influence significantly bacterial surface structures and the knockout mutant 176ΔNT017 could have modifications that impair its

ability to evade the host defense. Future studies will be addressed on characterization of outer membrane proteins and LPS of 176ΔNT017, to elucidate what are the components that influence the decrease of viability in presence of NHS.

6 – Discussion

The importance of LytM factors for bacterial physiology was already reported and their role in cell division was investigated in detail in *E. coli* [13,46,47]. In this study LytM proteins from Non typeable *H.influenzae* were identified and characterized for the first time and many new remarkable findings were carried out.

Deletions of single LytM genes in NTHi generate interesting phenotypes, mainly affecting cell morphology. In particular 176*ΔNT013* and 176*ΔNT022* show bacterial aggregation in liquid culture and aberrant cell splitting, confirming the role of these proteins in cell division machinery. The contribute of protein NT022 is very clear, in fact the respective knockout is able to duplicate but not to divide; NT013 knockout instead has a milder phenotype, the bacteria are able to split but their dimension and morphology are compromised. NT013 is probably still involved in the process, but its function could be more related to peptidoglycan biogenesis and rearrangement, as hypothesized for *E.coli* homologous YebA [13]. Protein NT017 seems to be not involved in NTHi cell division and this is a very surprising finding due to the essential role of the *E.coli* homologous EnvC in amidase activation.

Futher analysis of NTHi peptidoglycan was performed to evaluate the role of LytM factors in peptidoglycan remodeling during cell division. Comparison by NMR between purified peptidoglycans from WT and knockouts shows no major differences in amino acidic chains structure. This result correlates with previous studies on LytM mutants in other bacteria, in fact, similarity in peptidoglycan overall structure of WT and respective knockouts is probably due to spatial regulation of LytM proteins activation during cell cycle or to a redundancy of their functions [49-50].

Peptidoglycan cleavage assay confirms metalloprotease catalytic activity for protein NT013, it is able to cleave directly NTHi peptidoglycan, meanwhile no direct activity was assessed for NT017 and NT022. EnvC and NlpD (respectively NT017 and NT022 homologues) as reported before, are the regulators of amidases *E.coli* (AmiA, AmiB and AmiC). Since in *H.influenzae* genome is present only one Amidase (annotated as AmiB) and only 176*ΔNT022* has defects in cell division, we hypothesize an interaction between NT022 and AmiB (as observed in *E.coli*) and a loss of the activity of protein NT017 during evolution.

We found that the mutant 176*ΔNT013* and 176*ΔNT022* release more outer membrane vesicles with respect to the wild type strain. It is reasonable to think that this phenomenon is associated to the impaired cell division caused by the aberrant septum formation and defects in peptidoglycan rearrangement observed in these mutant strains. The correlation between LytM proteins and OMVs major release has not been highlighted before and our results suggest an involvement of LytM factors in bacterial membrane stability. OMVs purified from LytM knockouts are similar to the WT ones in terms of protein composition and reactogenicity. Only small differences came out in the amount of some proteins, this is probably due to the lack of each deleted LytM factor and it suggests a contribution of LytM proteins in other mechanisms out of cell splitting.

The localization of LytM proteins in the peripheral side of the bacterial cell and the indications that suggest the involvement of these factors in other functions in NTHi, prompted us to investigate on a possible role in pathogenesis. While NT013 seems to have no role in any of the aspects of pathogenesis that we have investigated, NT017 and NT022 are found to be related to some interesting phenotypes.

First of all, strain 176*ΔNT017* resulted to be affected both in *in vitro* adhesion to ephitelial cells and in serum resistance. Since NT017 is localized in the periplasm, these data suggest an indirect role of protein NT017 in NTHi pathogenesis. It is possible that the

lack of NT017 could affect the presence, the localization or the folding of specific NTHi surface determinants. Data on OMVs analysis indicate that there are several differences in proteins amount in the surface of NT017 mutant, moreover preliminary data shows that 176*ΔNT017* lacks of phosphorylcholine decoration (Fig. 21A), a component of LOS that is important for NTHi pathogenesis and that is fundamental for several process like: colonization [91], persistence [108, 109, 125, 126], resistance to antibiotics [63], evasion of the immune response and for the utilization of host receptors [127, 128].

The ability of *H. influenzae* to vary PCho expression, correlate with its ability both to persist on the mucosal surface (PCho+ phenotype) and to cause invasive infection by evading innate immunity mediated by CRP (PCho− phenotype). PCho- phenotype in *176ΔNT017* correlates with its attenuated adhesive capacity (Fig18-19), but it is in contrast with the increased susceptibility of the mutant to complement mediated killing (Fig.20).

Increased serum sensitivity in *176ΔNT017* could be probably related to the presence of defective surface structures that impair evasion from host defense, facilitating other ways of complement activation (e.g. alternative pathway activation mediated by factor H). Results shown in Fig. 21B demonstrated that factor H binding is compromised in the mutant and this finding could explain the lack of serum resistance in this strain.

The defective surface of 176*ΔNT017* mutant suggest us a possible activity of this protein in the LOS biogenesis or in promoting the correct folding and the exposure of outer membrane proteins.

For what concern NT022 instead, it has a strong contribute in biofilm formation; this protein is surface exposed and released in the supernatant and it is necessary for the bacterial community establishment. 176*ΔNT022* in fact is not able to form biofilm on plastic, probably not for the lack of NT022 activity, but for the aberrant morphology of knockout mutant itself. Antisera raised against NT022 will be used to evaluate if the block

of NT022 activity could result in a decrease of the formation or in the eradication of adherent biomass.

NT022 surface localization and its fundamental role for biofilm establishment highlight the importance of this protein in NTHi pathogenesis, NT022 is required for bacterial physiology and the block of its activity could be one of the strategies to eradicate NTHi infections. Moreover NT022 has a strong conservation among different strains and this is one of the key elements to consider this protein as a potential candidate for the development of a vaccine against NTHi. Further investigations on protective efficacy of this antigen and on strain coverage are needed to evaluate if NT022 could be the right target for a protein based vaccine against NTHi.

In conclusion, in this study we highlighted the physiological importance of LytM proteins in NTHi, confirming their role in cell division and in bacterial membrane stability (OMVs major release). Moreover, their contribution in pathogenesis is also underlined, NT017 in fact is fundamental for *in vitro* colonization and evasion from host defense.

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