Antimicrobial susceptibility and serum resistance in Haemophilus influenzae

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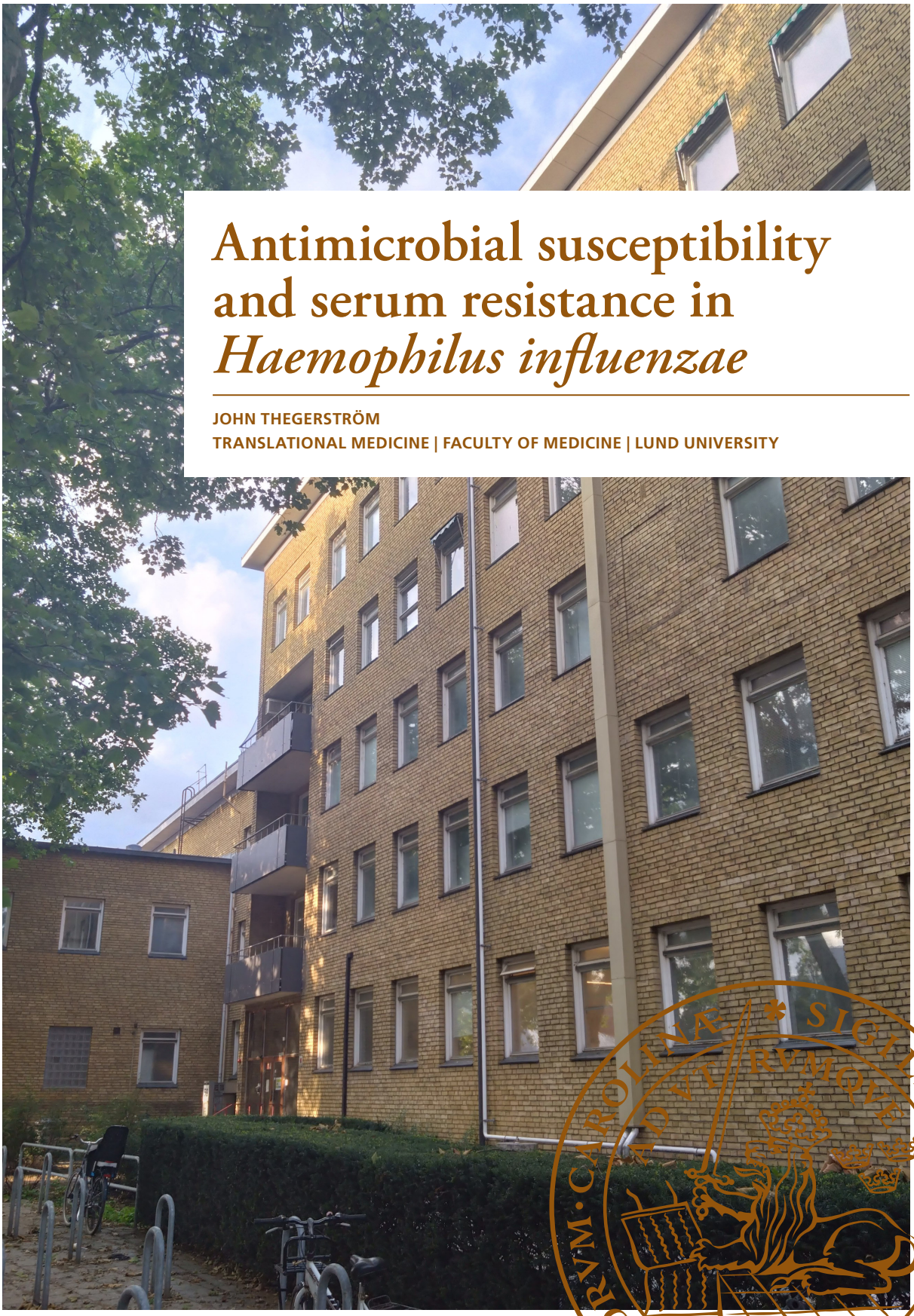

Antimicrobial susceptibility and serum resistance in *Haemophilus influenzae*

JOHN THEGERSTRÖM
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JOHN THEGERSTRÖM works as an infectious disease specialist at Skåne University Hospital in Malmö. His thesis includes studies on different aspects of antimicrobial susceptibility and immune escape in Haemophilus influenzae.

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Antimicrobial susceptibility and serum resistance in *Haemophilus influenzae*

John Thegerström

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Author: John Thegerström

Title and subtitle: Antimicrobial susceptibility and serum resistance in *Haemophilus influenzae*

Abstract:

What mechanisms lie behind antimicrobial resistance development in *Haemophilus influenzae*? What are their consequences for antibiotic treatment of this bacterial species? And how does *H. influenzae* evade the complement mediated killing of the human immune defense?

Haemophilus influenzae is a gram-negative bacterium that may be both a colonizer and a pathogen of the human respiratory tract. Aminopenicillins have historically been one of the preferred treatment options against infections caused by this bacterium. An increasing number of resistant isolates makes this regime less reliable. Non-beta-lactamase mediated resistance associated with alterations in penicillin binding protein 3 (PBP3) has emerged over the last decades and is of particular concern.

The effects of a recently identified amino acid substitution (Y528H) in PBP3 were investigated in Paper I. Isogenic mutants of a susceptible strain displaying this substitution became screening positive for beta-lactam resistance and increased their minimal inhibitory concentration (MIC) to aminopenicillins by one dilution-step.

In Paper II, a set of beta-lactamase negative *H. influenzae* isolates with similar genetic background and identical PBP3 sequences but variable MICs to aminopenicillins was investigated with whole genome sequencing. Amino acid substitutions in PBP2 associated with a higher MIC were identified. Isogenic mutants displaying these polymorphisms changed their MIC towards aminopenicillins with one-dilution-step.

In Paper III, no significantly detrimental effect on 30-day mortality could be seen when retrospectively comparing benzylpenicillin and beta-lactam antibiotics with a wider antibacterial spectrum as empirical treatment of lower respiratory tract infections eventually shown to be caused by *H. influenzae*.

Finally, in Paper IV, outer membrane protein 5 (P5) was defined as a ligand for human C4b-binding protein (C4BP) in nontypeable *H. influenzae* (NTHi). The P5-mediated recruitment of C4BP to the bacterial surface conferred increased resistance to serum.

In conclusion, additional factors such as polymorphisms in PBP2 may modulate aminopenicillin susceptibility levels in *H. influenzae* isolates with alterations in PBP3. Benzylpenicillin likely remains an appropriate empirical treatment option for mild to moderate community acquired pneumonia, including cases caused by *H. influenzae*. P5 is a ligand for human C4BP which significantly contributes to virulence.

Key words: *Haemophilus influenzae*, antimicrobial susceptibility, BLNAR, C4BP, NTHi, virulence

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Jag känner cellen, urdjuret inom mig; efter miljoner och åter miljoner år har jag återfunnit mig själv i en människa.

(Gunnar Ekelöf, Skärvor av en diktsamling, 1927)

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Paper I

Thegerström J, Matuschek E, Su YC, Riesbeck K, Resman F. A novel PBP3 substitution in *Haemophilus influenzae* confers reduced aminopenicillin susceptibility. BMC Microbiol. 2018 May 31;18(1):48.

Paper II

Thegerström J*, Su YC*, Venkatesh Rao V, Hoh CC, Khoo JS, Matuschek E, Engstrand L, Resman F, Riesbeck K. Novel resistance mechanism in beta-lactamase negative non-typeable *Haemophilus influenzae* with identical penicillin binding protein 3 but varying susceptibility to aminopenicillins. *Manuscript.*

* Contributed equally to this work.

Paper III

Thegerström J, Månsson V, Riesbeck K, Resman F. Benzylpenicillin versus wide-spectrum beta-lactam antibiotics as empirical treatment of *Haemophilus influenzae*-associated lower respiratory tract infections in adults; a retrospective propensity score-matched study. Eur J Clin Microbiol Infect Dis. 2018 Sep;37(9):1761-1775.

Paper IV

Thofte O, Bettoni S, Su YC, **Thegerström J**, Jonsson S, Mattsson E, Sandblad L, Martí S, Garmendia J, Blom AM, Riesbeck K. Nontypeable *Haemophilus influenzae* P5 Binds Human C4b-Binding Protein, Promoting Serum Resistance. J Immunol. 2021 Sep 15;207(6):1566-1577.

List of papers not included in this thesis

Resman F, **Thegerstrom J**, Mansson F, Ahl J, Tham J, Riesbeck K. The prevalence, population structure and screening test specificity of penicillin-susceptible *Staphylococcus aureus* bacteremia isolates in Malmo, Sweden. The Journal of infection. 2016;73(2):129-35.

Udden F, Filipe M, Reimer A, Paul M, Matuschek E, **Thegerstrom J**, Hammerschmidt S, Pelkonen T, Riesbeck K. Aerobic bacteria associated with chronic suppurative otitis media in Angola. Infectious diseases of poverty. 2018;7(1):42.

Su YC, Jalalvand F, **Thegerstrom J**, Riesbeck K. The Interplay Between Immune Response and Bacterial Infection in COPD: Focus Upon Non-typeable *Haemophilus influenzae*. Frontiers in immunology. 2018;9:2530.

Abbreviations

AECOPD	Acute exacerbation of chronic obstructive pulmonary disease
AOM	Acute otitis media
AST	Antimicrobial susceptibility testing
BHI	Brain heart infusion medium
bla	Beta-lactamase
BLNAR	Beta-lactamase negative, ampicillin resistant
BLNAS	Beta-lactamase negative, ampicillin susceptible
BLPACR	Beta-lactamase positive, amoxicillin clavulanate resistant
BLPAR	Beta-lactamase positive, ampicillin resistant
BMD	Broth microdilution
C4BP	C4b-binding protein
CAP	Community acquired pneumonia
cat	Chloramphenicol acetyltransferase
CCI	Charlson/Deyo comorbidity index
CI	Confidence interval
CLSI	Clinical Laboratory Standard Institute
COPD	Chronic obstructive pulmonary disease
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
ECOFF	Epidemiological cut-off
EF-Tu	Elongation factor thermo-unstable
Etest	Epsilometer test

EUCAST	European Committee on Antimicrobial Susceptibility Testing
FH	Factor H
fT > MIC	The amount of time in which free or non-protein-bound antimicrobial concentration exceeds the minimum inhibitory concentration
Fur	Ferric uptake regulators
gBLNAR	genomic beta-lactamase negative, ampicillin resistant
GlcNAc	N-acetylglucosamine
Hib	*Haemophilus influenzae* capsular type b
HGT	Horizontal gene transfer
HMW	High molecular weight protein (as in HMW1 and HMW2 adhesins or HMW penicillin binding proteins)
HTM	*Haemophilus* test medium
ICAM-1	Intracellular adhesion molecule 1
ICE	Integrative conjugative element
ICU	Intensive care unit
IRT	Inhibitor resistant TEM beta-lactamase
LDH	l-lactate dehydrogenase
LMW	Low molecular weight protein
LOS	Lipooligosaccharide
LRTI	Lower respiratory tract infection
MAC	Membrane attack complex
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MDR	Multi drug resistant
MH-F	Mueller Hinton fastidious media
MIC	Minimum inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MurNAc	N-acetylmuramic acid

NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NHS	Normal human serum
NordicAST	Nordic Committee on Antimicrobial Susceptibility Testing
NTHi	Nontypeable *Haemophilus influenzae*
OMP2	Outer membrane protein 2
OMV	Outer membrane vesicle
OR	Odds ratio
P5	Outer membrane protein P5
PBP	Penicillin binding protein (e.g. PBP1A, PBP1B, PBP2, PBP3 etc)
PcG	Penicillin G, benzylpenicillin
PcV	Penicillin V, phenoxymethylpenicillin
PCV	Pneumococcal conjugate vaccine
PD	Protein D
PE	Protein E
PF	Protein F
PK/PD	Pharmacokinetic/pharmacodynamic
PRP	Polyribosylribitol phosphate
QC	Quality control
rPBP3	Resistance mediated by amino acid substitutions in PBP3
SNP	Single nucleotide polymorphism
$t_{50\%}$	Time to 50% killing
t.i.d.	ter in die, three times a day (dosage)
USS	Uptake signal sequence
WGS	Whole genome sequencing
WSBL	Wide-spectrum beta-lactam
XDR	Extensively drug resistant

Introduction

In 1889, a new influenza pandemic was sweeping through the world. People called it the Russian flu, the first major outbreak had been in St. Petersburg (1). Thousands and thousands of people perished. Its origin was probably a novel variant of the influenza virus, possibly H3N8 (2). However, prospects to determine its cause there and then, in the late 19th century, were limited. Unlike in our most recent pandemic, there were no RespiFinderSmart22kits, no LightCycler 480 real-time PCR systems, no Transmission Electron Microscopy, to aid in the isolation and characterization of the infectious agent (3). There was not even a concept of viruses. Instead, it was the golden age of bacteriology. National heroes like Louis Pasteur and Robert Koch had a decade earlier revolutionized the view on what caused infectious diseases, from miasmas to microbes.

Inspired by their progress, many scientists therefore sought the etiological agent of influenza by looking for new bacteria. A couple of years into the pandemic, a disciple of Koch's, Richard Pfeiffer, examined respiratory secretions of affected victims under his microscope. He observed that all his 31 specimens from patients with symptoms of influenza, pneumonia and bronchial catarrh displayed large quantities of very tiny, gram-negative rodlets, whereas none of the 'numerous' controls did. Inoculation experiments showed that they caused disease in apes and rabbits, strengthening his theory that the isolated bacteria were the primal cause of influenza. He published his results in 1892 and initially won general acclaim (4). The bacterium was named *Bacillus influenzae* (5).

In the following decades, Pfeiffer would be proven wrong. It was shown that specimens from patients with influenza could transmit disease even though they had been cleared from bacteria (6). Eventually, the influenza virus was discovered as the causative agent (7). However, to this day, the observed bacterium retains the last part of its name, we now know it as *Haemophilus influenzae* (8).

Etymologically, *Haemophilus* is derived from Greek and means blood-loving, due to the organism's requirement for blood for adequate growth (1). The word influenza originates from 15th century Italy where an epidemic was attributed to the 'influence of the stars' (9). It has been used in the English language since the 18th century to describe certain respiratory tract infections (10). *Haemophilus influenzae* would thus be 'the blood loving bacterium influenced by the stars'.

Scientists generally seek to deconstruct astral influences into mechanistic explanations. Perhaps this is a reason as to why the strain of *H. influenzae* Rd KW20 would become the first free-living organism ever to have its entire genome

sequenced, approximately a century after the first description of this species (11). Indeed, substantial knowledge about *H. influenzae* has been generated over time. Some of it is summarized in the following pages.

Although not the cause of viral flu disease, *H. influenzae* is nevertheless a significant human pathogen. Many small children have suffered and died from meningitis, epiglottitis and septicaemia by this bacterium over the years. Attempts to manage these severe infections were initially made with serum, which had been efficient in the treatment of meningococcal meningitis (12). However, it eventually proved futile when used against *H. influenzae* (13). In 1928, Alexander Fleming discovered a new substance with the ability to inhibit the growth of several bacterial species. This 'mould-juice', as he would initially call it before settling for the less disgusting name of penicillin, did however not seem to affect the growth of *H. influenzae*. When publishing his results, the author chose not to highlight their colossal therapeutic potential in the title. Instead, one of medical history's most famous manuscripts would bear the title: '*On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to Their Use in the Isolation of* B. influenzae' (14). As to this date, there is nevertheless an ongoing discussion on the therapeutic effects of benzylpenicillin on infections caused by *H. influenzae* at higher concentrations. A very minor contribution to this debate is included in the present thesis. However, shortly after Flemings discovery, new antimicrobials, notably including the penicillin derivative ampicillin, were developed and administered for more effective treatment (15).

A different approach in combatting *H. influenzae* was undertaken in 1931 by American bacteriologist Margaret Pittman. She was the first to describe that encapsulated strains are the primary cause of severe invasive infections (16). Her work would more than 50 years later result in a vaccine against *H. influenzae* capsular type b and the implementation of a child immunization program in vast parts of the world, with a subsequent dramatic decline in severe infections in children. Since then many lives have been saved (17).

Despite these therapeutic and prophylactic progresses, several challenges still remain. After the introduction of the conjugate capsular vaccine, non-typeable *H. influenzae* remains an important pathogen, with the ability to escape both natural and induced immunity. It gives rise to an increasing number of invasive infections, mainly in the elderly and neonates (18). Since the 1970s, a steadily increasing number of isolates displaying antimicrobial resistance has been reported. Particularly reduced susceptibility to aminopenicillins presents a predicament, with new mechanisms of resistance emerging (19). This has led to the inclusion of ampicillin-resistant *H. influenzae* onto the 2017 WHO priority list of antibiotic resistant bacteria (20). In recent years, reports on multi-drug resistant strains have emerged (21, 22).

In 2023, in the years immediately after a new viral pandemic, this bacterial pathogen still poses intriguing questions to the scientific community, which

continuously seeks to decipher the secrets of ‘the blood loving bacterium influenced by the stars’.

Figure 1. The frontpage of a Parisian newspaper during the outbreak of the Russian flu in 1890. Bottom left panel features an illustration of two musicians performing the top hit song at the time: ‘L’influenza, tout l’monde l’a’ (‘Everybody’s got the flu’). Image from US National Library of Medicine, public domain.

Microbiological characteristics

Morphology, taxonomy, growth requirements and species identification

Haemophilus influenzae is a fastidious, facultatively anaerobic, gram-negative, pleomorphic rod-shaped coccobacillus (figure 2) (23). It belongs to the genus *Haemophilus* of the *Pasteurellacae* family (24). The genus includes nine species with host specificity for humans which in turn may be assembled into three related groups based on certain phenotypical traits; i) the *H. influenzae* group comprising *H. influenzae, H. aegyptius* and *H. haemolyticus*, ii) the *H. parainfluenzae* group comprising *H. parainfluenzae, H. parahaemolyticus, H. paraphrohaemolyticus, H. pittmaniae* and *H. sputorum* and finally iii) *H. ducreyi* which is more distantly related to the other species (24, 25).

H. influenzae is the type species and primary pathogen of the genus (26). Its clinical characteristics are described in greater detail as follows. *H. haemolyticus* is typically found as a commensal whereas *H. aegyptius* has been described as a frequent cause of conjunctivitis in hot climates (27). Virulent clones have also caused outbreaks of Brazilian purpuric fever, a condition characterized by purpura fulminans in combination with conjunctivitis (28). As for the species found in the *H. parainfluenzae* group, they are typically commensals with the important exception that *H. parainfluenzae* is the major species in the *Haemophilus* genus responsible for infective endocarditis (29). Finally, the more distantly related *H. ducreyi* is the pathogen causing the sexually transmitted disease chancroid (soft chancre or ulcus molle) (30).

The species in the *H. influenzae* group are characterized by their requirement of both factors X and V for growth (31). Factor X (haemin) is a crucial intermediate in the synthesis of respiratory cytochromes in bacteria (32). Respiratory cytochromes are not formed under anaerobic conditions and factor X dependency thus reduces significantly in *H. influenzae* during anaerobic growth (23). Factor V, which is a co-enzyme, can be supplied from nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). It is present in erythrocytes but must be released from the cells for optimal growth, making blood agar an insufficient medium (23). Instead, heat treated blood media, ‘chocolate agar’, is routinely used for cultivation (figure 2). Some Staphylococcal species produce and excrete NAD, giving rise to a ‘satellite phenomenon’ where colonies of *H. influenzae* may grow in proximity to staphylococcal colonies on V-factor deficient media. Individual discs with X and V factors may also be used for phenotypical identification of isolates belonging to the *H. influenzae* group and to discern them from isolates of the *H. parainfluenzae* group which typically only require V-factor (33, 34). Other criteria that traditionally help to distinguish one *Haemophilus*

species from another include the ability to lyse horse blood, the presence of catalase, and the pattern of sugar fermentation (26).

In modern clinical microbiology, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is commonly used for species identification. Briefly, in this analytical technique bacterial particles, mainly ribosomal proteins, are ionized and separated by their mass-to-charge ratio, and measured by the time it takes for the ions to travel to a detector at the end of a time-of-flight-tube in an electrostatic field (35). Since a given species has a unique set of protein content, the generated spectrum, often referred to as the mass fingerprint of the analyzed bacterial isolate, can be compared to a database with spectra from known isolates and type strains for species determination (36). The technique has been shown to reliably discriminate between *H. influenzae, H. haemolyticus* and *H. parainfluenzae* (37, 38). Other modern but more laborious techniques for accurate species determination include molecular methods such as sequencing of housekeeping or 16S-rRNA genes and whole genome sequencing (WGS) (24, 39)

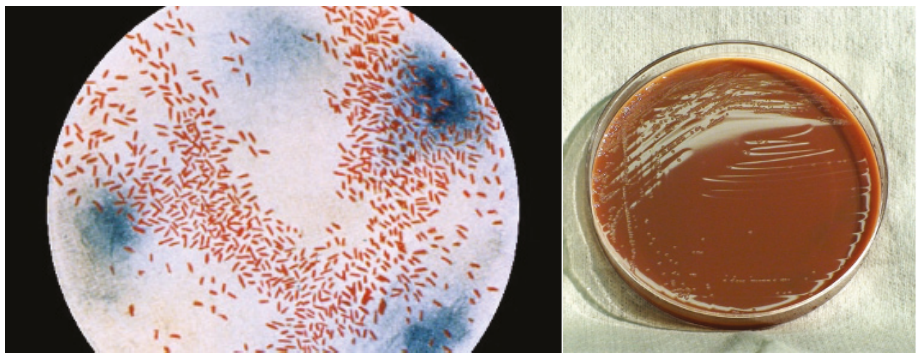


Figure 2. Morphology of *H. influenzae.*
Left panel: Gram stain of *H. influenzae* bacterial cells seen in light microscopy. Right panel: Colony morphology of *H. influenzae* cells when grown on chocolate agar. Images from public health image library, CDC. Public domain.

Biotyping and serotyping

H. influenzae can be subdivided into 8 biotypes based on 3 biochemical reactions, namely the presence of urease and ornithine decarboxylase and the production of indole (40). Biotype III also includes *H. aegyptius* and a clone of biotype IV has been associated with neonatal and post-partum infections (23, 41).

Subdivision may also be based on the presence and type of polysaccharide capsule. There are 6 types of encapsulated *H. influenzae* (denoted a-f) which can be identified using specific antisera (26). Repeating units of one of 6 different

disaccharides make up the bacterial capsule (42). The majority of isolates do not possess a capsule and can thus not be serotyped (23). They are designated as non-typeable *H. influenzae* (NTHi) . Encapsulated colonies are mucoid and larger in size than non-encapsulated ones when seen on agar plates, as famously described by Margaret Pittman as 'S strains' (smooth, i.e. capsulated) and 'R strains' (rough, i.e. non encapsulated) (16). Capsule typing is relevant as it is a major determinant of virulence. Since traditional serotyping is both laborious, user dependent, and prone to error due to false positive reactions of NTHi strains with antisera, a PCR based method with higher accuracy is generally preferred (43). It has recently also been demonstrated that MALDI-TOF MS can discriminate between strains with different capsular types (44).

Genomic characteristics

Horizontal gene transfer

In a bacterial species' population, there is, according to the distributed genome hypothesis, a core genome consisting of genes present in all strains, as well as a supragenome which encompass all genes available to the bacterial species (45). In *H. influenzae*, the supragenome has been estimated to span as many as 6000 genes, whereas the core genome contains approximately 1500 genes (45, 46). Most individual strains carry between 1600 and 2300 genes (47).

The genetic variability is due to the fact that a bacterial cell may acquire foreign genes by horizontal gene transfer (HGT). Parts of the supragenome is therefore also referred to as the horizontal gene pool and may consist not only of individual genes but of clusters of genomic islands located on mobile genetic elements such as plasmids, transposons and integrative conjugative elements (ICE) (48). The interchange of genetic material between *Haemophilus* isolates colonizing the airway aids them to adapt to environmental stress caused by for instance the host defense or antimicrobial therapy (49).

Three principal mechanisms for HGT have been described; transduction, conjugation and transformation (Figure 3) (50). In transduction, DNA is transferred by bacteriophages. Although four different bacteriophages have been identified in *H. influenzae*, none seems to be useful for transduction (26, 51). In conjugation DNA is transferred by direct cell to cell contact. This is the most common mechanism by which DNA is interchanged between bacterial cells and is classically executed through conjugative pili (50). In *H. influenzae*, a type IV secretion system seems particularly important for the propagation of genetic material through conjugation (48). Once DNA is transferred through conjugation, it can persist and replicate in the cytoplasm (e.g. plasmids) or integrate site-specifically into the

chromosome (e.g. ICEs which may lack their own origin of replication and therefore need to recombine with the chromosome) (50).

The third mode of HGT, transformation, plays a particular role in *H. influenzae.* Transformation denotes the ability of a bacterium to directly take up extracellular DNA from the environment (50). This capacity is also termed natural competence and is enhanced by nutrient limitation (52). Competence varies significantly between individual isolates, with the strain *H. influenzae* Rd KW20 (a serotype d derived strain lacking the actual capsule) being particularly efficient and therefore commonly used as an in vitro model for genetic analysis and manipulation (11, 53). As mentioned in the introduction, it was the first free-living organism to have its entire genome characterized (11).

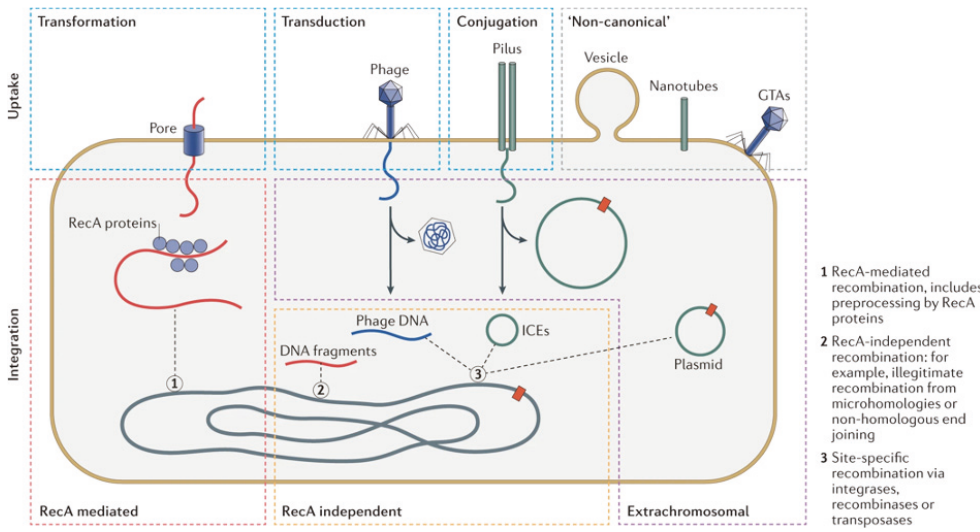


Figure 3. Principles of horizontal gene transfer in bacteria.
Transformation implies direct uptake of extracellular DNA from the environment that is subsequently incorporated into the bacterial chromosome through homologous recombination with the help of the RecA protein (left). In transduction, DNA is transferred by bacteriophages. In conjugation DNA is transferred via direct cell-to-cell contact through pili. The transferred DNA may linger in the cytoplasm as a plasmid or recombine with the chromosome (ICE). Image used with permission from the publisher (50).

The recognition and uptake of extracellular DNA is dependent on specific uptake signal sequences (USS) in the donor molecule (54). In *H. influenzae* the 9 bases 5'-AAGTGCGGT-3' have been identified as the probable USS. Particularly the four bases GCGG seem to be of importance (54, 55). This specific USS is common throughout the *H. influenzae* chromosome, appearing at 1465 sites with an average distance of 1224 bp in the 1.83 Mb genome of *H. influenzae* Rd. It occurs more

frequently in intergenic regions than in coding sequence (56). However, it is rarely seen in the DNA of bacteria from other genera than *Haemophilus,* which explains why transformation occurs more efficiently with donor DNA from another *Haemophilus* strain than from those of other genera (57).

Once internalized into the bacterial cell, the DNA is incorporated into the chromosome in a process called homologous recombination, creating mosaic gene patterns (50). This process is dependent on the RecA protein which binds to the foreign DNA and facilitates the search for homology in the recipient genome (Figure 3) (50, 58). The allelic variation caused by homologous recombination may involve parts of or even entire genes (50). Studies have shown that transformation in *H. influenzae* strains is more extensive than previously thought and may involve donor DNA segments as long as 16kb (59, 60).

Population structure

The population structure of *H. influenzae* has primarily been studied by molecular typing, initially with multilocus enzyme electrophoresis (MLEE) and subsequently with multilocus sequence typing (MLST) (61-65). In the MLST scheme for *H. influenzae*, the sequences of 7 housekeeping genes (*adk, atpG, frdB, fucK, mdh, pgi* and *recA*) are determined and based on their allelic profiles each isolate is assigned to a sequence type (63). The studies have shown that capsulated strains of *H. influenzae* are highly clonal whereas NTHi are more genetically diverse. Moreover, NTHi seemed more prone to recombinational events. Two distinct phylogenetic groups have been designated, with group I encompassing the majority of both capsular and NTHi isolates. Recent studies looking at the pan-genome of *H. influenzae* strains have confirmed a bipartite population with a higher degree of genetic diversity in NTHi strains (66, 67). One study found 10 times as many single nucleotide polymorphisms (SNP) in the core genome of NTHi compared to *H. influenzae* capsule type b (Hib) (67). There was nevertheless a discrete population structure also in NTHi strains supported by both core- and accessory genome analysis (66, 67).

Clinical manifestations

Carriage and transmission

H. influenzae is a colonizer of the human nasopharynx but is not part of the mouth flora. In rare occasions it can be isolated from the urogenital tract (26). No animal reservoir has been described, making humans the only known natural host (23). Transmission between humans occur through infected droplets (68). Carriage rates

of NTHi increase from infancy with an estimated 20% of children being colonized the first year of life (69). The bacterium is particularly common in children attending day-care facilities where it may be isolated from 30-40% of children (70, 71). Carriage in healthy adults is also reported at lower levels (72). Colonization is dynamic, with sequential carriage of various strains being reported in most studies rather than continuous carriage of the same strain (73, 74). Children can also be carriers of multiple NTHi strains concurrently (75). Co-colonization with *S. pneumoniae* and *M. catarrhalis* is frequently observed (76, 77).

Before the introduction of the Hib-vaccine, 3-5% of healthy school children were healthy carriers of capsule type b strains (78). The vaccine has markedly reduced the carriage rate, and colonization with encapsulated strains is now rare (79). The effects of the introduction of pneumococcal conjugate vaccines (PCV) in child immunization programs on NTHi carriage rates are uncertain. On one hand, the suppression of *Streptococcus pneumoniae* in the nasopharynx by these vaccines may give NTHi an opportunity to thrive. On the other hand, the 10-valent vaccine (PCV10) uses *H. influenzae* protein D as a carrier protein and may thus confer cross-immunity to NTHi strains, whereas other conjugate vaccines (7-, 13- and 20-valent) use different carrier proteins. Cross-sectional studies are conflicting, reporting both increasing and decreasing carriage rates after the introduction of PCV vaccines (80-82). A randomized controlled trial in 2013 showed no difference in carriage rates of NTHi after immunization with PCV7 or PCV10 (83).

Given that colonization of the upper respiratory tract is common, particularly in certain cohorts, this complicates the interpretation of the clinical significance of the isolation of *H. influenzae* from the airway of a patient presenting with respiratory symptoms.

Disease caused by encapsulated strains and effects of the Hib-vaccine

Before the widespread introduction of the Hib-vaccine from the late 1980s and onwards, severe infections caused by type b encapsulated strains was by far the most prominent clinical concern. Thus, when describing the clinical manifestations of *H. influenzae* disease, one may distinguish between a pre- and a post-vaccination era.

Hib disease is particularly associated with invasive infections, with meningitis being the most common focus, followed by in turn pneumonia, epiglottitis, septicemia of unknown origin, cellulitis, and osteoarthritis (84, 85). Hib infections may occur in any age group but over 90% occur in children less than 5 years of age (78). The greatest disease burden was previously seen in children aged between 4-18 months; too old to be protected by maternal antibodies, and too young to have mounted a natural immune response themselves (84). Children belonging to indigenous populations in North America and Oceania are particularly vulnerable to infections with encapsulated *H. influenzae*, probably due to unknown genetic factors (86, 87). Case fatality ratios for Hib disease reach on average an estimated 5% (88). In meningitis it has been reported to vary from 4.1% in the European region

to 27.6% in the African region (89). The risk of major sequelae such as hearing loss, seizures and intellectual impairment after Hib meningitis has been estimated at 9.5% (90). Epiglottitis generally affect older children with the peak incidence between 5 and 10 years of age. The mortality rate is lower, around 3% (88).

In the early 1990s, a marked impact on mortality and hospitalization rates due to Hib infections were observed following the introduction of routine infant immunization in many western countries (88, 91). In 2013, 183 of WHO's member states had implemented the use of conjugated Hib-vaccines into their immunization programs (78). There has been an estimated 90% reduction in Hib deaths globally from 2000 to 2015. However, approximately 30,000 children were still estimated to die from Hib infections in 2015. These casualties are almost entirely vaccine-preventable (92).

Invasive infections caused by other encapsulated strains, particularly serotype a and f, have been reported in selected geographical regions after the introduction of the Hib vaccine. (93, 94). Their clinical presentation resembles that of Hib (93, 94). However, there are no apparent signs of general serotype replacement of type b strains with other capsular types after the introduction of the vaccine (95). Instead, NTHi now stands for the vast majority of invasive infections caused by *H. influenzae* (96-100).

Disease caused by non-typeable *H. influenzae*

Infections with non-typable *H. influenzae* are generally milder in their clinical manifestation than those seen with encapsulated strains. They usually present as mucosal infections of the respiratory tract (18). However, an increasing number of invasive NTHi infections have been observed since the introduction of the Hib vaccine (96-100). Contrary to capsulated infections, invasive NTHi infections predominantly occur in the elderly population, with pneumonia being the most common focus (97, 101). There is also a peak in incidence in neonates (97). Predisposing conditions such as immunosuppression and underlying respiratory disease are common (97). A clonal outbreak of invasive infections mainly manifesting as septic arthritis was reported among persons living with HIV in metropolitan Atlanta, Georgia, in 2017-2018 (102). Fatality rates for invasive NTHi infections may reach 10-20%, equaling those seen in Invasive pneumococcal disease (IPD) (98).

Although less severe, mucosal infections by NTHi still cause considerable morbidity (18). Upper respiratory tract infections include acute otitis media (AOM), sinusitis and conjunctivitis (103). Since these infections are often poly-microbial, the role of NTHi in these infections may be both over- and under-reported (18). NTHi is together with *S. pneumoniae* one of the most common agents found in children with AOM and accounts for an estimated 25-35% of the episodes (104). It has also been designated as the most common cause of recurrent otitis media (105). Its prevalence increases in children who have received unsuccessful antimicrobial

therapy and in patients with bilateral AOM (106, 107). Co-presentation of infection with both conjunctivitis and AOM simultaneously seem to be particularly common for NTHi (108). As with carriage rates, the effect of PCV on AOM and sinusitis has been investigated, with trends of an increasing proportion of NTHi isolates being reported in some studies after the introduction of non-protein D carrying vaccines (80, 81, 109, 110). Moreover, a clinical trial has shown a 36% efficacy of PCV10 in reducing AOM caused by NTHi (111).

Lower respiratory tract mucosal infections by NTHi include acute exacerbations of chronic obstructive pulmonary disease (AECOPD) and community acquired pneumonia (CAP) (103). Since cultivable bacteria are seldom found in the lower airways of healthy individuals, it has previously been thought that the environment in healthy lungs is virtually sterile. However, the introduction of 16S rDNA based molecular diagnostics has shown that there is a distinct microbial community even in normal lungs, different from that of the upper respiratory tract (112). This has led to the hypothesis that there is a core lower airway microbiome which can be altered in the stable and acute phases of COPD (113). Notably, an increase in the relative abundance of *Haemophilus* spp. in general and NTHi in particular has been detected during both these phases using molecular as well as traditional culture methods (114).

Thus, NTHi may both play a role in COPD pathogenesis by sustaining chronic inflammation in the airways during stable disease, as well as by triggering exacerbations. Colonization with the same NTHi strain may persist for several months and even years in the lower airways of some COPD patients (115). Conversely, there is also a clear association between the acquisition of a new NTHi strain and AECOPD onset, indeed, adults with AECOPD associated with the acquisition of a new NTHi strain develop a specific immune response toward that strain (116, 117). Exacerbations may also be caused by multiple additional factors, mainly viral infections by human rhinovirus, influenza virus A and respiratory syncytial virus as well as other bacteria such as *S. pneumoniae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* (114). Nevertheless, NTHi remains the principal identifiable cause of COPD exacerbations, as is further evidenced by the fact that it can be isolated in protected brush specimens from the lower airways during this phase of the disease (103, 118).

The etiology of CAP is consistently difficult to determine due to the risk of contamination of samples from the upper airway. When culture-based methods are used, a bacterial etiology is only discovered in 30-40% of the cases. Traditionally, NTHi is considered the second most common bacterial cause of CAP after *S. pneumoniae* (119-123). Subsequent studies using molecular approaches report higher numbers of detection of etiology, and, interestingly, in some recent studies also a higher proportion of NTHi which sometimes is found as the most common agent (124-128). A yet unpublished study by our group using both traditional culture and molecular detection methods estimated that *H. influenzae* was associated with 16% of CAP cases among hospitalized adult patients in Skåne University Hospital,

Sweden, making it the second most common detected pathogen after *S. pneumoniae* (129). Risk factors for developing CAP with NTHi include COPD and advanced age (118, 130). Estimations on mortality for non-bacteremic CAP caused by *H. influenzae* are scarce, in one study it was 2 % for hospitalized patients (131). This is somewhat lower compared to the overall CAP case fatality ratio for hospitalized patients which is estimated at 4-18% depending on the setting (132, 133).

In Sweden, empirical antibiotic therapy recommendations for CAP in non-immunocompromised patients are based on disease severity, which is estimated using CRB-65 (134). CRB-65 is a scoring system based on clinical parameters including confusion/altered mental state, respiration rate >30, systolic blood pressure <90 mmHg and age >65 years (which thus gives a score between 0-4) (135). Hospitalized patients with mild- to moderate CAP (as estimated by a CRB-65 score ≤2) are recommended empirical intravenous treatment with benzylpenicillin, whereas patients with severe manifestations (CRB-65 ≥3) are recommended dual treatment with a beta-lactam and a macrolide/fluoroquinolone (134). Empirical treatment of mild- to moderate disease is thus primarily directed against *S. pneumoniae*, whereas the effects of benzylpenicillin on *H. influenzae* treatment outcome are less clear and will be discussed in further chapters.

Virulence and immunity

The ability of *H. influenzae* to survive and cause disease in the human body is a complex yet intriguing phenomenon that varies between strains. Most of the time, colonization does not result in infection and both host and pathogen factors are involved in disease development. Some of the principal mechanisms of *H. influenzae* pathogenesis are briefly summarized below.

Adhesion, invasion and persistence

In order to establish itself in the human airway, *H. influenzae* must first adhere to the respiratory mucosa. To achieve this, NTHi binds to a number of components in the extracellular matrix (ECM) of the respiratory epithelium, including laminin, vitronectin, fibronectin, collagen IV and proteoglycans (136). These components are particularly exposed by chronic inflammation or preceding viral infection (137). Several surface proteins on the outer membrane of NTHi have been shown to bind to such ECM components. A large number of proteins interact with laminin, including protein 4 (P4), protein E (PE), protein F (PF) and *Haemophilus* adhesion and penetration protein (Hap) (138-141). PE and PF are highly conserved and may also bind vitronectin (142, 143). Additional proteins recently identified to be involved in laminin interaction include elongation factor thermo-unstable (EF-Tu), l-lactate dehydrogenase (LDH), protein D (PD) and peptidoglycan associated

lipoprotein P6 (144). The redundancy of laminin binding proteins in *H. influenzae* suggests that binding to this ECM component is a key part of the pathogenesis (137). Other major adhesins include the surface-exposed high-molecular-weight proteins HMW1 and HMW2 which bind to the proteoglycan of the ECM (145). These are found in approximately 75% of NTHi isolates but not in encapsulated strains (146). The remaining proportion of NTHi isolates that do not display HMW1 or HMW2 at their surface instead express the *H. influenzae* adhesin (Hia) (147). Outer membrane proteins P2 and P5 have also been shown to bind human mucin (148, 149). Adherence may further be mediated through haemagglutinating pili, which are expressed by Hib strains as well as some NTHi strains (150). They are highly immunogenic and Hib isolates recovered from blood have generally lost their pili expression (151). It has also been shown that the type IV pilus on NTHi cells binds to intracellular adhesion molecule 1 (ICAM-1) on the surface of epithelial cells. ICAM-1 is upregulated in the human host following viral infection (152).

Once attached to the respiratory mucosa, *H. influenzae* can either persist as an innocuous colonizer, or progress by becoming more pathogenic and invade host respiratory cells (136, 153). Several adhesins, such as Hap, protein E and HMW1-2 may also be involved in host cell entry (154-156). Additionally, the gene *NTHI1441*, whose function remains unknown, have also recently been correlated to invasiveness (157). The ubiquitous PD has been shown to bind and facilitate entry into host mononuclear cells (158, 159). It may also damage the ciliated epithelium and thus disturb the mucociliary escalator (160). There is further evidence that NTHi may reside intracellularly in lymphoid tissue, notably in the tonsils (161). The role of IgA proteases in cellular invasion is discussed below.

When established on the respiratory epithelium, *H. influenzae* must also be able to persist in the host environment. To achieve this, it must overcome the nutrient limitations of the upper respiratory tract as well as the attacks of the host defense system. It must also compete with other respiratory tract pathogens such as *S. pneumoniae* (162). A key nutrient factor is the source of iron which is critical for survival, particularly since *H. influenzae* cannot synthesize its own haemin under aerobic conditions (23). In humans, iron is bound to transferrin in serum and lactoferrin in mucosal secretions, making the concentration of free iron too low for bacterial survival (163). *H. influenzae* has two transferrin binding proteins, Tpb1 and Tpb2, which can bind human transferrin under iron limitation (164). Once iron is captured, its uptake and utilization is cared for by a series of regulatory proteins termed ferric uptake regulators (Fur) (165). Haem and haemoglobin are sequestered by haemopexin and haptoglobin in the human body (166). Haem acquisition involves, among others, three outer membrane proteins, HxuA, HxuB, and HxuC, that bind haemoglobin-haptoglobin complexes and mediate the transfer of haem to the cytoplasm (167, 168). PE has also shown the ability to bind haemin at the cell surface (169).

There is emerging evidence that biofilm formation also facilitates the persistence of *H. influenzae* in human airways (170). Colonizing bacteria are seldom seen in a

free-living, planktonic form. Instead, aggregates of dead and living bacteria together with host cells forms what may be considered as a biofilm (137). Here, they may shielded from the actions of the immune system and from several antibiotics by reduced penetration of the agents in the biofilm matrix, and by a halt or reduction in the bacterial metabolism (171). Nutrient limitations seem able to induce a biofilm state in NTHi (172). The release of extracellular DNA also seems important for biofilm formation and stability, as does quorum sensing (173, 174). The effects of biofilms on antimicrobial susceptibility are discussed separately.

Immunity and immune escape

The host defense against *H. influenzae* include mechanical factors such as the mucociliary escalator, factors of innate immunity such as antimicrobial peptides, general inflammation, and the complement system, as well as factors of adaptive immunity such as secretory IgA1 and the production of other specific antibodies and effector cells (166).

The complement system is a first line of defense against invading pathogens, and a key part of innate immunity as well as an effector of adaptive immunity (175). The complement system may be activated through three different pathways: the classical, the alternative and the lectin pathway (Figure 4). The alternative and lectin pathways are activated by differences in bacterial membrane and envelope composition compared to the host. The classical pathway is instead activated by IgM and certain subclasses of IgG antibodies. Further downstream of all three pathways lies the formation of a C3 convertase which is required for deposition of C3b on the bacterial cell surface. The classical and lectin pathway results in the formation of the classical C3 convertase (C4bC2a) whereas the alternative pathway produces an alternative C3 convertase (C3bBb). C3b deposition is a crucial node in the complement cascade which enables the immune system to dispose of the bacteria through opsonization, phagocytosis and enhanced inflammation. Eventually, it leads to the recruitment of additional complement proteins in the terminal pathway and to the formation of the membrane attack complex (MAC) which directly lyse the bacterial cells (176). The complement system must be tightly regulated to avoid host tissue damage through overactivation. This is executed by a series of soluble and membrane-bound inhibitors. These complement regulatory proteins include C4b-binding protein (C4BP) which is the principal soluble inhibitor of the classical pathway, factor H (FH), which is the principal soluble inhibitor of the alternative pathway, and vitronectin which inhibits the terminal pathway (Figure 4) (175).

A paramount factor for the virulence of *H. influenzae* and for withstanding opsonophagocytosis and complement deposition is the polysaccharide capsule (177, 178). Capsular material is continuously shed from the cell surface and may also act as decoy for the host immune system (179). When isogenic capsular transformants (a-f) were nasally inoculated in a rat model, serotype b transformants consistently caused bacteremia, whereas type a and f transformants caused invasive disease in a

subset of the animals. Type c, d or e transformants did not give rise to any invasive infections (180). This is in good consonance with the epidemiology of invasive disease historically seen in humans. The Hib capsule consist of polyribosylribitol phosphate (PRP), a polymer of ribose and ribitole-5-phosphate (181). A thicker capsule can be produced if a Hib strain carries several copies of the capsule locus gene, which makes the isolate more resistant to complement (182). This conformation is generally seen in invasive Hib isolates (183).

Capsulated and NTHi strains additionally rely on two principal mechanisms for complement evasion; using branched lipooligosaccharide (LOS) on the bacterial surface as a camouflage, or hijacking of host complement regulatory proteins (184, 185). The LOS in *H. influenzae* resembles the lipopolysaccharide seen in *Enterobacterales* but lacks the O-antigen (186). It is a major virulence factor that enhances survival both in the nasopharynx and intravascularly and causes tissue damage. Phase variability (see below) of the LOS plays a vital role in the translocation from the nasopharynx to the bloodstream (187, 188). Tissue damage is triggered by its lipid A component which induces inflammation, while the oligosaccharide part is more involved in bacterial cell survival by complement escape (166). A large number of genes involved in LOS biosynthesis that are required for resistance to complement mediated killing and for survival in serum or in the presence of neutrophils have been identified (189-191). It has been shown that incorporation of phosphorylcholine and human derived N-acetylneuraminic acid into the LOS contributes to serum resistance in NTHi (192-194).

Several outer membrane proteins have been shown to bind complement regulatory proteins, thereby inhibiting the attacks of the complement system (185). Inhibition of the classical pathway by binding of C4BP to the bacterial surface is a strategy employed by many species including *S. pneumoniae*, *M. catarrhalis* and *Neisseria gonorrhea* (195-197). This faculty has also been shown in NTHi strains, which interact with complement control protein domains (CCP) 2 and 7 of C4BP (198). The bacterial ligand responsible for this is further investigated in the present study.

Inhibition of the lectin pathway has not been described in *H. influenzae* (185). As for the alternative pathway, factor H, which accelerates the decay of the alternative C3 convertase, can be bound by both capsulated and NTHi strains (185). In capsulated strains, protein H has been shown to be the ligand responsible for this interaction (199). NTHi strains do not express protein H, however, in vitro transformants of NTHi with this protein also increase their serum resistance. Instead, some NTHi have been shown to bind factor H through outer membrane protein P5 (200, 201). This ability varies significantly between strains and might be due to extensive sequence heterogeneity of the surface exposed loops of P5 (185).

Vitronectin does not only form part of the ECM but is also found in blood and inhibits the formation of the membrane attack complex (202). Adhesins binding to vitronectin such as PE, PF and P4 also confer serum resistance, and thus have a dual role in *H. influenzae* pathogenesis (185). Additionally, PE can bind human

plasminogen, which when activated serves as a protease and may hydrolyze key complement components such as C3b (203).

All these factors may influence the virulence of the bacteria, and complement resistance of NTHi has been linked to sepsis severity and increased invasiveness (204, 205).

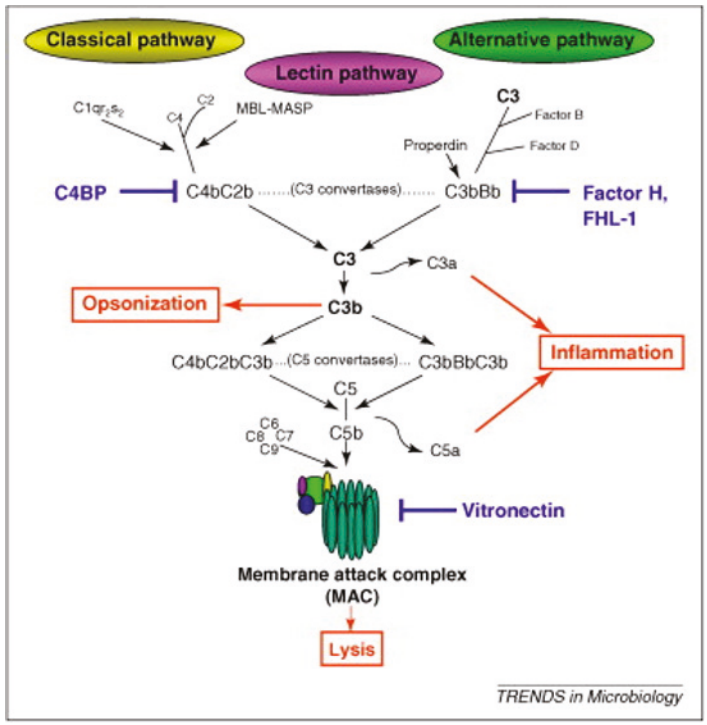


Figure 4. Overview of the complement system.
Pictured are the three principal pathways for activation as well as their main inhibitors bound by *H. influenzae*. Image used with permission from the publisher (202).

Beside the evasion of complement mediated killing, the defenses against secretory IgA1 have been extensively studied in *H. influenzae*. IgA1 is the predominant antibody on mucosal surfaces in the upper respiratory tract (206). It prevents colonization by binding the bacteria and simultaneously anchoring them to the mucus layer via its Fc region. Since the bacteria are retained within the respiratory mucus, their clearance by the mucociliary escalator is facilitated (166). It also agglutinates the bacteria and sterically hinders the interaction between adhesins and epithelial receptors (207). Both capsulated and non-encapulated *H. influenzae* secrete antigenically diverse IgA1 proteases which cleave the heavy chain of human

IgA1 in the hinge region (208, 209). Two genes encoding IgA1 proteases have been identified; *iga*A which is expressed in virtually all isolates, and *iga*B which is additionally expressed in about half of the NTHi strains (210, 211). The *iga*B gene has been found to be significantly less common in invasive compared to mucosal isolates but seems to be required for optimal intracellular persistance (210, 212, 213). The IgaA protease also seems to have a direct role in facilitating the invasion of host bronchial epithelial cells (212).

Another way to avoid the effects of adaptive immunity is to vary and modify the expression of molecules at the bacterial surface through a process called phase variation. This is described as a random process where a clonal bacterial population can present a heterogenous phenotype as a consequence of reversible genetic events (214). The result is that the expression of some immunogenic surface molecules such as adhesins can be turned on and off. Several mechanisms are involved, including slipped-strand mispairing, site-specific recombination and epigenetic regulation by DNA methylation (215). Important molecules that may undergo phase variation include hemagglutinating pili in Hib, the HMW-adhesins, IgA1 proteases and the LOS (187, 216-218). Beside phase variation, many of the outer membrane proteins expressed by NTHi are highly heterogenous and vary considerably between strains, which reduces specific antibody binding and hampers attempts to develop a NTHi vaccine (219).

Taken together, all these complicated escape mechanisms make immunity particularly against NTHi to a complex and incompletely understood phenomenon. Studies in adults with COPD and children with AOM have shown that the most prominent antibody response is directed at strain-specific determinants (117, 220). This may explain why recurrent infections are common even in immunocompetent hosts in these two conditions (23). An investigational vaccine against NTHi has been tested in a phase-2 clinical trial. The vaccine was based on the three antigens PD, PE and Pilin A. One year after the second dose, vaccine elicited antibody levels were still higher than at baseline (221).

Immunity against Hib disease is mediated by antibodies against PRP of the capsule. Maternally acquired antibody levels decline after birth and reach their lowest level at 18-24 months of age, making this age group most vulnerable to Hib disease. Gradually, PRP antibody levels rise with age due to gradual exposure, and severe Hib disease is seldom seen after the age of 6 years (23). In many regions of the world, herd immunity against Hib is now reached thanks to capsular conjugate vaccines as described above.

Antimicrobial susceptibility

Structure and classes of beta lactam antibiotics

Due to their safety profile and effect, beta-lactam antibiotics are the preferred treatment for a broad range of infections and are the most widely prescribed group of antimicrobial agents worldwide (222). They are defined by their common structure, the beta-lactam ring, a four-membered cyclic amide ring which is highly reactive and essential for their mode of action (223). It consists of one nitrogen and three carbon atoms and the bond between the nitrogen atom and the adjacent carbon atom with the carbonyl group is called the beta-lactam bond. Fused with the beta lactam ring is a secondary ring depending on whose structure four main groups are defined: penicillins, cephalosporins, carbapenems and monobactams (Figure 5) (224). The pharmacokinetic properties and antibacterial spectra of the beta-lactam antibiotics depend on this secondary ring as well as the addition of extra side chains to the core structure.

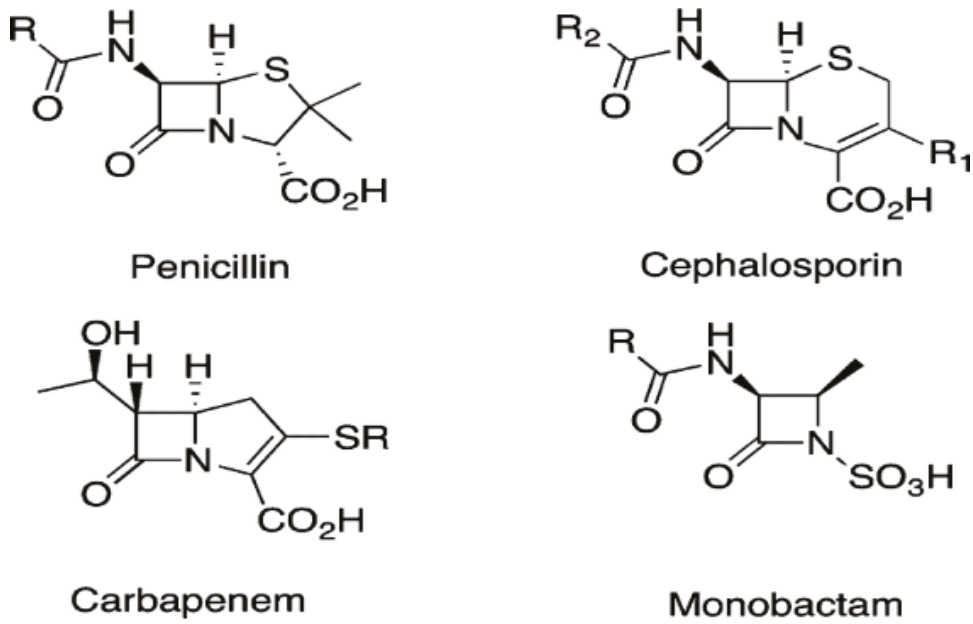


Figure 5. Structural formulas of beta-lactam antibiotics.
Top panel: Structural formulas of the four main classes of beta-lactam antibiotics. Image used with permission from the publishers (224).

Penicillins are characterized by a saturated five membered thiazolidine ring fused to the beta lactam ring (225). They can be further classified into five main categories depending on their side-chains (226). The naturally occurring, narrow-spectrum group of penicillins discovered by Fleming in 1928 comprise penicillin G (PcG, benzylpenicillin) and penicillin V (PcV, phenoxymethylpenicillin). It was the original formulation of penicillins in clinical use. The other four penicillin categories are semi-synthetic and are considered as extended spectrum agents. They include aminopenicillins (ampicillin and amoxicillin), ureidopenicillins (piperacillin), carboxypenicillin (ticarcillin) and penicillinase-stable penicillins (isoxazolylpenicillins). Particularly, aminopenicillins are characterized by an amino-group in the molecule and the prototype drug is ampicillin, commonly used for intravenous dosage (227). Amoxicillin has an additional hydroxyl group and superior bioavailability and is hence primarily used as a per oral drug (226, 228).

Beta-lactamase sensitive penicillins are sometimes combined with a beta-lactamase inhibitor, the most common ones being clavulanic acid, sulbactam and tazobactam. These inhibitors have structural similarities to penicillin and bind to beta-lactamase enzymes, inhibiting their activity by modifying their active site (226).

In cephalosporins, the beta-lactam ring is fused with an unsaturated six-membered sulfur-containing ring. Together, it is called a cephem nucleus. Cephalosporin antibiotics are divided into five different groups (called generations after their chronological discovery) with different antimicrobial activity dependent on structural changes of the cephem nucleus. They are generally penicillinase stable. Beside these five generations of cephalosporins, a new group of siderophore cephalosporins has been developed for the treatment of multi-resistant gram negative infections (226).

Carbapenems have their beta-lactam ring fused with an unsaturated 6 membered ring, which, in contrast to penicillins and cephalosporins, does not contain sulfur. They are stable against a board range of beta-lactamases including those with extended spectrum (ESBL). They have a broad antimicrobial activity and are hence often used as last line beta-lactam drugs in severe infections (229).

In contrast to the three other classes of beta-lactams, monobactams do not have a secondary ring fused to the beta-lactam ring. This is a small class of antibiotics with aztreonam as the principal agent. They exert stability to several beta-lactamases (230).

Beta-lactam activity against *H. influenzae*

Aminopenicillins have historically been the preferred treatment option for infections with *H. influenzae* and are still a first-hand choice for susceptible isolates (19). The activity of benzylpenicillin against *H. influenzae* has been a matter of debate. Time-killing experiments as well as Monte Carlo simulations of pharmacokinetic and

pharmacodynamical data (PK/PD) suggests a theoretical therapeutic effect of this agent, especially if a higher dose (e.g. 3 grams t.i.d.) is used (231, 232). However, one retrospective study has shown an increased mortality rate when PcG was used for treatment of *H. influenzae* bacteremia compared to wide-spectrum beta-lactams (233). PcV is not a recommended treatment option. Combinations of extended spectrum penicillins and beta-lactamase inhibitors such as amoxicillin – clavulanic acid and piperacillin – tazobactam are frequently used therapeutics. Most cephalosporins (except for the first-generation) have antimicrobial activity against *H. influenzae* and third generation cephalosporins are preferred empirical treatment options for severe infections such as meningitis. In the case of wide-spread beta-lactam resistance, carbapenems (particularly meropenem) remain a last line of choice (234).

Peptidoglycan synthesis and penicillin binding proteins (PBPs)

The main target for beta-lactam antibiotics is the bacterial peptidoglycan synthesis. The peptidoglycan is a key component of the cell wall which is essential to maintain cell shape and resist osmotic challenges (235). A functional synthesis is imperative for bacterial survival. In gram-negative bacteria such as *H. influenzae*, the peptidoglycan layer is located in the periplasm between the inner and outer phospholipid layer. The synthesis is best described for *Escherichia coli* but is thought to be common for most gram-negative bacteria (236, 237). It comprises three steps: first, activated nucleotide precursors are synthesized in the cytoplasm. These include N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). Throughout the synthesis, a pentapeptide chain is linked to MurNAc. Secondly, these precursors fuse to form a disaccharide-pentapeptide monomer peptidoglycan subunit on the inner leaflet of the cytoplasmic membrane. This monomer is then translocated to the periplasm, where the third and final step of peptidoglycan polymerization occurs (238).

It is this final step that may be disrupted by beta-lactam antibiotics such as penicillins, and key enzymes regulating this process are called penicillin binding proteins (PBP) since they are the targets for beta-lactam inactivation (figure 6) (239). Four specific reactions occur during the peptidoglycan polymerization: i) disaccharides are chained to form a glycan backbone in the *transglucosylase* reaction, ii) cross-linking of the pentapeptide chains through the *transpeptidase* reaction connects the polysaccharide chains to a mesh-like structure, iii) the removal of a D-alanine residue from some of the pentapeptide chains occurs before the latter reaction in a *carboxypeptidase* reaction and iv) existing cross-links are broken in the *endopeptidase* reaction to allow insertion of new peptidoglycan (236). All four reactions are catalyzed by penicillin binding proteins although additional enzymes also contribute especially in the endopeptidase reaction (240-242).

The penicillin binding domains of PBPs primarily catalyze the transpeptidase and carboxypeptidase reactions involved in the crosslinking of the pentapeptide chain

whereas the transglucosylase reaction is not affected by beta-lactams. The active site pocket, referred to as the transpeptidase domain, is dependent on three conserved amino acid motifs; SXXK, SXN and KTG. Particularly, the serine residue of the SXXK motif is the active residue essential for the catalytic reaction (241, 243). Beta-lactam antibiotics exert their activity by covalently binding to the active site pocket and thus inhibit the transpeptidase and carboxypeptidase reactions. This results in an imbalance of the peptidoglycan synthesis that eventually leads to morphological changes and cell lysis (238). These morphological changes of the bacterial cell wall depend on which PBP that is inhibited. For instance, in *E. coli* and other gram-negative bacteria, PBP1B and PBP3 are involved in the production of septal peptidoglycan during cell division and their inhibition leads to filamentation of the cells. On the other hand, PBP2 is involved in peptidoglycan synthesis during cell elongation and maintenance of the rod shape, and consequently its inhibition leads to the formation of spherical cells (236).

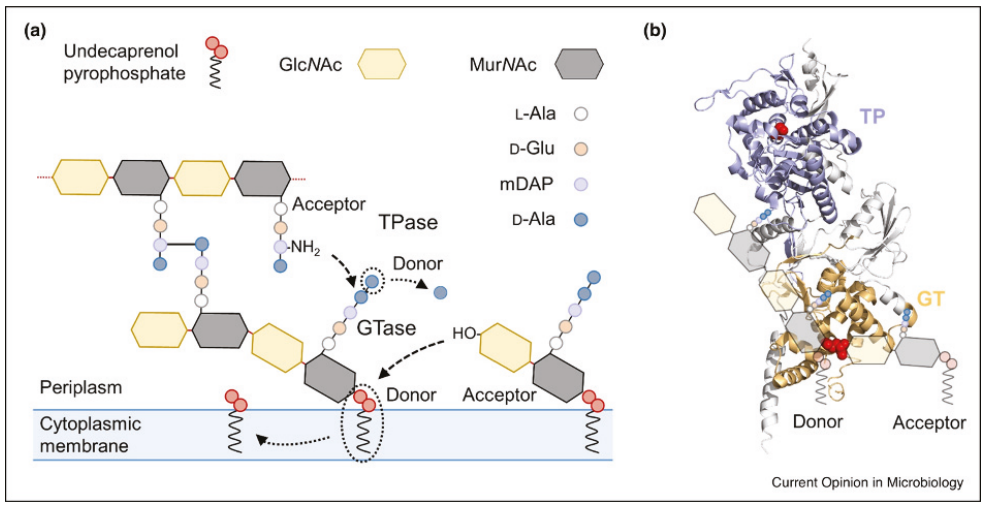


Figure 6. Peptidoglycan synthesis.
Illustration of the trans-peptidase and trans-glycosylase reactions involved in peptidoglycan synthesis (a). The crystal structure of PBP1B in *E. coli* with overlaid donor and acceptor substrates in the transglycosylase reaction (b). Image used with permission from the publisher (237).

Each bacterial species expresses a number of different PBPs which can be categorized based on their molecular weight in combination with which enzymatic activity they possess. High molecular weight (HMW) PBPs can be subdivided into two classes; class A exerts both transglucosylase and transpeptidase activity, whereas class B only displays transpeptidase activity. Low molecular weight

(LMW) PBPs are mainly involved in carboxypeptidase and endopeptidase activity (239, 243).

H. influenzae is considered to have eight penicillin binding proteins; two HMW-A PBPs (PBP1A and PBP1B), three HMW-B PBPs (PBP2, PBP3A, PBP3B) and three LMW-PBPs (PBP4, PBP5 and PBP6) (244-248). Of note, PBP3A and PBP3B are encoded by the same gene (*fts*I) and are sometimes referred to as just PBP3. It is thought that post-translational modulations account for the two different types of PBP3 (249). Older studies use a different nomenclature (PBP1-8) and the current terminology is based on the corresponding name of homologous PBPs in *E. coli* (250, 251).

Several previous studies have looked at the affinity of different beta-lactam agents for the various penicillin binding proteins of *H. influenzae*. The PBPs with the highest affinity to most beta-lactams are generally PBP1B and PBP3 (250, 252, 253). Specifically the high affinity of newer cephalosporins to PBP3 is well described (249, 254, 255). Some studies have also shown that the affinity is higher for PBP2 for some agents, such as amoxicillin, piperacillin and carbapenems (244, 249, 250, 255). One study reports that temperature affects the affinity of PBPs, and that PBP3 did not bind penicillin at 42°C (245). Moreover, when the bacterial cells are analyzed in a stationary growth phase, PBP3A and PBP4 are not always detected (252).

The essentiality of different PBPs for cell survival in *H. influenzae* have been investigated in two studies. In the first, knockout of the genes encoding PBP1B, PBP2 and PBP4 was lethal, whereas the effect of PBP3 was equivocal (256). In the second, only knockout of PBP3 showed a lethal effect on cells (257).

General physiological principles of antimicrobial resistance

There are four principal ways by which a bacterium can acquire resistance to an antibiotic agent. Enzymatic inactivation of an antibiotic can occur either by modification or degradation of the antibiotic molecule (258). In the latter reaction, hydrolysis of the functional group of the antibiotic renders it ineffective. This is exerted by for instance beta-lactamase (*bla*) enzymes. Resistance can also occur by target site alterations in which the antibiotic target is altered to the extent that binding of the antibiotic is significantly reduced while the target is still functional (259). In the context of beta-lactam resistance, such target-based resistance is conferred by alterations in the PBPs. Moreover, decreased influx of an antibiotic can be mediated by downregulation of porins on the bacterial outer membrane (260). Finally, active efflux can be facilitated by transmembrane pumps that export the antibiotic from its site of action, such as the periplasm (261). All these principal mechanisms have been described to confer beta-lactam resistance (262).

Table 1. Terminology of beta-lactam resistance in *H. influenzae*

Acronym	Name	Description
BLNAS	Beta-lactamase negative, ampicillin susceptible	Phenotypically susceptible to ampicillin,
BLPAR	Beta-lactamase positive, ampicillin resistant	Phenotypically resistant to ampicillin, nitrocefine positive
BLNAR	Beta-lactamase negative, ampicillin resistant	Phenotypically resistant to ampicillin, nitrocefine negative
BLPACR	Beta-lactamase positive, amoxicillin-clavulante resistant	Phenotypically resistant to amoxicillin-clavulanate, nitrocefine positive
gBLNAR	Genomic beta-lactamase negative, ampicillin resistant	Key amino acid substitutions in PBP3, nitrocefine negative
rPBP3	Resistance mediated by amino acid substitutions in PBP3	Key amino acid substitutions in PBP3 regardless of resistance phenotype and beta-lactamase production

Terminology of beta-lactam resistance in *H. influenzae*

In the case of beta-lactam resistance in *H. influenzae*, enzymatic degradation and target-based resistance are the most well characterized mechanisms of resistance, and will be discussed in greater detail as follows. In summary, resistance by enzymatic degradation is performed by beta-lactamases, whereas target-based resistance has particularly been linked to amino acid substitutions that alter the structure of PBP3 (19). The terminology used to describe these different types of resistance is summarized in Table 1. Since ampicillin has historically been the drug of choice for treatment, a classification system based on phenotypical ampicillin resistance is commonly used. Strains that are ampicillin resistant by the production of beta lactamases are termed BLPAR ('beta-lactamase positive, ampicillin resistant'), whereas ampicillin-resistant strains without beta-lactamase production are termed BLNAR ('beta-lactamase negative, ampicillin resistant'). Strains harbouring the two resistance types concomitantly are designated as BLPACR ('beta-lactamase positive, amoxicillin-clavulanate resistant') and finally, susceptible strains are referred to as BLNAS ('beta-lactamase negative, ampicillin susceptible') (19). However, this terminology only refers to phenotypical aminopenicillin resistance, and does not take into account neither the resistance to other beta-lactams, nor the fact that clinical breakpoints for ampicillin vary. Moreover, some strains with resistance-associated substitutions in their PBP3 may still be phenotypically susceptible to ampicillin as well as to other beta-lactams. To

describe these strains more accurately, the term gBLNAR ('genetic-BLNAR') has been suggested (263). Another term used to describe these isolates is rPBP3 (resistance mediated by alterations in PBP3) which denotes the presence of amino acid substitutions in PBP3 regardless of phenotypical resistance and presence or absence of beta-lactamases.

Beta-lactamase mediated resistance

Two different types of beta-lactamase enzymes have been described in *H. influenzae*, TEM and ROB. TEM was the first identified enzyme and accounts for up to 95% of beta-lactamase positive isolates globally (264). It has been shown to be identical with the TEM enzyme described in *E. coli* and interspecies transferability has been demonstrated, suggesting that it originates from Enterobacterales (265). The name TEM derives from the first patient (Temoneira) in Greece from whom it was isolated (266).

Classification of beta-lactamase enzymes can be based on structure (Ambler) or on substrate inhibitor profile (Bush-Jacoby-Medeiros) and TEM is categorized as Ambler class A and Bush-Jacoby-Medeiros class 2b (267). It is a serine beta-lactamase enzyme, and thus has a morphological resemblance with penicillin binding proteins which also use serine as their active residue (243). It attacks and hydrolyzes the beta-lactam bond (268).

Most TEM enzymes are TEM-1 with a minority being the variant of TEM-2 which has a substitution at amino acid position 37 (269). The bla_{TEM} gene is most frequently located on transposons (mobile genetic elements) which in turn are located on integrative conjugative elements (ICE, sometimes also referred to as large conjugate plasmids), meaning that the gene is transmitted horizontally through conjugation (direct bacteria – bacteria contact using secretion systems) and can be integrated into the bacterial chromosome (50, 270). These genetic elements often simultaneously contain genes that confer resistance to non-beta-lactam antibiotics such as chloramphenicol, tetracycline or kanamycin (271). ICEs that confer multidrug resistance in *H. influenzae* have been reported (49, 272). It has been shown that *H. parainfluenzae* carry the same family of large plasmids / ICE, which can be transferred between the two species, and may thus be a reservoir for beta-lactamase mediated resistance (271, 273). A minority of bla_{TEM} genes are instead located on small, non-conjugative plasmids (4 – 6 kb) where they are the only determinant of resistance (274).

Several different promoter variants have been described for the bla_{TEM} gene (275, 276). These variants have substitutions, deletions or insertions that enhance the promotor efficacy compared to the bla_{TEM} promotors seen in Enterobacterales (19). It has been hypothesized that since the outer membrane of *H. influenzae* is more permeable to beta-lactams than in Enterobacterales, these additional promotor enhancing mutations are needed to increase beta-lactamase production enough to

confer ampicillin resistance, which might explain why this resistance mechanism emerged later in *Haemophilus* than in Enterobacterales (277).

ROB-1 enzymes are also Ambler class A and Bush-Jacoby-Medeiros class 2b beta-lactamases. They are predominantly found in North America and account for approximately 5% of beta-lactamase positive isolates of *H. influenzae* globally (264). ROB has been detected in a number of species within the *Pasteurellacae* family and the gene is predominantly located on small plasmids ranging from 4 to 5 kB (19). There are reports of *H. influenzae* isolates with the concomitant presence of ROB-1 and TEM-1 enzymes (278).

Both ROB and TEM enzymes mediate high level resistance to the penicillin group of beta-lactams (including aminopenicillins) but can be inhibited by clavulanic acid, tazobactam and sulbactam. Most cephalosporins from the second generation and later are stable to degradation by these enzymes, as are the carbapenems (19). Several studies have found a correlation between increased antibiotic usage and the spread of beta-lactamases in *H. influenzae* (279, 280).

With the increasing spread of extended spectrum beta-lactamase (ESBL) mediated resistance in many gram negative species, there is concern that these enzymes would also appear in *H. influenzae* (19). So far, no clinical isolates with ESBL have been identified. In an in vitro study where the ESBL enzymes TEM-3, TEM-4 and TEM-5 were cloned and expressed in recombinant strains of *H. influenzae* a moderate increase in cefotaxime minimal inhibitory concentration (MIC) was observed (0.5 mg/L compared to 0.03 mg/L for the control strain expressing TEM-1) (281). Another in vitro study showed that when TEM-3-4-5 were expressed in strains that also harboured altered PBP3, cefotaxime resistance appeared (282). A report from South Africa describes two unrelated isolates of *H. parainfluenzae* displaying the ESBL enzyme TEM-15 and a cefotaxime MIC of >16 mg/L. Both isolates also had alterations in PBP3 and transformation of *H. influenzae* with TEM-15 did not increase the cefotaxime MIC to the levels of the original donor strain (283). Another study revealed that TEM-15 derived from *H. parainfluenzae* could confer cefotaxime resistance when expressed in vitro in susceptible strains of *H. influenzae* and *H. parainfluenzae* (284).

Yet another concern is the potential emergence of inhibitor resistant TEM beta-lactamases (IRT). So far, no such enzymes have been described in *H. influenzae.* However, two isolates of *H. parainfluenzae* with IRT enzymes TEM-34 and TEM-182 and a MIC of ≥8 mg/L for amoxicillin – clavulanate have been described (285). It is also hypothesized that IRT strains of *H. influenzae* may be mistaken for BLPACR strains upon routine testing (19).

Target-modified resistance – penicillin binding protein 3

Beside the production of beta-lactamases, the only well-characterized mechanism of beta-lactam resistance in *H. influenzae* is that of modifications in PBP3 (rPBP3).

Isolates carrying PBP3 with reduced affinity and elevated MIC to ampicillin were first described in the 1980s (244, 253). It could later also be shown that a low-affinity PBP3 was transformable to the reference strain *H. influenzae* Rd, thereby increasing its MIC (247). Furthermore, it was noted early on that strains with non-beta-lactamase mediated resistance could display reduced susceptibility not only to penicillins, but to a wider range of beta-lactams, including third generation cephalosporins (286).

Specific amino acid substitutions linked to ampicillin and cephalosporin resistance due to point mutations in the *fts*I gene encoding PBP3 were first presented by Ubukata and co-workers in 2001 (Figure 7). They were confirmed in recombinant strains of *H. influenzae* Rd (249). The same study showed that PBP3 harbouring these specific substitutions had reduced binding affinity to ampicillin. Moreover, 3D modelling showed that particular substitutions near the 379-SSN or 512-KTG motifs altered the active site of the enzyme. Isolates with substitutions near the SSN motif also displayed higher MICs towards cephalosporins (249). The role of these key amino acid substitutions has since been confirmed in several studies, both epidemiologically but also by the production of recombinant strains, site-directed mutagenesis and through trans-complementation (287-289). It has also been shown by competitive binding assays that affinity for aminopenicillins and certain cephalosporins is reduced compared to wild-type PBP3 (249, 288). Moreover, renewed theoretical 3D-modelling based on the crystal structure of PBP3 shows that substitutions may both sterically hinder the active site and directly interact with the side chains of beta-lactam antibiotics (290).

The specific amino acid substitutions with the strongest association to beta-lactam resistance are either R517H or N526K which are close to the KTG motif and are considered essential for beta-lactam resistance to occur. Virtually all BLNAR isolates have either of these substitutions, but no one has both (19). However, they are seldom seen as lone substitutions but act in concert with various additional substitutions in between the SSN and KTG motifs. They are principally associated with a low-grade aminopenicillin resistance whereas isolates generally remain susceptible to cephalosporins and carbapenems. Substitutions at S385T or L389F near the SSN motif confer a higher degree of aminopenicillin resistance as well as reduced susceptibility to a range of cephalosporins (249, 287, 288). Based on these key substitutions, different classification systems have been proposed. Common nomenclature is based on a ‘modified-Ubukata’ – system: isolates displaying R517H are termed ‘group I’ and N526K isolates ‘group II’ (249). Based on the additional SSN-near substitutions, ‘group III’ isolates harbour substitutions S385T and N526K whereas ‘group III-like’ isolates harbour S385T and R517H (21, 249). Finally, ‘group III +’ isolates (S385T, L389F and N526K) and ‘group III-like +’ isolates (S385T, L389F and R517H) contain the additional substitution L389F, which has been shown to elevate cephalosporin MIC even further when examined by site directed mutagenesis (287).

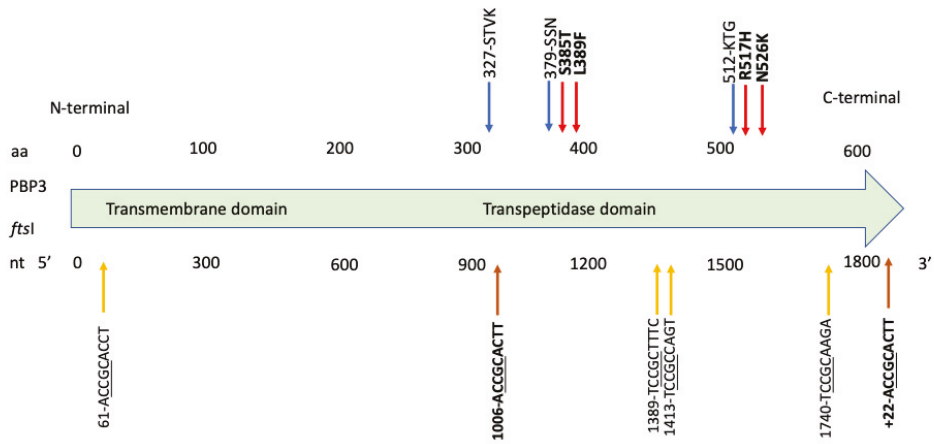


Figure 7. Schematic illustration of the *fts*I gene, encoding PBP3 in *H. influenzae*.
Blue arrows indicate the three catalytic domains involved in the transpeptidase reaction. Red arrows indicate key amino acid substitutions (bold text) linked to beta-lactam resistance. Brown arrows indicate full USS with consensus sequence (reversed, bold text). Yellow arrows indicate partial USS variants (reversed). The four key bases CCGC are underlined. Scales may vary. Image adapted from Hegstad *et. al.* under the terms of the Creative Commons Attribution 4.0 International License (49).

Group I and II isolates are sometimes referred to as low-BLNAR or low-rPBP3 strains, since their aminopenicillin MIC is less elevated than group III isolates. As for group II isolates, a range of associated substitutions between the SSN and KTG motifs have been described. Based on these associated substitutions, a subgrouping system (group IIa – IId) has been proposed by Dabernat *et. al.* (291). Skaare *et. al.* instead categorizes group II isolates into different 'PBP3 – types' (A-Q) based on additional substitutions (292, 293). The phenotypical implications of the associated mutations in the different subgroups on beta-lactam resistance is not clear, as the MIC levels are quite similar between the subgroups but still varies within them. A summary of the group- and subgroup defining substitutions of PBP3 and their associated MICs to aminopenicillins and cefotaxime is presented in Table 2.

Notably, some new PBP3 substitutions with additional potential of affecting beta-lactam susceptibility have been described in recent years. Substitutions G555E and Y557H have together been shown to induce cefotaxime resistance through transformation and site-directed mutagenesis in *H. influenzae* Rd. The substitutions were also able to individually increase MIC, but both needed to be present for resistance according to clinical break points to occur (294). Another research group recently identified a strong correlation between co-substitutions T532S + Y557H

and resistance to the fourth-generation cephalosporin cefepime in three isolates. The same group observed a moderate correlation between S385N + G416D and aztreonam resistance. Isogenic mutants of susceptible strains containing the same co-substitutions also showed increased MICs to these agents (295). In yet another study, two isolates with the amino acid insertion V525_N526insM as well as additional substitutions including Y528H, the latter of which is also investigated in this work, were reported to be non-susceptible to a range of carbapenems (296). Recombinant strains with this PBP3 genotype also displayed elevated MICs as well as reduced PBP3-affinity to carbapenems (297). The substitution Y528H has also been reported to appear together with the key substitution N526K in a clinical isolate which had an MIC of 1 mg/L for ampicillin but 16 mg/L for amoxicillin (298). The reason for this discrepancy of this is unclear. The same substitution has previously also been identified in two cefuroxime resistant isolates together with N526K and S357N (299).

Whereas a link between PBP3 substitutions and aminopenicillin and cephalosporin resistance is well established, the impact of PBP3 substitutions on resistance to other beta-lactams is less clear. The effect on piperacillin-tazobactam susceptibility seems to be marginal, and one study found it to be the most efficient beta-lactam agent against BLNAR and BLPACR strains (300). There also seems to be a conserved effect of meropenem against BLNAR isolates bearing the typical, group defining substitutions (234, 287). In contrast, imipenem heteroresistance has been shown to be transferrable between isolates carrying rPBP3 (301). Substitutions in PBP3 combined with altered influx and efflux has also been linked to imipenem heteroresistance (302). As mentioned above, some novel, less common, substitutions and insertions have also been shown to confer aztreonam and carbapenem resistance.

The global distribution of BLNAR isolates varies. Group III, high-rPBP3 isolates are predominantly found in Asia, particularly in Japan and South Korea where the use of oral cephalosporins for the treatment of respiratory tract infections is thought to drive resistance (303-306). In Europe, North America and Australia, rPBP3 mediated resistance is less common with low-BLNAR isolates from particularly group II dominating, although an increasing number of reports of resistant isolates appears (21, 292, 298, 307-310). rPBP3 isolates have also been identified in African countries, with recent reports from Tunisia and Cameroon (311, 312).

Table 2. Key amino acid substitutions in PBP3 and their respective group and MIC levels to amoxicillin, ampicillin and cefotaxime according to different studies.

Group[a]	Subgroup[b]	Type[c]	Group and subgroup defining substitutions	Amoxicillin MIC (mg/L)[d]	Ampicillin MIC (mg/L)[e]	Cefotaxime MIC (mg/L)[f]
I			R517H	1 - 16	0.5 - 16	0.01 - 0.25
II	Na	D, G*	N526K	1 - 16	0.5 - 4	0.03 - 0.125
	IIa	F	N526K	1 - 4	0.5 - 4	0.01 - 0.5
	IIb	A, C	N526K + A502V	1 - 8	0.5 - 12	0.01 - 0.25
	IIc	E	N526K + A502T	0.5 - 8	0.5 - 8	0.01 - 0.25
	IId	B	N526K + I449V	1 - 2	0.5 - 8	0.007 - 0.12
III			S385T + N526K	4	2 - 16	0.25 - 2
III-like			S385T + R517H		0.5 - 24	0.06 - 4
III+			S385T + L389F + N526K	0.5 - >64	0.5 - >64	0.5 - 2
III-like+			S385T + L389F + R517H	0.5 - >64	0.5 - >64	1

a) Group nomenclature based on PBP3 amino acid substitutions compared to *H. influenzae* Rd as provided by different authors (21, 249, 291, 313). **b)** Subgroup nomenclature based on PBP3 amino acid substitutions compared to *H. influenzae* Rd according to Dabernat (291) **c)** PBP3 types based on classification system by Skaare et. al. (293) **d)** references (291, 298, 305) **e)** references (70, 292, 293, 298, 305-307, 309, 310, 409) **f)** references (291-293, 309, 409)

*PBP3 type D contains additional substitutions D350N G490E A530S Type G contains additional substitutions V547I A554T A561E N569S according to Skaare *et. al.* (293)

In contrast to other bacterial species with PBP mediated resistance, such as *S. pneumoniae* where a small number of clones makes up for a large proportion of the resistant isolates worldwide, there seems to be a greater genetic diversity in strains of *H. influenzae* with rPBP3 (19). Nevertheless, spontaneous mutations in *fts*I followed by clonal expansion has been described as a key factor contributing to the emergence and spread of rPBP3 isolates (263, 292, 293, 313, 314). Clonal outbreaks with BLNAR strains have been described among cystic fibrosis patients, in hospital settings and in long-term healthcare facilities (291, 315, 316). However, it has been reported that similar alleles of *fts*I are distributed among several different sequence types or pulsotypes of *H. influenzae* (292, 293, 317, 318). This may be attributed to the fact that many strains are easily transformable by homologous recombination, and horizontal gene transfer may contribute to the diversity and spread of resistant isolates (60).

This fact is supported by several studies showing that the entire *fts*I gene can easily be transformed *in vitro* (249, 287, 288, 317). Moreover, comparison of mosaic sequences between different *Haemophilus* species has identified a homologous recombination between *fts*I in *H. haemolyticus* and *H. influenzae* (317, 319). This has been confirmed by the production of recombinant strains, suggesting that *H. haemolyticus* may also be a reservoir contributing to rPBP3 resistance (320). The *fts*I gene has also been shown to be transformable between different strains of *H. parainfluenzae* and site directed mutagenesis has demonstrated the relative contribution of amino acid substitutions at similar positions in PBP3 on aminopenicillin and cephalosporin resistance, but any interspecies transformation with *H. influenzae* has not been observed (284, 321).

Of note, recombinational events does not need to involve the whole *fts*I open reading frame, and events near the 3' end seem particularly common as many USS are located in this region (Figure 7) (319). Therefore, it is suggested that resistance conferring PBP3 may evolve either due to spontaneous point mutations, recombinational events, or both (292). To monitor the spread of this resistance mechanism, a combination of MLST and sequencing of *fts*I has been proposed (292).

Intriguingly, a potential correlation between rPBP3 and increased virulence has been suggested. One study demonstrated an increased ability of a group III rPBP3 strain to invade bronchial epithelial cells *in vitro* (322). The authors suggested a direct effect of rPBP3 as an adherence molecule. However, this has been contradicted by molecular epidemiological studies which show no direct correlation between rPBP3 and disease severity (323). Rather, it is considered that other, yet unknown, strain-associated virulence properties seen in specific sequence types may account for the more severe manifestations of some BLNAR infections (292).

As can be noted in Table 2, isolates within the different genotypical groups and subgroups still display a phenotypical variability in their MIC against beta-lactam agents. Indeed, even isolates with identical sequences of PBP3 belonging to the same sequence type group have been reported to have differing MICs to

aminopenicillins (324). Moreover, it has been noted that although transformant strains with rPBP3 increase their MIC, in many cases it does not reach the MIC of the original donor strain (249, 287, 288). However, when Yalçin and co-workers used trans-complementation to replace rPBP3 in BLNAR-strains with the wild-type PBP3 from *H. influenzae* Rd, ampicillin MIC decreased to virtually the same level as the wild-type strain (289). On the other hand, there are also reports of beta-lactamase negative isolates with wild-type PBP3 and phenotypical resistance towards amoxicillin (19, 325). Taken together, this variability in MIC within the rPBP3 groups has motivated the search for other factors, beside the known substitutions in PBP3, that affects susceptibility in beta-lactamase negative strains.

Target-modified resistance – the potential role of other penicillin binding proteins

One such potential factor of additional non-beta-lactamase mediated resistance would be similar alterations in other penicillin binding proteins than PBP3 that would reduce the affinity of beta-lactam antibiotics. Several previous studies have investigated such potential alterations but no clear link to resistance has been established. One study sequenced the genes for PBP1A, PBP1B and PBP2 in a set of BLNAR isolates with unusually high MICs for ampicillin, but could not correlate any substitutions to ampicillin resistance. No transformants with enhanced resistance could be obtained after transformation of *H. influenzae* Rd and two low-BLNAR strains with these other HMW-PBPs (288). In contrast, one older study found that a recombinant strain carrying low-affinity PBP2 and PBP3 had higher ampicillin MIC than a recombinant strain carrying low-affinity PBP3 alone (252). This study was performed before genetic sequencing was easily available, so no potential substitutions in PBP2 could be identified. As for LMW-PBPs, two studies have identified substitutions in the transmembrane domain of PBP4, but no obvious correlation to beta-lactam resistance was observed (299, 301).

In recent years, more wide-spread access to whole genome sequencing has made genome wide association studies more easily accessible. One such study found no association between PBP substitutions outside of PBP3 and beta-lactam resistance (326). One very recently published study did, however, see a correlation between substitutions at residue 518 in PBP2 and residue 225 in PBP6 and amoxicillin-clavulanate non-susceptibility. Furthermore, a substitution at residue 151 in PBP1A correlated with cefuroxime resistance. No isogenic mutants were produced to confirm the role of these substitutions (327).

Influx and efflux mediated resistance

Another potential factor that could theoretically confer additional resistance is the balance between influx and efflux over the bacterial outer membrane. The outer

membrane of *H. influenzae* is more permeable to hydrophilic drugs such as beta-lactam antibiotics compared to many other gram-negative bacteria (328, 329). Consequently, MICs to beta-lactam antibiotics are generally lower than in *Enterobacterales*. Outer membrane protein 2 (OMP2) is considered to be the only major porin in *H. influenzae* (330). One study found that OMP2 variants which conferred lower permeability were associated with increased ampicillin MIC (331). However, other studies contradict this finding (247, 288).

One of the main mediators of active drug efflux in *H. influenzae* is the AcrAB efflux pump, whose activity is regulated by the *acr*R gene (332). Dysregulation of this pump has in one study been linked to macrolide and rifampicin resistance, whereas no effect on beta-lactam MICs was seen (332). Another study which examined four low-rPBP3 isolates with unusually high MIC to ampicillin (≥ 8 mg/L) observed that all these isolates had nucleotide insertions in the *acrR* gene predicting an early termination of the reading frame (288). This was not seen in rPBP3 isolates with conventional ampicillin MIC (1 - 4 mg/L). Transformants with this *acr*R gene variant displayed significantly higher MIC levels than the recipient strains, and conversely, *acr*R knock out strains had significantly lower ampicillin MIC (288). These findings are contradicted by another study which also observed the same early termination in the *acr*R reading frame in several rPBP3 isolates, none of which had ampicillin MIC exceeding 2 mg/L (313).

Activity and acquired resistance of non-beta-lactam antibiotics in *H. influenzae*

There are several non-beta lactam agents with intrinsic activity and acquired resistance mechanisms to *H. influenzae*. These may be used for therapy in cases where aminopenicillins and other beta-lactams are inappropriate due to resistance or allergy.

Clinical breakpoints are established by both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory Standard Institute (CLSI) for tetracyclines, folic acid metabolism inhibitors, fluoroquinolones, chloramphenicol and rifampicin (333, 334). The latter is only recommended for prophylaxis, for instance for vulnerable household contacts exposed to a case of Hib disease (335). CLSI has also established clinical breakpoints for macrolide antibiotics, whereas EUCAST sees the clinical evidence for the efficacy of macrolides as conflicting due to high spontaneous cure rates in studies that have been performed on acute otitis media (333, 334). One study shows a lower bacteriological cure rate for the macrolide antibiotic azithromycin compared to amoxicillin-clavulanate (336). Another study comparing the same agents showed similar although not statistically significant results (337). Acquisition of further macrolide resistance is common and can be mediated by both efflux pumps, ribosomal methylase and alterations in ribosomal proteins and RNA (338-340).

Chloramphenicol was commonly recommended for the treatment of Hib meningitis before the introduction of third generation cephalosporins (341). Resistance to chloramphenicol is mainly conferred by the acquired enzyme chloramphenicol acetyltransferase, encoded by the *cat* gene (342). The *cat* gene is frequently found together with bla_{TEM} genes on ICEs (271).

The folic acid metabolism inhibitors of trimethoprim and sulfamethoxazole exert their antibacterial activity by inhibiting the activity of the enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively. These two enzymes both reduce reactions that lead to the production of tetrahydrofolate, which is essential for DNA replication and cell survival. Alternative variants of genes encoding DHFR and DHPS reduces the respective affinity of trimethoprim and sulfamethoxazole, and thus confer resistance to these agents (343, 344). Some of the altered genes may be carried on plasmids, whereas others are chromosomal (19, 344).

Tetracyclines inhibit bacterial protein synthesis by blocking the 30S subunit of the bacterial ribosome. Resistance is conferred by an efflux pump coded by the *tet*(B) gene, which is also frequently found on ICEs (271, 345). Fluoroquinolones act as inhibitors of the bacterial DNA replication. Quinolone resistance develops by sequential point mutations in the quinolone-resistance determining regions of DNA-gyrase (*gyr*A and *gyr*C) or topoisomerase IV (*par*C and *par*E) (346). These genes have been shown to be transferrable horizontally between strains of *H. influenzae* through transformation (347). Inter-species transformation from *H. haemolyticus* has also been demonstrated (348). Importantly, both fluoroquinolones and tetracyclines show intracellular accumulation, and thus possess activity against the portion of intracellularly located NTHi (349, 350).

Multidrug resistance

Multidrug resistance (MDR) is typically defined as acquired non-susceptibility to one agent in three different antibiotic classes (351). The term ‘extensively drug resistant’ (XDR) is used for bacterial isolates that display non-susceptibility to all but two or fewer antimicrobial categories (351). MDR strains of *H. influenzae* were first reported in West Germany in 1980 (352). Since, isolates displaying resistance to most relevant non-beta-lactam-agents, with or without concomitant resistance to beta-lactams, have been described (21, 338, 353, 354). The most frequently described ICE that mediates multi-drug resistance, ICE*Hin1056*, has *bla*TEM residing on one transposon and *tet*(B) and *cat* genes on another (270, 355). Conjugation of this ICE is enabled by a type IV secretion system, and it integrates into the chromosome of *H. influenzae* site-specifically into the $tRNA^{Leu}$ gene (48, 49, 356). It is transferred with a frequency between 10^{-1} and 10^{-2} (270). Recently, an ICE lacking *tet*(B) but instead displaying two genes that confer reduced susceptibility to macrolides and two others that confer resistance to

aminoglycosides in addition to *bla*$_{TEM}$ and *cat* was described (termed *TN7100*) (272).

The multi-drug resistance conferred by ICEs can be combined with other resistance determinants located on the bacterial chromosome, such as group III rPBP3 and genes for fluoroquinolone and trimethoprim – sulfamethoxazole resistance (49). One recent study found a significant correlation between BLNAR strains and low susceptibility to non-beta-lactams (357). Thus, a combination of spontaneous mutations and horizontal gene transfer through conjugation and transformation may in vivo give rise to clones bearing a large number of resistance determinants (49). Reports of such 'extensively-MDR' isolates have been published from Norway and Taiwan (21, 358). A related report on extensively-MDR *H. parainfluenzae* has also been published (359).

Additional factors that may modulate the antibiotic effect against *H. influenzae*

Antimicrobial resistance is defined in relation to *in vitro* variables such as MIC and zone diameter. These are based on a fixed bacterial inoculum in a predefined medium. However, other conditions may interfere with the bacterial cell growth both *in vivo* and *in vitro*, which may also affect the antimicrobial treatment outcome. One such factor is biofilm formation, which may occur in the case of chronic airway infection in COPD patients as well as in AOM infections (170, 360). Studies *in vitro* have shown that NTHi isolates residing in a biofilm are protected against the effects of several antibiotics, particularly aminopenicillins (361, 362). The best antimicrobial biofilm activity *in vitro* was seen with a combination of rifampicin and ciprofloxacin (361). Conversely, it has been shown that biofilm destabilizing agents such as EDTA and DNaseI enhance the susceptibility to aminopenicillins in a NTHi biofilm (363). It has also been suggested that sub-inhibitory concentrations of beta-lactam antibiotics stimulate biofilm formation in NTHi by upregulating carbohydrate metabolism (364). In a study on NTHi isolates from patients presenting with AOM and initial treatment failure, 89.6% of the isolates were shown to be able to form biofilm. No significant correlation between rPBP3 and biofilm formation could be observed (365). Another study showed no association between AOM treatment failure and biofilm formation and a significantly negative association between rPBP3 and biofilm was found (366).

An additional factor that may affect the treatment outcome is the size of the bacterial inoculum. Substantial *in vitro* inoculum effects have been noted for aminopenicillins (particularly in beta-lactamase producing strains) and cephalosporins with regards to *H. influenzae* (367, 368). However, no animal or human studies have been performed to evaluate the clinical significance of this (368).

In the case of colonization or polymicrobial infections, it has also been shown *in vitro* that beta-lactamase negative strains of *H. influenzae* can be protected from the lethal effects of amoxicillin by the release of outer membrane vesicles (OMVs) containing beta-lactamase from *Moraxella catarrhalis* (369). Conversely, it has been shown that beta-lactamase producing strains of *H. influenzae* can shelter *Streptococcus pyogenes* from the bactericidal activity of amoxicillin through the release of OMVs (370).

Additionally, it has been shown in *E. coli* that antibiotic tolerance to ampicillin may precede actual resistance (371). Antibiotic tolerance is defined as the ability of bacterial cells to survive a transient exposure to antibiotics at concentrations that would otherwise be lethal (372). This is achieved by entering a quiescent state when antibiotics are around in high concentrations and requires the acquisition of particular mutations compared to wild-type strains (373). A related phenomenon is that of persistence, where a subpopulation of a clonal bacterial population has the ability to transiently survive exposure to antibiotics at high concentrations (374). The phenomena of tolerance and persistence and their relation to treatment failure and resistance development are poorly studied in *H. influenzae.* Nevertheless, it has been reported that the activity of ampicillin on killing of *H. influenzae* cells paradoxically decreased rapidly at concentrations 2-4 times higher than MIC (375, 376). Paradoxically reduced killing at higher antibiotic concentrations is known as the Eagle effect and may be related to similar mechanisms as those seen in persistence (377, 378).

Clinical breakpoints and antimicrobial susceptibility testing

In Europe, three susceptibility testing categories are defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (333):

(S) – 'Susceptible, standard dosing regimen'. This category is used when there is a high likelihood of treatment success with a standard dosing of an agent.

(I) – 'Susceptible, increased exposure'. This category is used when there is a high likelihood of treatment success when exposure to an agent is increased either by modified dosage or concentration at the site of infection.

(R) – 'Resistant'. This category is used when there is a high likelihood of treatment failure even after increased exposure to an agent.

In the United States, the (S) and (R) categories are used similarly by the Clinical Laboratory Standard Institute (CLSI) whereas the (I) category stands for 'Intermediate' which also includes a buffer zone for inherent variability in testing methods and suggests that response rates may be slower than for susceptible

isolates. A separate category called SDD, meaning 'Susceptible, Dose-Dependent' is also used (334).

To categorize bacterial isolates into any of these categories, clinical breakpoints are employed. Based on the MIC distribution of a specific antimicrobial agent to a specific bacterial population, an epidemiological cut-off (ECOFF) is set (379). This is defined as the highest MIC of the wild-type population of the bacteria that lack phenotypically detectable acquired resistance mechanisms (380). To establish clinical breakpoints, the ECOFF is evaluated together with other variables such as pharmacodynamic targets (e.g. %fT>MIC), pharmacokinetic data of the antimicrobial agent, Monte-Carlo simulations of target attainment rates and ideally also results from clinical studies (381). Hence, the clinical breakpoints may differ from the ECOFF, as they seek to determine whether a therapeutic effect may be expected for a specific agent rather than identifying the upper end of the wild-type bacterial distribution. Current clinical breakpoints for *H. influenzae* as well as other bacterial species are set by EUCAST in Europe and CLSI in the United states (333, 334). In Sweden, the Nordic Committee on Antimicrobial Susceptibility Testing (NordicAST) set clinical breakpoints based on the EUCAST guidelines (382). Clinical breakpoints may be determined based on MIC distributions, but can also be based on disc diffusion diameter, since this is a less laborious method of susceptibility testing commonly used in clinical laboratories (383).

The gold standard method for AST is MIC determination by broth dilution in which a serial dilution of a specific antimicrobial agent is tested against a fixed bacterial inoculum (384). Guidelines for broth microdilution (BMD) are published by both EUCAST and CLSI (385, 386). Quality controls (QC) should be used and a QC MIC of ±1 dilution steps within target are accepted (387). Therefore, the susceptibility of an organism to a specific agent my differ by two dilution steps in two independent tests even by the gold standard method of BMD. In clinical laboratories, gradient tests (Epsilometer test, E-test) are commonly used instead of BMD for MIC determination since they require less labor. The performance of gradient tests should always be evaluated against the gold standard method and may vary significantly (388).

A more commonly used routine method for clinical laboratorial testing of AST is the disc diffusion method where a paper disc infused with an antimicrobial agent is placed on an agar plate first inoculated with bacteria. A concentration gradient is formed around the disc and an inhibition zone is seen in the case of a susceptible bacterial strain growing on the plate (384). The zone diameter is correlated to the MIC value of the strain and clinical breakpoints can be established for zone diameters just as readily as for MICs (383). This is currently the susceptibility test with the lowest cost (388).

Phenotypical susceptibility testing of *H. influenzae*

Different media are used when testing the susceptibility of the fastidiously growing bacteria of *H. influenzae* compared to when testing other species. Whereas regular Mueller Hinton medium is used for other gram-negative bacteria such as *Enterobacterales*, a supplemented medium needs to be used when testing *H. influenzae*. EUCAST recommends the use of Mueller Hinton medium supplemented with 5% lysed or defibrinated horse blood and 20 mg/L β-NAD (MH-F broth or agar) (389). CLSI instead recommends the use of *Haemophilus* Test Medium (HTM) which consists of Mueller Hinton agar or broth supplemented with 5 g/L of yeast extract as well as 15 mg/L of haematin and β-NAD respectively (390). The bacterial inoculum used for broth microdilution corresponds to approximately 5×10^5 CFU / mL by EUCAST methodology (385).

To determine the susceptibility of *H. influenzae* to beta-lactam antibiotics in routine clinical laboratories, a specific algorithm based on disc diffusion has been developed by EUCAST (333). Firstly, screening for beta-lactam resistance by disc diffusion with a 1 U PcG disc is performed. For isolates displaying zone diameters ≥12 mm, this excludes beta-lactam resistance and the isolate can, without further testing, be reported as susceptible to all beta-lactam agents for which there are clinical breakpoints, with the exception for oral amoxicillin, oral amoxicillin-clavulanic acid and oral cefuroxime which are reported 'susceptible, increased exposure' (I). Isolates that are screened as resistant (zone diameter <12 mm) need to be further tested for the presence of a beta-lactamase, usually by using a chromogenic substrate such as nitrocefin (391). Beta-lactamase negative isolates with a zone diameter <12 mm are suspected to have acquired resistance by substitutions in PBP3 (rPBP3) and need to be tested against individual beta-lactam agents and interpreted according to clinical break points, as their susceptibility levels are variable.

Beta-lactamase positive isolates need to be tested further for the possibility of concurrent presence of PBP3 substitutions. This is performed by a second disc diffusion assay with amoxicillin – clavulanic acid (2 and 1 μg respectively). Isolates with a zone diameter ≥15 mm are considered to have beta-lactamase production as the sole mechanism of resistance. These isolates are reported as resistant to ampicillin, amoxicillin, and piperacillin but susceptible to aminopenicillins and piperacillin combined with a beta-lactamase inhibitor as well as to cephalosporins and carbapenems. Finally, beta-lactamase positive isolates with a zone diameter <15 mm for amoxicillin – clavulanic acid need to be tested individually to all agents with clinical break points, since they may also harbour substitutions in PBP3.

The 1 U PcG disc diffusion screen has been shown to correctly identify the presence of PBP3 substitutions with an accuracy of 96% in two separate studies (392, 393). In another study, a high sensitivity but lower specificity (100 and 70% respectively) for the identification of beta-lactamase positive isolates with PBP3

substitutions with the 2 / 1 μg amoxicillin-clavulanate disc was observed (394). A more recent small study showed somewhat lower numbers for both discs (395). It is important to note that the clinical breakpoints for PcG zone diameter only apply as screening for general beta-lactam resistance and not as break points for susceptibility of the agent of benzylpenicillin itself. Furthermore, given that some isolates with rPBP3 substitutions are not phenotypically BLNAR upon further testing with BMD or gradient tests (see below), the high accuracy of the screening test in identifying rPBP3 isolates does not automatically translate into a good discrimination for phenotypical resistance. In fact, given a specificity of 70% and a prevalence of 10-20%, the positive predictive value of the test is quite low despite almost perfect sensitivity. Since clinical laboratories do not always test screening positive isolates further, there is a risk of over-reporting of phenotypically resistant isolates in those cases.

Susceptibility testing of *H. influenzae* to aminopenicillins has been deemed particularly challenging (396, 397). For determination of susceptibility according to MIC, clinical breakpoints for *H. influenzae* are defined as R >1 mg/L for ampicillin and R >2 mg/L for amoxicillin (with or without clavulanic acid) by EUCAST. Isolates with lower MIC are reported as susceptible for ampicillin (except for in meningitis) and susceptible with increased exposure for amoxicillin (I, recommended dosage of 750 mg t.i.d.) (333). As for breakpoints defined by CLSI, resistance to ampicillin is set at ≥ 4mg/L. There is also an intermediate category of isolates with MIC 2 mg/L while isolates displaying an MIC ≤ 1 mg/L are categorized as susceptible. No breakpoints for amoxicillin as a sole agent are established by CLSI, however, for amoxicillin in combination with clavulanic acid they are set to R ≥ 8/4 mg/L and S ≤ 4/2 mg/L (334). The breakpoints set by the two organizations are debated in terms of clinical relevance. CLSI breakpoints were primarily set to distinguish beta-lactamase producing isolates from non-producing isolates and may thus have a lower accuracy for identifying rPBP3 (397). The breakpoints for amoxicillin set by EUCAST are based upon the ECOFF, but also on pharmacokinetic modelling which show that due to a saturable absorption, 40% *f*T > MIC is not always reached even at concentrations of 750 mg t.i.d. or higher, when the MIC exceeds 1 mg/L (398). The consequence is that the breakpoint splits the MIC distribution of the rPBP3 group, meaning that these isolates can be categorized as both susceptible and resistant by MIC. Therefore, although the presence of PBP3 substitutions has a high sensitivity for identifying aminopenicillin resistant isolates, their positive predictive value for finding true BLNAR isolates may be as low as 55% as reported in one study (399). Another study reported that even in group III rPBP3 isolates, as many as 44% were phenotypically classified as susceptible to aminopenicillins (326).

In clinical routine, gradient tests are commonly used for MIC determination. Several studies have highlighted the fact that these tests may both over- and underestimate the MIC of aminopenicillins in *H. influenzae*, leading to the misclassification of isolates (392, 394, 397, 400). Particularly, it has been proposed

that gradient tests may overestimate the MIC in rPBP3 isolates (401). In contrast, one study found an excellent correlation between gradient tests and BMD (402).

Epidemiology of antimicrobial resistance in *H. influenzae*

To assess any general trends in the levels of antimicrobial resistance in *H. influenzae* globally is difficult. Particularly for aminopenicillin resistance, regional variations are considerable, and may be affected by which clinical breakpoints are employed in different studies, as well as whether the studies report phenotypical resistance levels or the prevalence of genetic resistance determinants such as rPBP3. Since the first reports on ampicillin resistance were published in 1974, the proportion of ampicillin resistant isolates has increased continuously until the mid 1990s (403, 404). More recently, one global study encompassing the years 2004 – 2012 found beta-lactamase production in 20.2% of *H. influenzae* isolates, with the highest numbers in Asia/Pacific Rim and North America. There was a significant decrease in the prevalence of beta-lactamase producing isolates during the study period (405). Only 1.5% of the global isolates were considered beta-lactamase negative, ampicillin resistant. The study uses CLSI breakpoints, which may explain the low number of BLNAR isolates. They were most frequently found in Asia and least common in the Middle East and North America (405). Similar proportions of aminopenicillin resistance have been reported in the United States (406). As noted earlier, the prevalence of BLNAR isolates in Japan and South Korea is considerably higher, with numbers reaching up to 60% (22, 303-305, 357, 407). Numbers in Europe are somewhere in between those seen in America and Southeast Asia, but reports on an increasing proportion of BLNAR isolates are continuously published (275, 292, 307, 308, 318, 408-410)

In Sweden, annual reports on antimicrobial resistance in *H. influenzae* collected from blood and nasopharynx are published by the Public Health Agency of Sweden and National Veterinary Institute ('Swedres-Svarm'). This voluntary surveillance uses results collected from 22 regional clinical laboratories through a system called Svebar since 2015 and onwards. The system does not differentiate between the different types of beta-lactam resistance, but reports numbers of ampicillin resistance at 28.2% as of 2022 (Figure 8). The number of isolates that are positive in the 1 U PcG screen are higher, ranging from 32.3% for invasive isolates to 36.6% for nasopharyngeal isolates (411).

Tetracycline resistance rates in *H. influenzae* are still low, generally below 5% (412). A similarly low number of resistant isolates is also seen for fluoroquinolones although reports of quinolone resistant strains have recently been published particularly in Asia and Europe (354, 413, 414). Previously, quinolone resistant isolates were exclusively reported in adults, since these agents are not used in children due to the risk for adverse effects (354). However, a recent study reports the isolation of a quinolone resistant *H. haemolyticus* strain in a child previously unexposed to this agent (415).

Resistance rates for trimethoprim - sulfamethoxazole are considerably higher, with up to a third of the isolates displaying resistance in Europe (410, 411, 416). This poses a challenge specifically in pediatric patients, where this often is the only suitable non-beta-lactam agent due to the risk for adverse effects seen with tetracyclines and fluoroquinolones as well as the uncertain effect of macrolides. Therefore, a high level of trimethoprim – sulfamethoxazole resistance in combination with an increasing number of BLNAR strains may cause a situation where infections with *H. influenzae* will be difficult to treat in children.

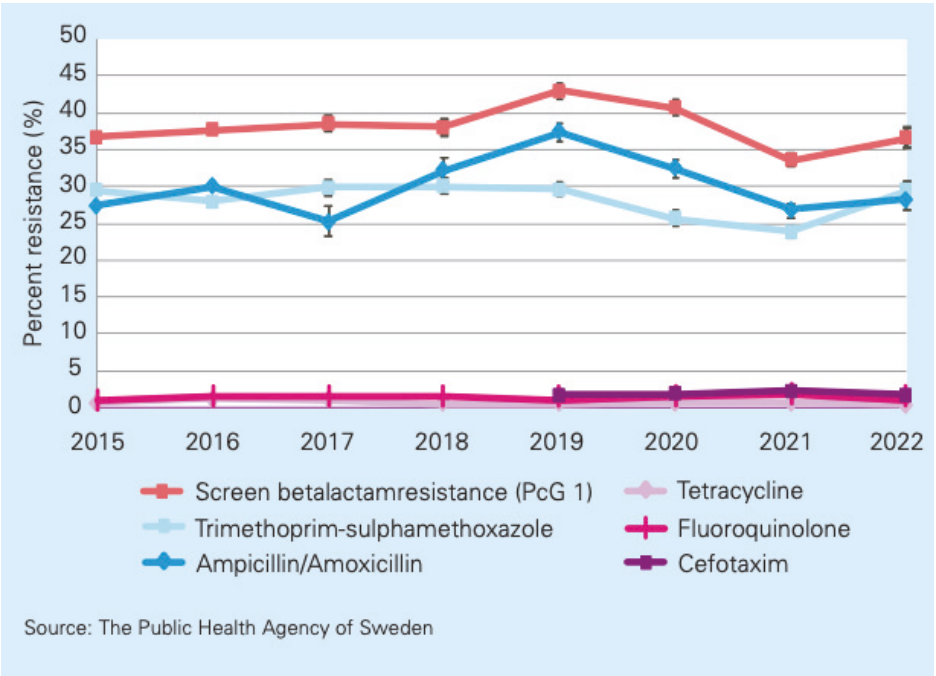


Figure 8. Antibiotic resistance levels in *H. influenzae* in Sweden.
Resistance levels in *H. influenzae* isolated from nasopharynx between 2015 and 2022 in Sweden. Image used with permission from the publisher (411).

Aims

The overall aims of this thesis were:

- To examine the role of a newly observed amino acid substitution in PBP3 of *H. influenzae* in aminopenicillin resistance.
- To investigate a set of clonally related *H. influenzae* isolates with identical PBP3 sequences but varying MICs to aminopenicillins with the purpose of identifying additional factors contributing to aminopenicillin susceptibility.
- To compare the clinical outcome of empirical treatment with benzylpenicillin and wide-spectrum beta-lactam antibiotics in lower respiratory tract infections caused by *H. influenzae*.
- To identify the ligand in NTHi responsible for binding of human C4BP.

Rationale

Paper I

PBP3 mediated resistance to aminopenicillins is characterized by key amino acid substitutions near the 379-SSN and 512-KTG motifs (Table 2). Particularly, substitutions R517H and N526K are present in virtually all BLNAR isolates (19). Although the evidence for a correlation between these two substitutions and aminopenicillin resistance is strong, their causality as single resistance determinants is less certain. When introduced into the wild type PBP3 of *H. influenzae* Rd by site directed mutagenesis, neither of these two substitutions did by their own confer resistance according to clinical break points, although MIC levels to aminopenicillins increased by one dilution step (287).

In the present study, we identified a beta-lactamase negative isolate of *H. influenzae* that lacks these substitutions associated with resistance, but which was nevertheless categorized as aminopenicillin resistant upon initial disc diffusion screening and by MIC-determination with gradient tests. Sequencing of the *fts*I gene instead revealed an alternative substitution near the KTG-motif; Y528H. The effects of this particular substitution on aminopenicillin susceptibility and bacterial fitness were further investigated.

Paper II

Isolates of *H. influenzae* with non-beta-lactamase mediated resistance may show varying degrees of susceptibility to aminopenicillins, even when displaying similar or even identical amino acid sequences in PBP3 (Table 2) (298, 313, 324). This variation in MIC may be of the order of 1-4 dilution steps, even when repeated testing is performed. It is a small but relevant variation since the current clinical breakpoints by EUCAST split the rPBP3 group, implying that some isolates with key amino acid substitutions in PBP3 are categorized as resistant, whereas others are classified as susceptible (333, 334). Moreover, transformation assays of susceptible strains with mutated *fts*I do not always yield recombinants with the same MIC as the non-susceptible donor strain (249, 287, 288). This suggests a potential presence of additional mechanisms that modulate the resistance phenotype. Such

mechanisms may have clinical significance as even a small additive effect on the MIC may cause a rPBP3 isolate to surpass clinical breakpoints.

Our group has previously described a set of isolates belonging to the same clonal group (ST14CC) with identical PBP3 (PBP3 type IIb /A). It was noted that their MIC to aminopenicillins still differed (MIC 0.5-4 mg/L for amoxicillin upon initial testing), despite their close genetic relatedness and similar PBP3 sequence (324). In this study, we selected a subset of these isolates with the purpose of finding additional causes for their variation in MIC.

Paper III

In Sweden as well as in other Scandinavian countries, high dose benzylpenicillin is the recommended empirical intravenous treatment for mild to moderate forms of CAP (CRB-65 score ≤2 and no concomitant immunosuppression or sepsis) (134). After the introduction of PCV in the child immunization program, there is concern that an increasing portion of CAP may be caused by *H. influenzae* (122, 124, 125). Although the effects of benzylpenicillin on the treatment outcome of infections with *S. pneumoniae* are well documented, their effect on *H. influenzae* is debated (121). There are no clinical breakpoints established by EUCAST, which refers to lack of evidence from clinical studies (333). PK/PD modelling and time killing experiments show a theoretical effect of this agent (231, 232). However, a retrospective study on benzylpenicillin as definite treatment of invasive infections showed a higher mortality compared to broad-spectrum penicillins and other beta-lactams (233).

Given this uncertain effect and the potential increase in CAP caused by *H. influenzae,* we wanted to determine whether benzylpenicillin can still be safely used as empirical treatment for CAP. In the present study we compared the effects of benzylpenicillin as empirical treatment of *H. influenzae* lower respiratory tract infections to those seen with beta-lactams with a wider spectrum in a retrospective, propensity-score matched study.

Paper IV

Recruitment of the human complement regulatory protein C4BP to the bacterial surface is a well-known survival strategy for a vast array of bacterial pathogens. The bacteria are thereby sheltered from opsonization and experience diminished phagocytosis by immune cells. The lysis of gram-negative bacteria through the formation of the MAC is blocked since C4BP is an inhibitor of the classical and lectin pathways (175). It acts as a co-factor in factor I mediated proteolysis of C4b which eventually leads to the degradation of the classical C3-convertase, C4bC2a.

It is a large (500 kDa) glycoprotein made up of 7 α-chains and one β-chain. These in turn encompass 8 and 3 complement control protein (CCP) domains, respectively (417).

Ligands responsible for binding of C4BP have been identified in for example *S. pneumoniae, M. catarrhalis* and *N. gonorrhea* but not conclusively in NTHi (195-197). It has, however, previously been shown that NTHi recruits C4BP to its surface, thereby increasing its serum resistance (198). Here, we seek to define the outer membrane protein P5 as the ligand responsible for this binding. We also explore the effect of a therapeutical C4BP-IgM fusion protein on NTHi serum resistance.

Material and methods

Patient data, clinical definitions and statistical methods (Paper III)

Patients with positive cultures of *H. influenzae* in Skåne county were identified through the records at the Clinical microbiology laboratories in Malmö/Lund (Sweden). Two case definitions were applied: (1) bacteremia with *H. influenzae* in an adult individual with a lower respiratory tract infection (LRTI) between 1997 – 2016, and (2) the isolation of *H. influenzae* in a respiratory tract sample from an adult individual hospitalized with a LRTI between 2015-2016. Patients in whom the concomitant presence of another respiratory tract pathogen could be shown were excluded.

Descriptive patient data recorded from the hospital medical records included age, sex, immunosuppression and comorbidities according to the Charlson/Deyo comorbidity index (CCI) (418). Descriptive data of the ongoing infection included sepsis severity score (as defined by sepsis-2 criteria), maximal concentration of CRP, the CRB-65 score, and admittance to an intensive care unit (ICU) (419). Regarding the outcome of the infection, 30-day mortality, 30-day readmission rates, and early clinical response (clinical stability) were registered. The FDA-definition of early clinical response comprises absence of fever, tachycardia, hypotension or tachypnoea on day 4 following hospital admission (420). Patients were subsequently sorted into three categories depending on which empirical antibiotic therapy they received: (1) benzylpenicillin, (2) another beta-lactam antibiotic with clinical breakpoints against *H. influenzae* and (3) non-beta-lactam agents. Group (2) is also referred to as patients receiving wide-spectrum beta-lactam (WSBL) treatment. Clinical outcomes were compared between group (1) and (2). Patients that concomitantly received another antibiotic with activity against *H. influenzae* were excluded.

Baseline statistics between the empirical treatment groups were compared using Chi^2-test for categorical variables and the Mann-Whitney U-test or Kruskal-Wallis test for continuous variables. Crude and adjusted logistic regression analysis was performed for the three primary outcomes between group (1) and (2). Multivariate models were fitted using the purposeful selection algorithm (421). *P*-values ≤ 0.05 were considered statistically significant.

Given the assumption that there would be an indication bias making the two compared groups inherently different, a propensity matched score analysis was also performed. A logistic regression with the treatment groups as outcome was used to calculate the propensity scores. Covariates included age (categorized), sex, ICU-care, maximum CRP, CCI, bacteremia, immune suppression and sepsis severity score. CRB-65 score was excluded in this analysis due to high number of missing values. A 1-1 nearest neighbor matching without replacement was then performed with a caliper of 0.2. After ensuring that the groups were balanced, treatment outcome was finally assessed in a full cohort logistic regression as well as in a conditional logistic regression model of matched pairs.

Laboratory methods (Paper I, II and IV)

The identity of all *H. influenzae* isolates was confirmed by MALDI-TOF MS. NTHi 3655 was a kind gift from Dr. R. Munson (Ohio State University, Columbus, OH) (422). Strains were grown at 37°C in 5% CO_2 on chocolate agar or in brain heart infusion (BHI) broth supplemented with 10 mg/L each of NAD and hemin unless stated otherwise. AST was performed at the EUCAST development laboratory (Växjö, Sweden) with disc diffusion and broth microdilution according to the EUCAST algorithm and ISO standard 20776-1 (333, 385). Zone diameters and MIC levels were interpreted according to EUCAST clinical breakpoints (333).

Paper I

A clinical *H. influenzae* blood isolate (NTHi 93-57485) collected as part of routine clinical diagnostics in Kronoberg county (Sweden) was subjected to AST at the laboratory of clinical microbiology in Växjö, Sweden. Upon initial AST with Etest (bioMérieux, Marcy l'Etoile, France), the MIC for amoxicillin was 2 mg/L, classifying it as resistant according to EUCAST breakpoints at the time (2011). Sequencing of the *fts*I gene did not reveal any of the previously described key amino acid substitutions. Instead, a novel substitution, Y528H, was identified.

The substitution Y528H was introduced into a susceptible isolate through site-directed mutagenesis. As a recipient strain, NTHi 3655 was chosen since it has a wild-type PBP3 similar to the one seen in *H. influenzae* Rd KW20 and its whole genome sequence has been published (accession no. AAZF01000004.1). The wild-type *fts*I gene of NTHi 3655 was amplified by PCR and subsequently cloned into the pCR-XL-TOPO vector using the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA). Thereafter, the recombinant plasmid construct was transformed into *E. coli* TOP10 cells. Site directed mutagenesis using the specific primers (as stated in Paper I, Table 2) was performed using Pfu Turbo DNA polymerase (Agilent, Santa Clara, CA) and the PCR product was digested for 1 h at 37°C using *Dpn*I

(Thermo Scientific, Waltham, MA). The mutated *fts*I gene was amplified by PCR and transformed into NTHi 3655 by using the protocol by Poje and Redfield (423). Transformants were selected on supplemented BHI agar with increasing concentrations of ampicillin and their *fts*I gene was amplified and sent for sequencing (Eurofins Genomics, Ebersberg, Germany) to verify that it contained the desired substitution. The generated transformant, named NTHi 3655-PBP3^{Y528H} was subjected to AST together with wild type NTHi 3655 and the clinical isolate NTHi93-57485. Growth curves for NTHi 3655-PBP3^{Y528H} and NTHi 93-57485 were registered and the *acr*R gene was sequenced for both isolates.

Paper II

A set of 24 *H. influenzae* isolates collected between 2010 and 2012 from upper respiratory tract specimens and belonging to the same sequence type group (ST14CC-PBP3 IIb/A) as previously described by Månsson *et. al.* were selected for further analyses based on their initial amoxicillin MIC (324). Shotgun genome sequencing of the isolates was performed at the Department of Microbiology, Tumor and Cell Biology (Karolinska Institutet, Sweden) by using Illumina 100-bp paired-end technology (HiSeq 2500; Illumina, CA). Draft genome assembly and data analysis of the sequencing reads was carried out at Codon Genomics Sdn. Bhd. (Selangor, Malaysia). BLAST analysis of predicted genes against available protein databases was performed for functional annotation.

Mutants of *H. influenzae* strains with modified PBP1b (encoded by *mrc*B) and PBP2 (encoded by *mrd*A) were constructed. Briefly, recombinant plasmids containing the *mrc*B or *mrd*A genes (flanked by 500 bp of their upstream and downstream region) fused with *ble*O (encoding for zeocine resistance) or *cat* (encoding for chloramphenicol resistance), respectively, were purified from overnight cultures of *E. coli*. Linear DNA fragments of the constructs were amplified by PCR and subsequently transformed into recipient strains of *H. influenzae* according to the protocol by Poje and Redfield (423). Isogenic mutants were selected on chocolate agar plates containing zeocine or chloramphenicol. Double mutants containing both modified PBP1b and PBP2 were also manufactured by a first transformation with the *mrc*B-*ble*O construct and a second transformation with the *mrd*A-*cat* construct. AST of clinical isolates and isogenic mutants was performed as described above.

Paper IV

Non-typeable *H. influenzae* collected from the upper respiratory tract were obtained from the Clinical microbiology laboratories, Laboratory medicine (at the time both Malmö and Lund, Sweden). Invasive isolates from blood and cerebrospinal fluid had previously been characterized by Resman *et. al.* (100, 307). Tonsil specimens

were obtained from patients undergoing tonsillectomy at Skåne University Hospital (161). NTHi samples from the lower respiratory tract were provided by the microbiology department of Hospital Universitari Bellvitge (Barcelona, Spain) and were collected from patients with COPD stage II-IV.

To identify the C4BP ligand in NTHi 3655, outer membrane fractions of an overnight culture were extracted and separated by two-dimensional SDS-PAGE as previously described (142, 144). Proteins on the gel were then transferred onto a 0.45-mm Immobilon-PTM PVDF membrane (EMD Millipore, Bedford, MA) and incubated with 100 μg C4BP. After detection with primary and secondary antibodies, C4BP binding protein spots were excised and analyzed by nano-liquid chromatography tandem mass spectrometry and MALDI-TOF MS.

P5 deficient mutants of NTHi 3655 and NTHi KR271 (a blood isolate that is more serum resistant than NTHi 3655) were constructed by replacement of the *ompP5* gene with the chloramphenicol acetyltransferase gene (*cat*) by overlapping PCR as previously described (142). Heterologous expression of P5 derived from NTHi 3655 and NTHi KR271 on the surface of *E. coli* was performed by cloning their respective *ompP5* genes into the pET-16 vector (Novagen) using restriction enzymes and subsequent transformation of *E. coli* DH5α-cells for plasmid propagation. The serum sensitive laboratory strain *E. coli* BL21 (DE3) was transformed and induced with 1 mM isopropyl β-D-thiogalactoside.

The binding of C4BP and expression of P5 on the surface of bacterial cells was measured by flow cytometry. Serum resistance was assessed in a serum bactericidal assay where pooled normal human serum (NHS) obtained from healthy volunteers with informed consent was incubated with aliquots of bacteria (log-phase) in $DGVB^{++}$ media. At given time points, 100 μl aliquots were plated on chocolate agar (NTHi) or Luria Betani (LB) agar (*E. coli*). The plates were incubated overnight and the percentage of survival was calculated. A separate bactericidal assay was performed with fusion proteins C4BP-IgG and C4BP-IgM, respectively.

Ethical considerations

The study design of Paper III was reviewed and approved by the Regional Ethical Review Board in Lund, Sweden (Dnr 2016/743, addendum 18-391). In paper IV, tonsil specimens were obtained from Skåne University Hospital (ethics approval number BD46/2007). Paper I and II did not involve patient data and no ethical permit was needed.

Results

Paper I

The mutant produced by site directed mutagenesis, NTHi 3655-PBP3^{Y528H}, had an identical sequence of the PBP3 transpeptidase domain compared with the clinical isolate NTHi 93-57485. Thus, the substitution Y528H was introduced into a wild-type PBP3. This rendered the transformant to become screening-positive for beta-lactam resistance according to the EUCAST disc diffusion algorithm (Table 3). MIC for ampicillin increased from ≤0.25 mg/L for the recipient strain to 0.5 mg/L for the transformant. A similar increase in amoxicillin MIC from 0.5 mg/L to 1 mg/L was observed. Upon retesting of the clinical isolate NTHi 93-57485 with BMD, it was noted that it had higher MIC for ampicillin (1 mg/L) and cefuroxime (4 mg/L) compared to the transformant NTHi3655-PBP3^{Y528H} with the same PBP3 genotype. Moreover, the zone diameter of PcG was smaller (6 mm) compared to NTHi 3655-PBP3^{Y528H} (11 mm).

All isolates were categorized as susceptible to aminopenicillins by BMD, contrary to the initial Etest performed with NTHi 93-57485 which had classified it as resistant to amoxicillin at the time being (MIC 2 mg/L). All isolates were negative in the nitrocefin test and none had any frame shift insertions in the *acr*R gene. Growth curves showed a tendency for slower growth of NTHi 93-57485 but no difference between the transformant and its parental strain.

Table 3. Results of screening for β-lactam resistance and susceptibility testing to various beta-lactam antibiotics by BMD in Paper I.

Strain ID	Geno type	Zone diam eter PCG 1U (mm)	Screening phenotype[a]	Suscept ibility to amino-penicilli ns[b]	MIC[c] amoxi cillin	MIC ampi cillin	MIC amoxi cillin clavul anic acid	MIC cefota xime	MIC ceftria xone	MIC cefuro xime	MIC imipe nem	MIC merop enem	Beta-lacta mase
NTHi 3655	wild type	16	Susceptible	Suscepti ble	0,5	≤0.25	≤0.25	≤0.015	≤0.015	1	0,5	0,06	Negati ve
NTHi3655-PBP3^{Y528H}	Y528H	11	Resistant	Suscepti ble	1	0,5	1	≤0.015	≤0.015	1	1	0,06	Negati ve
NTHi93-57485	Y528H	6	Resistant	Suscepti ble	1	1	1	0,06	≤0.015	4	0,5	0,06	Negati ve

a As interpreted by EUCAST clinical breakpoints for benzylpenicillin 1 U screening with a zone diameter < 12 mm categorized as resistant

b Interpreted by EUCAST clinical breakpoints where ampicillin MIC > 1 mg/ L and Amoxicillin MIC > 2 mg/ L are categorized as resistant

c MIC in mg/ L determined by BMD

Paper II

A series of *H. influenzae* clinical isolates clonal group ST14CC-PBP3 IIb/A (*n*=24) were selected for genotypical and phenotypical comparison of aminopenicillin resistance. They all shared the same amino acid sequence in the transpeptidase domain of their PBP3 (substitutions D350N, M377I, A502V, N526K, V547I, and N569S compared to *H. influenzae* Rd). Upon renewed susceptibility testing, they were all screened as potentially resistant to beta-lactam antibiotics by the EUCAST disc diffusion algorithm. All isolates were nitrocefin negative except for one which had intentionally been included as a control for comparative purposes. When subjected to BMD, MIC ranged between 0.5-4 mg/L for amoxicillin and 0.5-2 mg/L for ampicillin (with the beta-lactamase positive isolate being excluded). MIC levels for other beta-lactam agents did not differ between the isolates and they were all resistant to trimethoprim-sulfamethoxazole, in concordance with their ST14 origin.

A shot-gun genomic analysis of the 24 isolates was conducted. Potential genes contributing to resistance were examined in a BLAST analysis which revealed conserved protein sequences of AcrR regulators, AcrAB-efflux pumps and the main membrane porins P2 and P5. The sequences of PBP3 were confirmed as being identical between all the isolates and only the nitrocefin positive isolate expressed the bla_{TEM}-gene encoding a beta-lactamase. However, the protein sequences of PBP1B and PBP2 were not conserved between the isolates. We noticed that some isolates with a higher aminopenicillin MIC displayed particular amino acid substitutions. For instance, isolate NTHi KR5003, with an MIC of 2 mg/L for amoxicillin, harboured substitutions A32V, G411V, and T569S in PBP1B, and G538V in PBP2. Furthermore, isolate NTHi KR5014, with an amoxicillin MIC of 4 mg/L, displayed substitutions T710A in PBP1B, and substitutions T60M and T537I in PBP2.

To test the relationship between these alterations and aminopenicillin susceptibility, we used *H. influenzae* Rd KW20 as a host for heterological expression of PBP1B and PBP2 from isolates KR5003 and KR5014. *H. influenzae* Rd mutants expressing PBP1B from the two isolates did not change their MIC to aminopenicillins. As for mutants expressing PBP2 from KR5003/KR5014, they increased the geometrical mean of their MIC to aminopenicillins by one dilution-step (e.g. 0.5 mg/L for amoxicillin compared to 0.25 mg/L in Rd). However, no decrease in PcG or ampicillin zone diameter was observed (Table 4). Double mutants of *H. influenzae* Rd expressing both PBP1B and PBP2 from KR5003 and KR5014 were manufactured to assess any possible synergistic action of the two proteins. The double mutants increased their MIC values to approximately the same levels as those seen in single mutants with PBP2 only, suggesting that the observed amino acid substitutions in PBP1B did not affect aminopenicillin susceptibility, neither individually nor in concert with the substitutions in seen in PBP2.

In order to further assess the contribution of the amino acid substitutions in PBP2 on aminopenicillin MIC as observed in KR5003 and KR5014, we switched their endogenous PBP2 to the wild-type PBP2 of *H. influenzae* Rd. The generated mutants decreased the geometrical mean of their MIC for ampicillin and amoxicillin by at least one dilution step (Table 4). Importantly, a reciprocal increase in the zone diameter for ampicillin was observed. No change in zone diameter for PcG was noted, consistent with the mutants still harboring their endogenous PBP3 type IIb/A. Furthermore, replacement with a wild-type PBP2 caused the mutants to be categorized as susceptible instead of resistant to ampicillin by EUCAST breakpoints for both MIC and zone diameter. Additionally, isolate KR5014 transitioned from being resistant to susceptible (upon increased exposure, 'I') towards amoxicillin by the same breakpoints.

Table 4. Summary of transformant strains of PBP1b and PBP2 in Paper II.

		Broth Microdilution MIC[a] (mg/L)			Disc diffusion zone diameter[a] (mm)	
Strain	**Status[b]**	**Amoxicillin**	**Ampicillin**	**Cefotaxime**	**PcG**	**Ampicillin**
Rd KW20	Wildtype (reference PBPs)	0.25 (I)	0.12 (S)	≤0.015(S)	19.6 (S)	22.9 (S)
Rd-PBP1b^{KR5003}	Mutant	0.25 (I)	0.12 (S)	≤0.015(S)	19.3 (S)	23.6 (S)
Rd-PBP2^{KR5003}	Mutant	0.50 (I)	0.25 (S)	≤0.015(S)	19.0 (S)	23.6 (S)
Rd-PBP1b/2^{KR5003}	Double mutant	0.50 (I)	0.15 (S)	≤0.015(S)	19.0 (S)	23.3 (S)
Rd-PBP1b^{KR5014}	Mutant	0.25 (I)	0.12 (S)	≤0.015(S)	20.3 (S)	24.0 (S)
Rd-PBP2^{KR5014}	Mutant *	0.50 (I)	0.25 (S)	≤0.015(S)	18.0 (S)	23.3 (S)
Rd-PBP1B/2^{KR5014}	Double mutant **	0.50 (I)	0.25 (S)	≤0.015(S)	18.3 (S)	22.9 (S)
KR5003	Wildtype	2.00 (I)	2.00 (R)	0.06(S)	6.00 (R)	11.7 (R)
KR5003- PBP2Rd	Mutant	1.00 (I)	1.00 (S)	0.06(S)	6.00 (R)	18.6 (S)
KR5014	Wildtype	3.17 (R)	2.00 (R)	0.06(S)	6.00 (R)	6.6 (R)
KR5014-PBP2Rd	Mutant	0.63 (I)	1.00 (S)	0.06(S)	6.00 (R)	20.3 (S)

[a]Susceptibility according to EUCAST clinical breakpoint: (S) – susceptible (ampicillin zone diameter ≥18 mm, ampicillin MIC ≤1, amoxicillin MIC ≤0.001), (I) – susceptible, increased exposure (amoxicillin MIC 0.001 – 2 for a dose of 750 mg tid), (R) – resistant (Ampicillin zone diameter <18 mm, ampicillin MIC >1, amoxicillin MIC >2). MICs and zone diameters are given as geometrical means of three individual experiments.

[b]Wild-type strains were used as recipient for *mrc*B or/and *mrd*A from other strains to generate mutants.

* Extra amino acid residue substitution M437I in PBP2 unintentionally spontaneously introduced during the transformation process.

** Extra amino acid residue substitution P321L in PBP2 unintentionally spontaneously introduced during the transformation process

Paper III

140 individuals with *H. influenzae* bacteremia and 341 patients with positive airway cultures and LRTI were identified. Thus, 481 patients were included for comparison of antimicrobial therapy (Figure 9). Their median age was 75 years (interquartile range 66-84 years) and 25% of the patients (*n*=120) had COPD. CAP was the most common infectious diagnosis (*n*=418, 87%), followed by AECOPD (*n*=39, 8%). All cause 30-day mortality was 9% (12% in the bacteremia group and 7% in the group with positive respiratory samples).

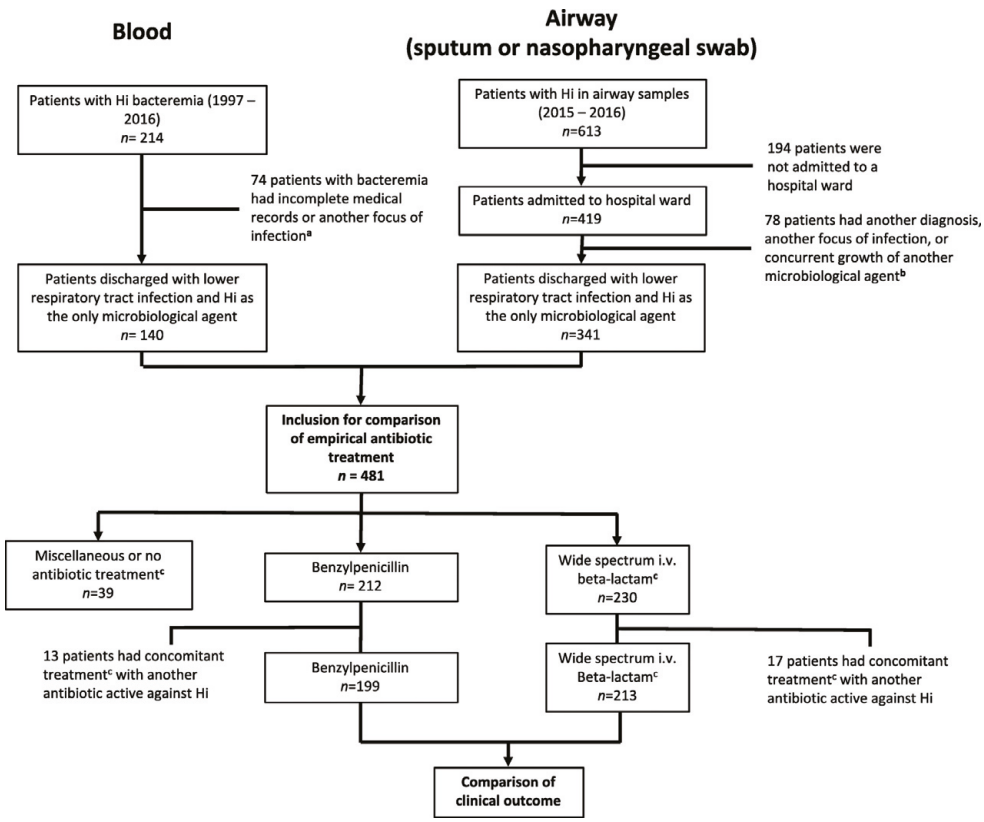


Figure 9. Cases included in the study in Paper III.
Flowchart depicting the number of included and excluded patients in the study as well as the reason for exclusion. Hi – *Haemophilus influenzae*. [a,b] see Paper III, appendix Table 6. [c] see Paper III, appendix table 7.

In total 199 patients had received empiric antimicrobial monotherapy with benzylpenicillin whereas 213 patients received a wide-spectrum beta-lactam monotherapy, most commonly cefotaxime (*n*=162, 76%), piperacillin-tazobactam (*n*=18, 8.5%) and cefuroxime (*n*=16, 7.5%). Patients receiving WSBL therapy had significantly higher sepsis severity scores and CCI, and were more frequently admitted to an ICU. A significantly larger proportion of patients in the WSBL group also had bacteremia. 30-day mortality was higher in this group (*n*=28, 13%) compared to in the benzylpenicillin group (*n*=11, 5%). In a univariate logistic regression analysis, this difference was statistically significant. However, in a fitted multivariate analysis adjusted for age, sepsis severity and CCI score, no statistically significant difference in mortality between the two groups remained (OR 2.03, 95% CI 0.91–4.50, p =0.082). In contrast, early clinical response rates were higher in the WSBL group (*n*=170, 84%) than in the benzylpenicillin group (*n*=153, 81%). This difference was statistically significant after analysis with a multivariate regression model adjusted for age, bacteremia, sepsis severity and CCI (OR 2.28, 95% CI 1.21–4.31, p = 0.011). No difference was seen between the two antibiotic groups in terms of 30-day readmission rates.

The observed differences in outcome between the two groups remained when analyzed in a propensity score matching. The matched cohort consisted of 151 patients in each treatment group and resulted in the omission of the most severely ill patients in the WSBL group. Nevertheless, mortality was still higher in this group (*n*=18, 12%) compared to in the group receiving benzylpenicillin (*n*=9, 6%). When comparing the two matched groups in a full cohort logistic regression model and in a conditional regression model, mortality was still higher but without any statistical significance (OR 2.14, 95% CI 0.93–4.92, p = 0.075 and 1.89, 95% CI 0.84–4.23, *p* = 0.12, respectively). Early clinical response rates remained significantly higher in the WSBL group and no difference in 30-day readmission rates was seen (Table 5).

Finally, infection with a beta-lactamase producing isolate was shown to cause significant effect modification between treatment group and early clinical response (the interaction term for beta-lactamase × treatment group (benzylpenicillin as reference): β = 3.12, 95%CI 0.82–5.43, *p* = 0.008). No such effect modification could be observed neither for isolates with rPBP3 mediated resistance (the interaction term for rPBP3 × treatment group (benzylpenicillin as reference): β = 0.74, 95% CI − 1.05–2.54, *p* = 0.42), nor for any of the other outcomes with the two resistance mechanisms.

Table 5. Outcomes stratified by treatment group in the propensity-matched cohort in Paper III.

Outcome	n (%)		logistic regression, OR (95%CI)			conditional logistic regression, OR (95%CI)		
	Bensyl-penicillin	WSBL	Bensyl-penicillin	WSBL	p-value	Bensyl-penicillin	WSBL	p-value
30-day all cause mortality, (n=300)	9/150 (6.0%)	18/150 (12.0%)	1 (ref)	2.14 (0.93-4.92)	0.075	1 (ref)	1.89 (0.84-4.23)	0.12
30-day all cause hospital readmission, (n=275)	24/143 (16.8%)	21/132 (15.9%)	1 (ref)	0.94 (0.49-1.78)	0.85	1 (ref)	0.89 (0.46-1.72	0.74
Early clinical response at day 4 (n=292)	119/146 (81.5%)	132/146 (90.4%)	1 (ref)	2.14 (1.07-4.27)	0.031	1 (ref)	2.5 (1.20-5.21)	0.014

Paper IV

To identify the outer membrane protein(s) responsible for C4BP binding in *H. influenzae*, the membrane fraction of NTHi3655 was extracted and separated by a two-dimensional SDS-PAGE and subsequently probed with C4BP in a far-Western blot. The C4BP binding spots were then excised and analysis by mass spectrometry identified the binding spot as NTHi3655 Omp P5.

In downstream experiments, the clinical strain NTHi KR271 was also included for further analysis since it had previously been found to be more serum resistant than NTHi 3655. P5 consists of 8 transmembrane domains forming 4 outer surface loops. A set of synthetic peptides corresponding to these outer loops of P5 derived from NTHi 3655 and NTHi KR 271 was generated. Biolayer interferometry revealed a dose dependent binding of C4BP to outer surface loop 2 of both isolates.

The expression of P5 and binding of purified C4BP was then measured by flow cytometry in a series of clinical isolates. A significant positive correlation between P5 expression and C4BP binding was noted (Spearman correlation analysis: $r = 0.48$, $p < 0.001$). It was additionally observed that isolates obtained from tonsil specimens, or the lower respiratory tract bound significantly higher levels of C4BP compared to isolates from the upper respiratory tract or invasive isolates (Figure 10).

P5 deficient mutants of NTHi 3655 and NTHi KR271 were subsequently produced. Mutant and parental strains were compared by scanning- and transmission electron microscopy. The morphology of their cell walls was similar. The P5 deficient transformants bound significantly less purified C4BP compared to their parental strains when analysed by flow cytometry ($p < 0.001$). Notably, a significantly higher degree of binding was observed for NTHi KR271 compared with NTHi 3655. Moreover, the P5 deficient mutant NTHi KR271Δ*OmpP5* retained approximately 40% of its C4BP-binding capacity, in contrast to NTHi 3655Δ*OmpP5*, suggesting the putative presence of an additional C4BP binding factor in NTHi KR271. When incubated in NHS, C4BP binding was still significantly lower for the mutants compared to the wild type strains. Conversely, the mutants bound 4- to 5-fold higher levels of C3d at their surface, suggesting that the diminished binding of C4BP resulted in increased complement deposition at their surface.

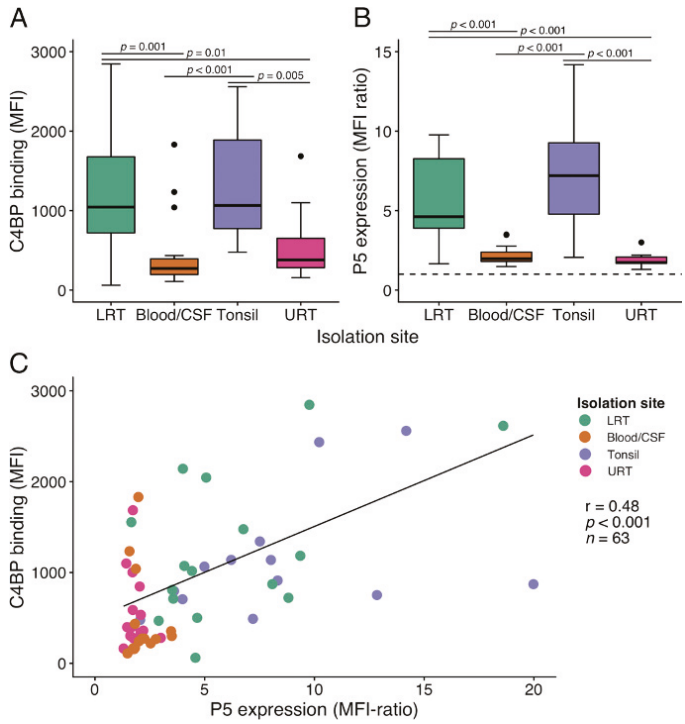
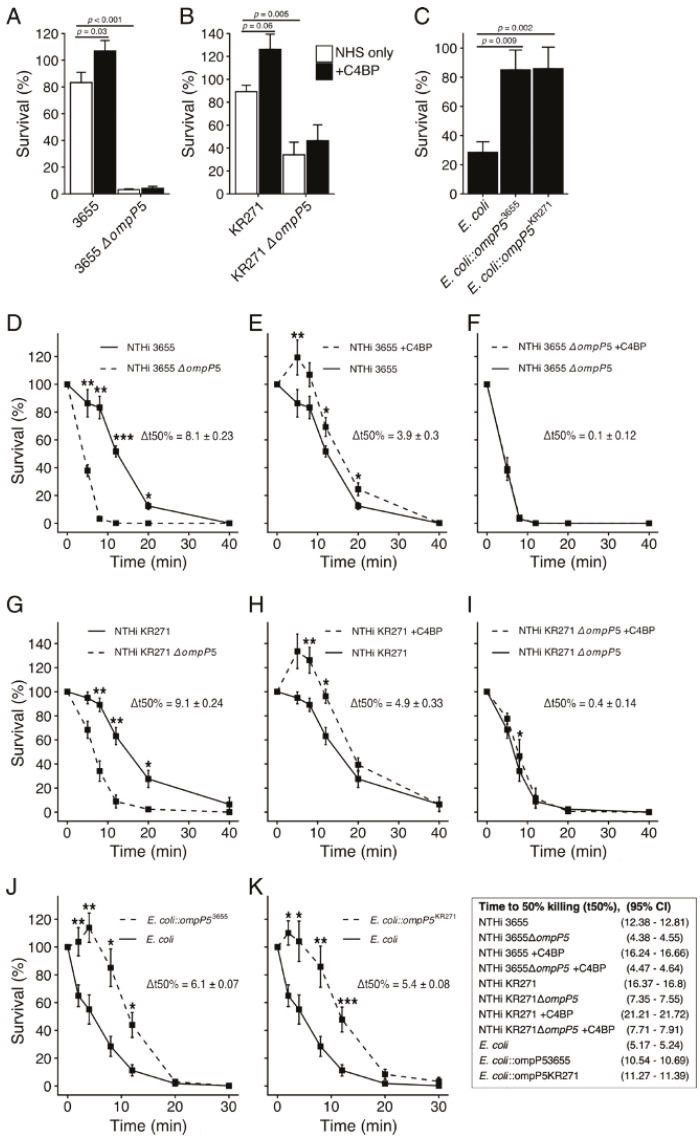


Figure 10. P5 expression and C4BP binding in clinical isolates in Paper IV.
Upper panels: Box plot showing binding of human C4BP to NTHi isolates from the lower respiratory tract, blood/CSF, tonsils and the upper respiratory tract (A) and P5 expression of the corresponding isolates (B) as measured by Flow-cytometry. Lower panel: Spearman correlation analysis of C4BP binding versus P5 expression on clinical isolates of NTHi (C).

To assess the impact of P5-mediated recruitment of C4BP on serum resistance, serum bactericidal assays were performed. It was shown that the P5 deficient transformants displayed significantly lower serum resistance across multiple time points. Preincubation with C4BP resulted in significantly higher levels of serum resistance for wild type strains but not for any of the two transformants (Figure 11). Heterologous expression of P5 on the surface of *E. coli* rendered the bacteria significantly more resistant to NHS.

NTHi 3655 and NTHi KR271 were also incubated with fusion proteins of C4BP-IgM and C4BP-IgG to see whether the antibody Fc-domain could promote complement activation and MAC formation. The binding capacity of these fusion proteins was similar to that seen with full length C4BP when assessed by flow cytometry. However, neither of the two fusion proteins did significantly promote bactericidal activity when tested in a bactericidal assay.



Time to 50% killing (t50%),	(95% CI)
NTHi 3655	(12.38 - 12.81)
NTHi 3655Δ*ompP5*	(4.38 - 4.55)
NTHi 3655 +C4BP	(16.24 - 16.66)
NTHi 3655Δ*ompP5* +C4BP	(4.47 - 4.64)
NTHi KR271	(16.37 - 16.8)
NTHi KR271Δ*ompP5*	(7.35 - 7.55)
NTHi KR271 +C4BP	(21.21 - 21.72)
NTHi KR271Δ*ompP5* +C4BP	(7.71 - 7.91)
E. coli	(5.17 - 5.24)
E. coli::ompP53655	(10.54 - 10.69)
E. coli::ompP5KR271	(11.27 - 11.39)

Figure 11. Serum bactericidal assays in Paper IV.

Panel A and B: Serum resistance of strains NTHi 3655, NTHi 3655Δ*OmpP5,* NTHi KR271 and NTHi KR271Δ*OmpP5* after 8 min incubation in NHS, with or without preincubation with C4BP. **Panel C**: Serum resistance of naive *E. coli* BL21(DE3), *E. coli::ompP5*^{3655}, and *E. coli::ompP5*^{KR271} after 8 min of incubation in NHS. **Panel D – F**: Serum resistance of NTHi 3655 and NTHi 3655Δ*OmpP5* at various time-points, with or without preincubation with C4BP. **Panel G – I**: Serum resistance of NTHi KR271 and NTHi KR271Δ*OmpP5* at various time-points, with or without preincubation with C4BP. **Panel J and K:** Serum resistance of naive *E. coli* BL21(DE3), *E. coli::ompP5*^{3655}, and *E. coli::ompP5*^{KR271} at various time-points. N.B., NHS concentrations are strain optimized (2% for NTHi 3655, 7.5% for NTHi KR271 and 0.5% for strains of *E. coli*) but comparable between wild-type and recombinant strains.

Discussion

Paper I

In paper I, we show that the introduction of a novel amino acid substitution, Y528H, into PBP3 of a fully susceptible isolate of *H. influenzae* rendered it screening-positive for beta-lactam resistance by the EUCAST disc diffusion algorithm. It also increased the transformant's MIC for ampicillin and amoxicillin by one dilution step. This was however not enough to make the transformant categorized as resistant by clinical breakpoints. The original clinical isolate displaying this substitution still had a higher MIC for ampicillin and cefuroxime as well as a smaller PcG zone diameter than the transformant. However, upon retesting with BMD, the clinical isolate was not categorized as resistant to aminopenicillins, in contrast to the results of the original clinical AST. The latter was performed with a gradient test, which has previously been suggested to overestimate aminopenicillin MIC in isolates with rPBP3 (401).

The effects of the introduction of Y528H into a wild-type PBP3 resemble those seen when Osaki *et. al.* employed site-directed mutagenesis to introduce the key amino acid substitutions R517H and N526K into the PBP3 of *H. influenzae* Rd. This also resulted in a one-step increase in the MIC of ampicillin. Both substitutions N526K and Y528H imply that the original residue is replaced by an amino acid with a positively charged side chain (lysine and histidine, respectively). This may sterically hinder the binding of the antibiotic agent to the active site pocket (290). Given these conformational changes as well as the proximity of the two substitutions to each other as well as to the 512-KTG-motif, their similar effects on ampicillin MIC seem reasonable. However, although the transpeptidase sequence of PBP3 is identical between NTHi 3655 and *H. influenzae* Rd, they have different genetic backgrounds. Therefore, the effect of our transformation is not directly comparable with those seen in other studies, as the effect of an amino acid substitution is contingent on the genetic background. Moreover, N526K seldom appears as a lone substitution, and may mediate resistance in concert with additional substitutions seen between the SSN and KTG motifs.

Despite the similar outcome in resistance levels, the N526K substitution is distinctly more common than Y528H in sequenced isolates, occurring in the vast majority of isolates with rPBP3 mediated resistance. At the time of publication, Y528H had only been described in one previous study, where it appeared in two

cefuroxime resistant isolates together with substitutions S357N and N526K (299). Since then, it has also been identified in two isolates together with the insertion V525_N526insM. Recombinants with this PBP3 conformation displayed decreased susceptibility to a range of carbapenems (297). Y528H has also been noted together with N526K in an isolate susceptible to ampicillin but with unusually high MIC for amoxicillin (MIC 16 mg/L) (298). No transgenic mutants were manufactured in that study.

The reason as to why Y528H is a much more rare substitution remains to be elucidated. No clear effects on bacterial growth rates was observed when we compared the transformant to its parental strain, but other potential manifestations of reduced bacterial fitness have not been investigated. Furthermore, it cannot be completely excluded that the studied substitution is less prone to selection by antibiotic pressure due to a lower efficiency.

The introduction of Y528H into a susceptible isolate did not restore the PcG zone diameter or ampicillin MIC levels to those seen in the original clinical isolate. This suggests the possibility of another, yet uncharacterized, factor other than rPBP3, beta-lactamase production or AcrAB dysregulation contributing to resistance in this isolate. Interestingly, growth curves showed a slower growth rate for NTHi 93-57485 compared to NTHi 3655 and its transformant. In other bacterial species, a reduced fitness in terms of delayed growth has been linked to antimicrobial resistance (424).

A limitation to our study is the fact that the observed differences in zone diameter and MIC are small and therefore prone to error due to natural variation in AST. Nevertheless, transformants could be obtained using ampicillin as selection marker, strengthening the fact that a veritable difference in susceptibility was present. Although transformation did not confer resistance according to clinical breakpoints, it nevertheless caused the isolate to be screened as resistant, and substitution Y528H may thus significantly affect the outcome of routine clinical susceptibility testing in *H. influenzae*.

Paper II

A possible association between amino acid changes in PBP1B and PBP2 and aminopenicillin MICs was identified in Paper II through whole genome sequencing of a set of isolates of *H. influenzae* with identical PBP3 sequences. After the production of isogenic mutants, no effect of substitutions in PBP1B on aminopenicillin MIC could be confirmed. However, isogenic mutants of PBP2 displayed a change of one-dilution-step in their MIC. This effect was particularly evident when the endogenous PBP2 of two ST14CC-PBP3 IIb/A strains was replaced by the wild-type PBP2 of *H. influenzae* Rd, which lead to both a decrease in MIC and a reciprocal increase in ampicillin zone diameter. The consequences of

introducing PBP2 polymorphisms from the two ST14CC-PBP3 IIb/A strains into the genetic background of *H. influenzae* Rd were more ambiguous, with aminopenicillin MICs still increasing by one-dilution step, while zone diameters remained unchanged. We therefore suggest a potential additive effect of amino acid substitutions in PBP2 on aminopenicillin MIC particularly when combined with a rPBP3 genotype, at least in the genetic background of ST14CC-PBP3 IIb/A.

Although the observed differences in MIC are small, they may be of clinical relevance since current breakpoints by EUCAST split the rPBP3 group. Indeed, one of the isolates (NTHi KR5014) shifted its resistance phenotype for amoxicillin from resistant to susceptible upon increased exposure subsequent to transformation with PBP2 from *H. influenzae* Rd. Population pharmacokinetic models have shown that target attainment rates for therapeutical levels of amoxicillin decrease once MIC exceeds 1 mg/L, even upon an increased dosing regimen of 750 mg t.i.d. (398, 425).

To identify which specific substitutions in PBP2 that may affect aminopenicillin MIC, further studies using site directed mutagenesis would be needed. As it is uncertain whether PBP2 substitutions have an effect in a different genetic background than ST14CC-PBP3 IIb/A, this should also be elucidated in future works. Hypothetically, both a direct interaction between protein polymorphisms in PBP2 and rPBP3 type IIb/A, or an epistatic interaction between PBP2 substitutions and the general genetic background of ST14CC, could justify the observed changes in MIC. To further discriminate between these two putative explanations, genome wide association studies or transformations of strains belonging to different sequence types would be of value. The production of double mutants of *H. influenzae* Rd containing polymorphisms in both PBP2 and PBP3 could theoretically also clarify this.

As in Paper I, the fact that the studied differences in MIC are small and subject to day-to-day variations in AST poses a challenge when interpreting our results. In order to obtain double-mutants as well as mutants with a negative effect on MIC, selection was made not with aminopenicillins but rather with chloramphenicol and zeocine. Nevertheless, the MIC differences were confirmed by triplicate testing, and in case of the transformants of ST14CC-PBP3 IIb/A strains, also by a reciprocal shift in disc diffusion zone diameter.

The role of non-PBP3 related factors in aminopenicillin resistance in beta-lactamase negative isolates of *H. influenzae* has previously been investigated in several other studies, but the contribution of other penicillin-binding proteins remains unclear. A recently published study in which a series of resistant isolates had undergone whole genome sequencing reported an association between substitution A518T in PBP2 and amoxicillin-clavulanate non-susceptibility (327). However, in another recent similar study no such association with any other penicillin binding protein than PBP3 was found (326). Isogenic mutants of PBP2 have been produced in two previous studies. In a study from 2004, no mutants with increased aminopenicillin resistance could be obtained after transformation of PBP1B or PBP2 into *H. influenzae* Rd in addition to two BLNAR strains. MIC

variability was instead explained by an early termination in the *acr*R gene (288). A study from 1990 in which Mendelman *et. al.* transformed DNA from BLNAR strains into an ampicillin susceptible strain showed that two out of three transformants displayed a reduced binding affinity for PBP2 to penicillin, as well as a reduced binding affinity for PBP3 present in all three transformants. Despite any sequencing of the *mrd*A or *fts*I genes was not performed in this study, its results are potentially in line with ours.

Thus, although PBP3 remains the principal determinant for non-beta-lactamase mediated resistance in *H. influenzae*, we suggest that amino acid alterations in PBP2 may modulate this resistance in a clinically important way.

Paper III

In paper III, we show that after adjustment for potential confounders, empirical monotherapy with benzylpenicillin is not associated with an increased 30-day mortality compared to treatment with a wide-spectrum beta-lactam. The overall all-cause mortality in our study was 9%, with a higher number of casualties occurring in the bacteremia cohort (12%). This is in line with previous studies reporting fatality rates between 8 and 22% in patients with *H. influenzae* bacteremia (98, 100, 233). For patients with respiratory tract infection without concurrent bacteremia, the mortality rate of 7% was slightly higher than in another study on CAP caused by *H. influenzae* (2%) (131).

A previous retrospective study by Thønnings *et. al.* from 2012 has investigated the outcome of treatment of *H. influenzae* bacteremia with benzylpenicillin on mortality (233). In this study, definite as opposed to empirical treatment was the main predictor studied. Nevertheless, an analysis of patients receiving benzylpenicillin as empirical treatment was also performed and showed a trend towards a higher mortality in this group, bordering statistical significance (p=0.06). However, due to a low number of cases (37 patients received benzylpenicillin as empirical therapy, 15 of whom in combination with gentamicin), no adjustment for covariates was performed. Furthermore, the study had a higher overall mortality rate (22%) and included all causes of bacteremia, not only respiratory tract infections. In the benzylpenicillin group, 30% of the patients had an unknown site of infection.

Another retrospective study compared mortality rates in CAP (with CRB-65 scores ≤2) regardless of etiological agent between patients treated with PcG or PcV and those treated with WSBL. No significant difference in outcome was seen (121). *H. influenzae* accounted for 7.1% of the cases with a CRB-65 score ≤1. However, no subgroup analysis of the outcome of infections with this specific bacterial species was presented.

Our study comprises a medium size cohort and cases were identified from positive cultures instead of clinical diagnosis. This reduces the risk of a bias when the cohort

was formed. Outcomes were analyzed by two different statistical methods, multivariate logistic regression and propensity score matching, which both showed similar results despite caveats with both techniques. Nevertheless, the retrospective design of our study always causes a risk of residual indication bias in the different treatment groups. This is a probable explanation for the implausible fact that mortality was significantly higher in the WSBL group in a crude analysis. Statistical adjustment for covariates reduced this difference, however it still bordered significance, suggesting that residual confounding was present. The best way to overcome this would be to perform a prospective study. However, since the etiological agent is unknown at the initiation of treatment, such a study would be difficult to carry out in a clinical setting. Inclusion of more covariates for statistical adjustment could also have been considered, but this would have entailed a risk of over-fitting. Due to a relatively low mortality rate (14% in the WSBL group), the power of our study was also limited to detect an odds ratio of approximately 2 (assuming α=0.05 β=0.8 and a two-sided test of significance).

In contrast to 30-day mortality, early clinical response rates were significantly higher in the WSBL group. This could to a large extent be explained by infections with a beta-lactamase producing isolate. Still, response-levels were high (>80%) in both groups, and after excluding beta-lactamase positive isolates, no significant difference between the groups remained. As most of the antibiotics in the WSBL group are stable to TEM-1 beta-lactamases, the result is quite intuitive. Interestingly, no effect modification was seen for patients infected with rPBP3 isolates regarding early clinical response. These isolates are generally susceptible to the agents in the WSBL whereas the effects of aminopenicillins and benzylpenicillin are highly variable as discussed previously.

The use of a higher dose of benzylpenicillin (3 g t.i.d.) is recommended as empirical treatment of CAP in current Swedish guidelines (134). This is supported by PK/PD data showing considerably higher target attainment rates for this regimen (232). The present study did not allow for statistical comparison of the outcome between different dosing regimens, as the number of outcomes was too low. However, a tendency towards fewer casualties was seen in the high dose group (*n*=4, 3% for a dose of 3 g t.i.d.) compared with the low dose group (*n*=7, 8% for a dose of 1 g t.i.d. or lower).

To summarize, our results do not contradict the current Swedish recommendations of treating mild to moderate CAP in immunocompetent individuals with a CRB-65 score ≤2 with high dose benzylpenicillin, even in the light of a potential increase in the fraction of cases caused by *H. influenzae*. Definite treatment should instead include an agent for which clinical breakpoints are established.

Paper IV

In paper IV, we define outer membrane protein P5 in NTHi as a ligand for human C4BP. NTHi mutant strains devoid of P5 bound significantly less C4BP and had a significantly lowered resistance to serum as evidenced by a lower $t_{50\%}$. Recombinant strains of *E. coli* expressing P5 at their surface conversely bound a significantly higher amount of C4BP and displayed a higher resistance to serum than the wild type bacterium. A correlation between P5 expression and the amount of C4BP-binding was observed in a set of clinical strains. Isolates from the lower respiratory tract and tonsils bound significantly higher amounts of C4BP than invasive or nasopharyngeal isolates.

Although P5 knockouts of both NTHi 3655 and NTHi KR271 displayed significantly reduced C4BP binding, some C4BP binding remained in NTHi KR271. This suggests the potential of additional C4BP binding factors in this isolate. This was mirrored by a higher serum resistance for NTHi KR271 (both wild-type and knockout) compared to NTHi 3655. However, when P5 from the two strains were expressed in *E. coli*, the effects on C4BP binding and serum resistance was similar. This suggests that the increased binding seen in NTHi KR271 is not due to variations in the sequence of P5 outer membrane loops but rather to the effects of other surface proteins. Since only two NTHi isolates were studied in detail, it can nevertheless not be ruled out that polymorphisms in P5 may affect the binding efficacy in other strains.

In good agreement with our previous study, no difference in C4BP binding was observed between blood and nasopharyngeal isolates (204). However, isolates from the lower respiratory tract of COPD patients and from tonsil specimens were also included in the present study. These showed both an increased P5 expression and increased C4BP binding compared with invasive and nasopharyngeal isolates. Prolonged colonization is often present at these sites, and the immunological pressure may be different, which in part could explain this variability in expression. Moreover, P5 is also involved in other stages of NTHi pathogenesis, such as cell adhesion, which may be more important in the lower airways or the tonsillar tissue (426). Nakamura *et. al.* observed a higher level of serum resistance in NTHi isolates from the lower airways in COPD patients compared to nasopharyngeal isolates from children, consistent with the differences in C4BP binding found in this study (189). Additionally, isolates from the lower respiratory tract and tonsils showed a greater variability in their P5 expression and C4BP binding. In COPD patients, it has been shown that colonization with the same strain NTHi strain in the lungs may persist for months up to years (115). Therefore, the variability observed in our study may potentially reflect different time stages of colonization.

We also investigated whether the two C4BP fusion proteins C4BP-IgM and C4BP-IgG could induce complement mediated killing of NTHi. Fusion proteins of factor H has previously shown promising results in eliminating *S. pyogenes* and *N. gonorrhea* through complement activation (427, 428). The C4BP-IgM fusion

protein has additionally been shown to kill *N. gonorrhea in vitro* and in a mouse model (429). However, no bactericidal activity was observed in the present study. The binding of the C4BP fusion proteins were markedly lower in NTHi than in *N. gonorrhea*, which may explain this lack of activity.

P5 has also been shown to interact with factor H in some NTHi strains, thereby inhibiting the alternative pathway (200, 201). It is thus a versatile protein which may inhibit the complement system at different stages as well as being involved in NTHi virulence by promoting adhesion. It could be a target for novel approaches of *H. influenzae* elimination strategies such as C4BP fusion proteins if an increased binding affinity can be obtained.

Concluding remarks and future perspectives

With an augmenting number of *H. influenzae* isolates displaying non-beta-lactamase mediated beta-lactam resistance, the need to better comprehend the underlying mechanisms behind this type of resistance becomes greater. In the present thesis, we seek to demonstrate that additional factors other than beta-lactamase production and the previously described key amino acid substitutions in PBP3 may modulate aminopenicillin susceptibility levels in a clinically relevant way. Such factors may include novel substitutions in both PBP2 and PBP3. Their presence may potentially affect the outcome of both the diagnostic screening for resistance as well as the clinical therapeutic response. Without denying the central role of previously described substitutions in PBP3, further studies should also focus on confirming the role of these additional factors as well as identifying others involved in beta-lactam resistance. With whole genome sequencing becoming ever more accessible, genome wide association studies among resistant *H. influenzae* isolates could represent an opportunity to further identify such elements. To confirm their impact, additional experimental studies producing isogenic mutants in different genetical backgrounds may be needed.

The emergence of new mechanisms of resistance highlights the need to confirm the efficacy of current treatment options with commonly used antibiotic regimens towards infections caused by *H. influenzae*. Here, we conclude that benzylpenicillin is still a safe empirical treatment option for mild to moderate CAP compared to beta-lactam antibiotics with a wider antibacterial spectrum, even when *H. influenzae* is the etiological agent. Interestingly, an effect modification of resistant isolates on treatment outcome was only evident for beta-lactamase producing isolates, but not for isolates with altered PBP3. Since the aminopenicillin MIC distribution of the latter isolates is divided by the clinical breakpoints set by EUCAST, their response to treatment is debated. Clinical data on the outcome of infections with rPBP3 expressing isolates are scarce and would be needed to be complemented. However, to obtain such data in a prospective manner is challenging in a bacterium that has both a commensal and pathogenic profile, and that likely causes a number of self-limiting infections in the upper respiratory tract. Large studies would be required to obtain confident results. Further retrospective studies looking at potential effect modifications by this resistance mechanism when an aminopenicillin is used as treatment could be of some value. Moreover, if a clear role of non-PBP3 mediated

resistance factors in aminopenicillin resistance can be established, improved diagnostic screening algorithms separating treatable isolates from truly resistant ones may be developed.

In the long run, the ever increasing global burden of antibiotic resistance stresses the need to come up with new, innovative ways of combatting bacterial infections. To achieve this, basic understanding on how bacteria such as *H. influenzae* cause disease and evade the immune system is needed. The interaction of outer membrane protein P5 with human C4BP is an example of one such elemental factor that may be further used in the development of new antibacterial agents or vaccines.

Populärvetenskaplig sammanfattning

Hur utvecklar en bakterie resistens mot antibiotika? Vad får antibiotikaresistens för konsekvens för hur vi behandlar vanliga infektioner såsom lunginflammationer? Och hur kan en bakterie undkomma attacker från människokroppens immunförsvar? Det är olika aspekter av dessa tre frågor som utreds i denna avhandling.

Bakterien i fråga heter *Haemophilus influenzae*. Namnet till trots så orsakar den inte influensa, även om man först trodde det när den upptäcktes i slutet av 1800-talet. Inte desto mindre är det en betydelsefull bakterie som kan leva i människans luftvägar, särskilt hos barn i förskoleåldern. Ofta ger den bara upphov till ett ofarligt bärarskap, men hos särskilt mottagliga individer kan den orsaka besvärlig sjukdom. Historiskt sett så har infektioner med en viss undergrupp av *H. influenzae,* kapseltyp b, orsakat svår sjukdom hos små barn. Efter att man i stora delar av världen på 1990-talet introducerade ett vaccin mot denna kapseltyp i det allmänna barnvaccinationsprogrammet så har antalet allvarliga infektioner minskat. I dagsläget dominerar istället s.k. icke-typbar *H. influenzae* (NTHi) som kan orsaka sjukdom i luftvägarna hos äldre individer, ofta med nedsatt lungfunktion som vid kroniskt obstruktiv lungsjukdom (KOL). Småbarn kan drabbas av öroninflammationer. I allvarliga fall så kan nyfödda och äldre sköra individer med nedsatt immunförsvar utveckla sepsis (’blodförgiftning’).

Sedan 70-talet så har ett ökande antal bakterier börjat uppvisa olika former av resistens mot vanliga antibiotikapreparat. Särskilt resistens mot en ofta använd undergrupp av penicilliner (som exempelvis innefattar preparatet amoxicillin) har på senare år fått ökad uppmärksamhet. Denna resistens kan orsakas av att bakterierna producerar enzymer som bryter ned antibiotikan. Alternativt kan resistens utvecklas genom att bakterien förändrar sina molekyler (proteiner) som antibiotikan binder till. Om dessa förändras tillräckligt mycket så kan antibiotikan inte längre fästa till dem och utöva sin bakteriedödande effekt.

Förändringar i sådana penicillinbindande proteiner ligger till grund för undersökningarna i artikel I och II. Sedan tidigare har man visat att om vissa specifika byggstenar (aminosyror) i proteinerna byts ut så är detta kopplat till en ökad resistens. I artikel I bytte vi på konstgjord väg ut en aminosyra på en specifik, ej tidigare beskriven, plats i ett penicillinbindande protein på en antibiotikakänslig *Haemophilus*. Detta gjorde så att bakteriens känslighet för amoxicillin minskade. I artikel IV undersöks istället 24 isolat av *H. influenzae* som alla är identiska avseende tidigare beskrivna molekylära resistensmekanismer men vars känslighet för amoxicillin ändå skiljer sig åt. Genom att kartlägga bakteriernas arvsmassa

identifierade vi nya skiften av aminosyror i ett helt annat penicillinbindande protein än det som hittills främst varit kopplat till resistens. Vi såg en möjlig koppling mellan aminosyre-förändringarna i det nya proteinet och en förhöjd resistensnivå. Genom att på konstgjord väg förändra detta protein i olika isolat av *H. influenzae* så kunde vi se att känsligheten för amoxicillin också ändrades. Skillnaderna i antibiotikakänslighet var inte stora, men om de uppträder tillsammans med de förändringar som finns beskrivna sedan tidigare så kan de ändå vara tillräckliga för att avgöra om bakterien är behandlingsbar med amoxicillin eller inte.

I artikel III undersöktes behandlingseffekten av ’vanligt hederligt penicillin’ (bensylpenicillin) på luftvägsinfektioner orsakade av *H. influenzae*. Ända sedan det upptäcktes år 1928 av Alexander Fleming så har bensylpenicillins effekt mot *H. influenzae* varit omdebatterad. Mot bakgrund av en tilltagande antibiotikaresistens samt det faktum att en allt större andel av lunginflammationer idag potentiellt skulle kunna orsakas av *H. influenzae* så ville vi ytterligare försöka utvärdera dess effekt. Det vi specifikt ville undersöka var om bensylpenicillin fortfarande kan användas som behandling i det första stadiet av en sjukhuskrävande luftvägsinfektion, innan man vet vilken bakterie som har orsakat den (s.k. empirisk behandling), även om det senare när provsvaren trillar in efter ett par dagar skulle visa sig att det var *H. influenzae* som var boven i dramat. Vi gick igenom 412 journaler från patienter som vårdats på sjukhus med *H. influenzae* i luftvägarna och jämförde utgången hos de patienter som empiriskt behandlats med bensylpenicillin mot de som behandlats med besläktade preparat men med en mer väletablerad effekt mot denna bakterie. Vi såg ingen skillnad mellan grupperna i dödlighet efter 30 dagar eller i återinläggningsfrekvens vid behandling av mild till måttlig sjukhuskrävande luftvägsinfektion.

I artikel IV så undersöktes istället hur vissa stammar av *H. influenzae* kan undvika avdödning av vårt immunförsvar. En viktig del av människans immunsystem är det s.k. komplementsystemet, som består av olika proteiner i blodet som kan känna igen och attackera bakterier. För att undvika att dessa proteiner även attackerar vår egen kropp så finns det andra proteiner som dämpar komplementsystemets aktivitet. Sedan tidigare vet man att *H. influenzae* kan utnyttja detta genom att binda sådana dämpande proteiner till sin yta, med följden att komplementsystemet ’lugnar ner sig’ och inte angriper bakterien. Ett specifikt sådant protein som bakterien utnyttjar heter C4BP. Tidigare har det varit okänt hur bakterien kan fånga upp detta på sin yta. I artikel III visar vi att detta sker genom ett protein på bakteriens yttermembran som heter P5. Vi visar också att om man på konstgjord väg tar bort P5 från bakteriens yta så binder den mindre eller inget C4BP alls. Bakterien avdödas då snabbare när den kommer i kontakt med mänskligt serum (den del av blodet där komplementsystemet finns).

Sammanfattningsvis tyder de data som presenteras i denna avhandling på att andra skiften av aminosyror, än de som tidigare har beskrivits, i penicillinbindnande proteiner hos *H. influenzae* kan modifiera bakteriens känslighet för vissa antibiotika såsom amoxicillin. Vidare stödjer våra data nuvarande svenska

behandlingsrekommendationer som består i att använda bensylpenicillin som empirisk behandling av milda till måttliga sjukhuskrävande lunginflammationer. Slutligen visar vi att *H. influenzae* kan undvika avdödning av människans komplementsystem genom att med hjälp av protein P5 binda humant C4BP till sin yta.

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