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Title:

Serum resistance and phase variation of a nasopharyngeal non-typeable *Haemophilus influenzae* isolate

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ABSTRACT

Haemophilus influenzae harbours a complex array of factors to resist human complement attack. As non-typeable *H. influenzae* (NTHi) strains do not possess a capsule, their serum resistance mainly depends on other mechanisms including LOS decoration. In this report, we describe the identification of a highly serum resistant, nasopharyngeal isolate (NTHi23) by screening a collection of 77 clinical isolates. For NTHi23, we defined the MLST sequence type 1133, which matches the profile of a previously published invasive NTHi isolate. A detailed genetic analysis revealed that NTHi23 shares several complement evading mechanisms with invasive disease isolates. These mechanisms include the functional expression of a retrograde phospholipid trafficking system and the presumable decoration of the LOS structure with sialic acid. By screening the NTHi23 population for spontaneous decreased serum resistance, we identified a clone, which was about 10^3 -fold more sensitive to complement-mediated killing. Genome-wide analysis of this isolate revealed a phase variation in the N⁷-terminal region of *lpsA*, leading to a truncated version of the glycosyltransferase (LpsA). We further showed that a *lpsA* NTHi23 knockout mutant exhibits a decreased invasion rate into human alveolar basal epithelial cells. Since only a small proportion of the NTHi23 population expressed the serum sensitive phenotype, resulting from *lpsA* phase-off, we conclude that the nasopharyngeal environment selected for a population expressing the intact and functional glycosyltransferase.

INTRODUCTION

The Gram-negative bacterium *Haemophilus influenzae* is a human-specific pathogen, which can be subdivided into encapsulated and non-encapsulated strains. The latter are usually referred to as non-typeable *H. influenzae* (NTHi) strains (Pittman, 1931). Encapsulated *H. influenzae* strains, foremost of type b capsule (Hib), are responsible for a variety of severe invasive diseases, and are mostly observed in infants and children, causing epiglottitis, sepsis, and meningitis. After the introduction of an effective vaccination strategy against type b capsule strains in the early 1990s, invasive diseases decreased substantially in populations in which the vaccine was applied (Kelly et al., 2004; Ulanova, 2013). Since Hib-vaccine is type b capsule specific, it does not protect against NTHi caused diseases. NTHi strains are mostly assigned to the commensals of the respiratory tract, and are mainly producing local disease in the upper and lower respiratory tract, such as acute otitis media, sinusitis, and bronchitis. Additionally, they cause exacerbations in chronic diseases such as asthma and chronic obstructive pulmonary disease (COPD). Since the introduction of the Hib vaccine and the resulting decrease in Hib infections, more attention was attracted by NTHi strains. Thereby, genotypic and phenotypic differences were characterized trying to define clades of invasive isolates (Erwin et al., 2005; Erwin et al., 2008). Increasing numbers of reports indicate that NTHi cause invasive diseases, which are as serious as those caused by encapsulated strains and which occur in infants, children and in adults. Hence, there is evidence for a shift from invasive diseases caused by encapsulated strains to those caused by NTHi infections as monitored in vaccinated areas (Giufre et al., 2011; Rubach et al., 2011).

Human pathogens, including *H. influenzae*, need to constantly cope with the various defence mechanisms of the immune system. *H. influenzae* is susceptible to complement mediated killing and in order to infect its human host it has to counteract this otherwise lethal attack (Zola et al., 2009).

H. influenzae strains have developed several sophisticated strategies to evade the complement system. The genetic locus of the polysaccharide capsule of typeable strains was shown to be frequently amplified in clinical isolates, which was associated with decreased complement mediated killing (Noel et al., 1996). Furthermore, diverse outer membrane proteins were shown to be involved in complement resistance. In 2014, Rosadini et al. identified outer membrane protein P5 as an essential factor in serum resistance. They showed that P5 decreases binding of IgM antibodies and thereby is interfering with classical complement activation. Additionally, P5 can bind factor H, which inhibits the alternative pathway of complement-mediated lysis (Rosadini et al., 2014). Another outer membrane protein, namely adhesion protein E (PE), was shown to bind vitronectin, an important inhibitor of the membrane attack complex. Thus, *H. influenzae* lacking PE was rendered more susceptible towards complement mediated killing (Hallstrom et al., 2009). This implicates another strategy employed by *H. influenzae*, which is attracting complement regulatory factors to hinder the complement cascade to proceed. In addition to binding factor H and vitronectin (Hallstrom et al., 2009; Hallstrom et al., 2006; Hallstrom et al., 2008), NTHi decrease classical complement activation by sequestering C4b binding protein (Hallstrom et al., 2007).

Furthermore, phase variable LOS structures have been shown to play a major role in resistance to the classical as well as the alternative pathway of complement activation. In 1993, High et al. identified *lic2A* as a phase-variable gene, facilitating the Gal(1-4)beta Gal extension to the LOS structure (High et al., 1993) Not only *lic2A* but also *lgtC* and *lex2A* were shown to switch from phase-off to phase-on during passage through human serum. LgtC as well as Lex2A independently contribute to better survival by shielding conserved LOS inner core structures (Clark et al., 2013).

Due to the phase variability of LOS, a distinct *H. influenzae* population comprises different phase variants with different structures and modifications. The respective host condition might select for a modification-specific population while clearing the other. The phase variable structure phosphorylcholine (ChoP) represents one example, where one environment selects for phase-on while another environment selects for phase-off (Weiser et al., 1998). ChoP is recognized by C-

reactive protein (CRP), which in turn leads to the activation of the complement system. Environments with high levels of CRP select for *lic1* phase-off variants, which lack the ChoP moiety (Clark et al., 2012; Tong et al., 2000; Weiser et al., 1998). In other environments this modification seems to be advantageous. Therefore, the phase variability of the *lic1* locus provides an efficient mean to frequently turn off and on gene expression, depending on the environment *H. influenzae* faces. This is also suggested for other phase variable loci, which are associated with high mutation rates and will need further investigations (Clark et al., 2013).

As surveillance programs report a steady increase in invasive disease due to non-typeable strains (Giufre et al., 2011; Rubach et al., 2011), we conducted a detailed analysis of a strain collection consisting of invasive and non-invasive isolates with regards to their serum resistance profile. Our findings highlight that there is no observable correlation between serum resistance and invasiveness. Interestingly, our studies identified a highly serum resistant isolate (NTHi23), which was derived from a healthy donor during a routine examination. In addition, we revealed that the high serum resistance of the isolate depends on the phase variable gene *lpsA*, involved in LOS synthesis and enhanced invasion into epithelial cells.

MATERIALS AND METHODS

Ethics statement. Human blood was obtained from five healthy adults in accordance with the Ethical Review Committee of the University of Graz (39/31/63 ex 2012/13). All subjects provided written consent.

Culturing methods and growth conditions. Clinical NTHi isolates used during this study were obtained from AGES-Graz (Agentur Gesundheit und Ernährungssicherheit / Zentrum fuer lebensmittelbedingte Infektionskrankheiten) and the Institute for Hygiene and Microbiology (University of Würzburg, Germany). 46 strains are classified as invasive and 31 as non-invasive isolates. Among them, NTHi23 was isolated from a 6 year-old female during routine examination (personal communication Dr. Georg Steindl, AGES Graz) and was analysed in detail. All generated, isogenic mutants are listed in Table 1. *H. influenzae* strains were grown aerobically at 37°C in brain heart infusion (BHI) supplemented with 10 µg/ml NAD and hemin-solution, containing 20 µg/ml hemin, 20 µg/ml L-histidine and 0.08% triethanolamine (sBHI). To select for chloramphenicol^r (Cm, 2 µg/ml) or kanamycin^r (Km, 50 µg/ml) corresponding resistance gene cassettes were applied (see below). A549 cells (human alveolar lung adenocarcinoma cells; ATCC CCL87) were cultured in DMEM supplemented with 10% fetal calf serum at 37 °C with 5% CO₂.

Construction of deletion mutants. Deletion mutants were constructed as previously described (Lichtenegger et al., 2014), using an overlap extension PCR. Briefly, the target gene was exchanged by a Cm or Km resistance cassette obtained from plasmid pAKcat (Kraiss et al., 1998) or pKan π (Oka et al., 1981), respectively. To amplify the three fragments, six oligonucleotide primers (x₁ – x₆) were applied with x corresponding to the gene of interest. The upstream fragment was generated using x₁ and x₂, the antibiotic resistance cassette using x₃ and x₄ and the downstream fragment using x₅ and x₆. Oligonucleotides are listed in Table 2. The final PCR fragment was transformed into strain NTHi23, applying a modification of the static aerobic method established by Gromkova et al. (Gromkova et al., 1989). One single colony was suspended in 200

μl sBHI (supplemented BHI medium) and incubated with DNA at 30°C overnight. Positive transformants were selected using agar plates containing the appropriate antibiotic.

***lpsA* complementation.** The *lpsA* mutation was chromosomally restored according to a previously described method (Lichtenegger et al., 2014). The *lpsA* gene was fused to a Km resistance cassette by overlap extension PCR and the final fragment was transformed into NTHi23 Δ *lpsA* by the above-described transformation procedure.

Serum bactericidal assays. Bactericidal assays were carried out as previously described (Lichtenegger et al., 2014). Serum from 5 healthy volunteers served as a source of antibodies and complement factors. Cells were grown to mid-log phase under shaking conditions at 180 rpm and transferred to Hank's buffer. Normal human serum was applied in concentrations varying from 3-35%. After incubation for 45 min at 37°C with rotation, cells were plated on sBHI to determine viable cell counts. In parallel either a non-serum or heat-inactivated control was performed. Heat-inactivated serum was generated at 56°C for 30 min.

High-throughput bactericidal assays. High-throughput assays were performed in 96 well polystyrene plates. Isolates were grown to mid-log phase in static liquid cultures. Mid-log phase cultures were shifted to Hank's buffer containing 20% of human serum. After incubation at 37°C for 45 min alamarBlue cell viability reagent array was added to detect metabolic activity, indicating viable and metabolic active bacterial cells. Viable cells reduce the active component of alamarBlue (resazurin) to resorufin, which is fluorescent. Fluorescence was monitored 2 h after addition of the reagent in a plate-based fluorescence spectrometer (FLUOstar, OMEGA, BMG Labtech). The fluorescence signal, we observed for the heat-inactivated serum control, was set to 100%.

Invasion assay. Adhesion and invasion assays were performed as previously described (Tchoupa et al., 2015). 24 hours prior to infection A549 cells were seeded in 12 well plates at a density of 3×10^5 cells per well. Cells were infected with *H. influenzae* NTHi23 wt strain or the *lpsA* mutant at a

multiplicity of infection (MOI) of 100. Two hours after infection the bacteria containing medium was replaced by cell culture medium containing 100 µg/ml gentamicin to efficiently kill extracellular bacteria. After a 1 hour incubation step, the number of intracellular bacteria was determined. Consequently, A549 cells were lysed using PBS containing 0.5% saponin for 15 minutes. To determine viable bacterial cell counts, the lysates were plated on sBHI agar plates at the appropriate dilutions. The number of internalized bacteria was normalized to the bacterial input.

Adhesion assay. Adhesion assays were performed in parallel to the invasion assay and according to (Voges et al., 2012). Consequently, A549 cells were seeded 24 hours prior to infection at a cell density of 3×10^5 cells per well. Cells were infected with *H. influenzae* NTHi23 wt strain or the *lpsA* mutant at a multiplicity of infection (MOI) of 100. Two hours after infection the bacteria containing supernatant was removed and cells were washed twice with PBS and lysed with PBS containing 0.5% saponin for 15 minutes. To determine viable bacterial cell counts, the lysates were plated on sBHI agar plates at the appropriate dilutions. The number of adherent bacteria was calculated by subtracting the number of intracellular bacteria from the in parallel-performed invasion assay from the number of viable bacteria determined during the adhesion assay. The number of adherent bacteria was normalized to the bacterial input.

MLST analysis. MLST analysis was performed according to the MLST *H. influenzae* database (<http://haemophilus.mlst.net>). Briefly, defined fragments of seven *H. influenzae* housekeeping genes, namely *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi* and *recA*, were amplified with the oligonucleotides listed in Table 2. After sequencing the amplified fragments (Table S1), the encoding gene sequences were entered in the MLST database and the allele number for each gene was obtained. The MLST database provides a sequence type in consequence of the allele profile and subsequently enables you to identify genetic matches.

Genome sequencing. NTHi23 and NTHi23_variant genome sequences were determined by using the Illumina technology. Libraries were constructed by using the Illumina Nextera XT kit. Sequencing was performed on an Illumina MiSeq, 140 bases single read run. Sequences were

assembled with Velvet (DOI 10.1101/gr.074492.107) and an optimized K value. Differences (SNP and short INDELS) between the two isolates were determined by aligning reads of strains NTHi23_variant on the contigs of strain NTHi23 using BRESEQ (DOI 10.1007/978-1-4939-0554-6_12). Contig sequences of strain NTHi23 were deposited at EMBL (Genome project pending).

RESULTS AND DISCUSSION

Serum resistance does not coincide with invasiveness. Although non-typeable *H. influenzae* strains usually cause mucosal infections, they are increasingly reported to cause invasive disease (Giufre et al., 2011; Rubach et al., 2011). In general, serum resistance is suggested to facilitate the spread of bacterial pathogens in the human body. In the present study we investigated 77 clinical NTHi strains, isolated from various infection sites, for their ability to survive in 20 % human serum. The isolates were collected from patients suffering from various diseases (lymphadenitis, meningitis, pneumonia, mastoiditis, endophthalmitis, sepsis, and sinusitis). We blotted and compared the data for invasive (n = 46) and non-invasive obtained isolates (n = 31) Figure 1 to elucidate a potential correlation between serum resistance and invasiveness. As there was no significant difference between the two groups, the hypothesis of a correlation between serum resistance and invasiveness cannot be confirmed. These results are in agreement with a study by Hallström et al., which suggests that serum resistance is equally important for strains causing upper respiratory tract infections and strains causing sepsis. Nevertheless, they found that serum resistance correlates with the severity of the disease and might therefore become increasingly important once the infection is established (Hallstrom and Riesbeck, 2010). We extend and support this analysis by characterizing NTHi strains from multiple infection sites and conclude that high serum resistance is not an exclusive marker of invasive strains.

An NTHi isolate displays a similar serum resistance profile when compared to an encapsulated strain. In the course of the performed high-throughput assay (see Material and Methods), we identified a recognizable highly serum resistant strain NTHi23, which was isolated from the nasopharynx of an asymptomatic 6-years old female patient. In Figure 2 we compare the serum survival rate of NTHi23 and the well-characterized type b strain, Hib Eagan (Noel et al., 1996; Sutton et al., 1982). The polysaccharide capsule of the typeable strain represents an important

protective coat against complement-mediated attack (Cerquetti et al., 2005; Corn et al., 1993). Although, the obtained survival data between the two strains show no significant differences, NTHi23 tends to display even higher survival in 35% human serum than the type b strain. Therefore, these findings provide further evidence that NTHi strains have developed sophisticated strategies for complement evasion, compensating for the lack of a capsule.

MLST analysis reveals an exact sequence type match of NTHi23 with a previously identified disease isolate. Highly serum resistant NTHi strains, although having the potential to cause disease, also persist in a commensal state (Hallstrom et al., 2010). To further unravel the nature and origin of NTHi23, we performed an MLST analysis (Table S1). This analysis revealed that NTHi23 displays an exact sequence type match with NTHi strain Hi391, isolated from a patient suffering from invasive disease (Giufre et al., 2011). This finding supports the hypothesis, that host factors play a prominent role in the outcome of a *H. influenzae* infection. According to this hypothesis, a similar isolate can reside as a commensal in the nasopharynx of one patient, while having the potential to cause severe disease in another patient. NTHi strains are more likely to cause disease in immunocompromised patients. However, an invasive outcome of an *H. influenzae* infection not always correlates with the immune status of the human carrier (Hallstrom et al., 2010). This indicates that other factors are involved in the clinical phenotype of an infection. These factors might include virulence factors of the particular strain and potentially pre-existing antibodies of the host due to prior contact to other *H. influenzae* strains (Hallstrom et al., 2010). Taken together, this analysis strengthens the hypothesis that the invasiveness and virulence of a particular strain relies on a complex array of bacterial as well as host factors.

Pathways, previously associated with serum resistance, play a crucial role in the serum resistant phenotype of strain NTHi23. Several mechanisms and pathways contributing to serum resistance were unravelled recently. These mechanisms include the incorporation of sialic acid into the LOS structure (Figueira et al., 2007), the overexpression of a retrograde phospholipid trafficking system (Malinverni and Silhavy, 2009; Nakamura et al., 2011) and the expression of the hypothetical protein Hi0461 (Langereis et al., 2012; Nizet et al., 1996). In order to obtain a comprehensive overview of serum resistance mechanisms for NTHi23 a detailed characterization was conducted.

H. influenzae lacks the ability to *de novo* synthesize sialic acid and therefore relies on the uptake of external sources (Severi et al., 2005). A deletion of *siaP* inactivates the tripartite sialic acid transport system (SiaPQM) and subsequently sialic acid attachment to the LOS structure. LOS modification with sialic acid has been attributed with a critical role in resistance to complement mediated killing by the alternative pathway (Figueira et al., 2007). Figure 3 shows that a *siaP*-mutant displays decreased survival in 20% human serum, when compared to the wt strain. This finding shows that SiaP function plays a vital role in the serum resistance of strain NTHi23.

In addition to LOS decoration with sialic acid, LOS compactness, which is influenced by the VacJ/Yrb ABC transport system, impacts the serum resistance of NTHi23. Not only a *siaP*-mutant, but also an *yrbE*-mutant shows decreased serum resistance (Figure 3) in this strain background. YrbE is part of a retrograde phospholipid trafficking system, comprising at least six proteins: VacJ, YrbC, YrbD, YrbE, YrbB, YrbF. The model for its involvement in serum resistance suggests that blocking the lipid trafficking system leads to an accumulation of phospholipids in the outer membrane and subsequently to the destabilization of intermolecular LOS interactions. This in turn exposes epitopes, which are recognized by bactericidal antibodies, leading to antibody-mediated killing. Strains, which were isolated from lower respiratory tract infections display increased expression of VacJ and YrbE (Malinverni and Silhavy, 2009; Nakamura et al., 2011). The observation that a *yrbE*-mutant is more susceptible to complement attack in NTHi23 suggests that

also in this strain LOS epitopes are recognized by NHS antibodies upon an alteration of the outer membrane phospholipids:LOS ratio.

As evident from Figure 3, a *Hi0461*- mutant also displays decreased serum resistance in the NTHi23 background. As shown previously, a deletion of the R2866_0112 gene, the homolog of Rd KW-20 *Hi0461*, leads to increased IgM binding and subsequently to an increase in serum sensitivity (Langereis et al., 2012). R2866_0112 mutants express various numbers of truncated LOS glycoforms. Furthermore, a prominent glycoform is absent and therefore it is tempting to suggest that R2866_0112/ *Hi0461* does not encode a transferase, which is directly involved in LOS biosynthesis (Langereis et al., 2012; Nizet et al., 1996).

In conclusion, our data demonstrate that the described mechanisms for serum resistance can be extended to our strain, strengthening the hypothesis that the underlying mechanisms can be considered general evasion strategies of *H. influenzae*. In addition the characterization of the three single mutants provides a first insight into the serum resistance mechanisms of strain NTHi23.

A subpopulation of the NTHi23 population displays altered serum resistance. Clark and colleagues hypothesized that bacteria evade killing by the host immune system through selective enrichment of resistant bacteria from a mixed population (Clark et al., 2013). Assuming that the isolated NTHi23 population also displays differences in their serum resistance status, we hypothesized that individual clones would express differences in their survival when incubated in human serum. The identification of more serum susceptible variants could contribute to unravel mechanisms involved in the serum resistance of the investigated NTHi strain. Therefore, 60 individual clones were tested in a high-throughput assay for their survival in human serum. In the course of this study, we identified a variant of the strain (NTHi23_variant), which was nearly 1000-fold more sensitive than the mixed population. To validate the obtained results, we tested the variant in our standar serum assay, which confirmed the serum sensitive phenotype (Figure 4).

These results show that variations in serum resistance levels exist within the NTHi23 population. The observation, that only one out of 60 shows decreased serum resistance suggests that the *in vivo* situation, in this case the nasopharynx of a child, selected for serum resistant variants.

The NTHi23 population is enriched in *lpsA* phase-on. To determine the genetic basis of the serum sensitive phenotype of NTHi23_variant, the whole genome of the wt and the variant strain was sequenced. The sequence analysis revealed a 5 base insertion (GCACA) in *lpsA*. LpsA belongs to the family of GT-A glycosyltransferases and mediates the transfer of the initial hexose to Hep_{III} (Deadman et al., 2006). Interestingly, NTHi23 displays a short sequence repeat (5 nts per repeat) at the 5' end of the *lpsA* coding region. A seven repeat unit in strain NTHi23 leads to the expression of a functional protein. The five base pair insertion leads to a frame shift mutation in *lpsA* and subsequently to the formation of a preliminary stop codon and the expression of a truncated, most likely non-functional protein. Phase variations, resulting from slipped-strand mispairing, are frequently found in genes encoding LOS modifications, but were not comprehensively described for the *lpsA* gene.

To validate and investigate the observed *lpsA* phenotype, we constructed a deletion mutant and complementation strain for the phase variable gene. Figure 5 shows that the deletion strain displays similar serum sensitivity as observed for the phase-off variant. Reintroducing the phase-on variant of the *lpsA* gene in the deletion mutant complemented serum sensitivity. Collectively these data suggest that the serum sensitive phenotype of NTHi23_variant can presumably be attributed solely to the expression of a truncated version of LpsA.

The NTHi23 Δ *lpsA* mutant displays decreased invasion of epithelial cells. NTHi23 was isolated from a 6-year old carrier showing no signs of disease. To further determine the biological relevance of the *lpsA* phase variation and the fact that the phase-on variant was predominantly represented in

the isolated NTHi23 population, we performed an invasion and adhesion assay. The wt and the *lpsA* mutant adhered equally well to epithelial cells (Figure 6A). In contrast, the *lpsA* mutant was significantly attenuated in cell invasion (Figure 6B). This observation correlates with the observed serum sensitive phenotype of *lpsA*. The results of both assays might shed light on the *in vivo* selective pressure towards the *lpsA* phase-on variant in the nasopharynx of the human host. Whether the *lpsA* associated modification of the LOS structure plays a role in receptor mediated internalization into human epithelial cells remains elusive and needs further investigation.

At least four alleles of *lpsA* exist with the presence of one excluding the presence of the others. Differences in the amino acid sequence, encoded by the different alleles, determine whether a Glc or Gal is added in a β 1-2 or β 1-3 linkage. One single amino acid residue determines which hexose is added to Hep_{III}. Threonine leads to the addition of galactose whereas cysteine, alanine or methionine leads to the addition of a glucose moiety (Deadman et al., 2006). For NTHi23 the amino acid sequence predicts that *lpsA* displays glucosyltransferase activity as a cysteine is found at the amino acid position, which determines the attached residue. The nature of the initial hexose, substituted at Hep_{III}, has an important influence on bacterial survival. About 70% of NTHi strains express Hep_{III}-Glc, which is suggested to be recognized by naturally occurring IgM antibodies in turn leading to phagocytosis by human neutrophils (Langereis and Weiser, 2014). A Gal residue at this position, which is frequently found in the LOS of invasive strains, is not recognized by these IgM antibodies and seems therefore advantageous for invasive disease. Nevertheless, most of the strains investigated in the study by Langereis et al. express Hep_{III}-Glc. Therefore, it is tempting to suggest that this structure, although recognized by naturally occurring antibodies, is advantageous in some microenvironments (Langereis and Weiser, 2014). A similar phenomenon is observed for the presence of ChoP in the LOS of *H. influenzae* strains, which leads to high serum resistance during nasopharyngeal colonization (Clark et al., 2012; Poole et al., 2013), but results in complement activation in the bloodstream due to binding of circulating CRP (Weiser et al., 1998).

Additionally, HepIII-Glc is an acceptor for further extensions (e.g. the addition of a galactose moiety mediated by Lic2A), whereas Gal is suggested to be a terminal moiety. Additional substituents lead to shielding of the immune-dominant epitope HepIII-Glc and experimentally prevent IgM binding and phagocytosis (Langereis and Weiser, 2014). The fact that several USS sequences are located upstream and downstream of the *lpsA* gene leads to the assumption that this gene could be subject to horizontal gene transfer and might be readily exchanged between strains. This could represent an additional important tool to adapt to different niches within the human host as it is suggested that the addition of either a Glc or Gal potentially mediate survival in different niches (Langereis and Weiser, 2014).

The *lpsA* gene displays both intra- and interstrain variability. Intrastrain variation, which is defined as a difference in mother and progeny cells, arises from the phase variability of *lpsA*. According to a Nucleotide BLAST analysis, phase variable *lpsA* seems to be found only in a small proportion of NTHi strains. This potentially arises from the fact that it represents a glycosyltransferase mediating the initial substitution of one of the conserved heptoses. Interstrain specificity results from the different alleles found in distinct strains. In conclusion, *lpsA* plays a crucial role in the diversity and composition of the LOS molecule, which in turn mediates colonization efficiency and virulence.

Strain NTHi23 represents a highly serum resistant clinical isolate, which did not cause disease in a 6-year old female patient at the time of isolation. Nevertheless, it comprises several sophisticated strategies to cope with the immune system with LpsA presumably playing a crucial role. The phase-off switch in NTHi23_variant strengthens this hypothesis. Expressing a truncated LpsA protein, the NTHi23_variant most likely loses the ability to mediate LOS substitutions at Hep_{III}. This subsequently leads to the accessibility of LOS core structures, which are suggested to be immune-dominant epitopes (Clark et al., 2013). In addition, the modification of the LOS core structure by LpsA seems to play a significant role in the internalization of NTHi23 in human epithelial cells. The *lpsA* phase-off strain (NTHi23_variant) represented just a small proportion of the population (one out of sixty). This observation suggests that the predominant phenotype (*lpsA*

phase-on) was enriched during nasopharyngeal colonization. Interestingly, Poole and colleagues (Clark et al., 2012; Poole et al., 2013) monitored phase-variation during experimental colonization in the nasopharynx of healthy human volunteers with strain NTHi2019Str^R over a several days period. They observed that a significant number of isolates switched, e.g. shown for *licA* and *igaB* from phase-off to phase-on during a 4-6 days period. The authors conclude that these genes might play a role in early nasopharynx colonization. This might be also the case for the *lpsA* gene, thereby contributing to an efficient nasopharyngeal colonization. These observations suggest that the nasopharynx of healthy subjects can provide an environment, which favours the selection of phase-on variants of various genes. In addition, we cannot exclude, that the child acquired the NTHi strain from a diseased person (family, presumable kindergarten or school).

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Table 1: Bacterial strains used

	Genotype and Description	Reference
Hib strain Eagan	Encapsulated serotype b strain obtained from A. Wright	(Anderson et al., 1972)
NTHi23	NTHi nasopharyngeal, clinical isolate	Agentur fuer Gesundheit und Ernaehrungssicherheit GmbH (AGES)
NTHi23 Δ <i>siaP</i>	<i>siaP</i> replaced with Cm ^R cassette	This study
NTHi23 Δ <i>yrbE</i>	<i>yrbE</i> replaced with Cm ^R cassette	This study
NTHi23 Δ <i>Hi0461</i>	<i>Hi0461</i> replaced with Km ^R cassette	This study
NTHi23 Δ <i>lpsA</i>	<i>lpsA</i> replaced with Cm ^R cassette	This study
NTHi23 Δ <i>lpsA lpsA</i> ⁺	chromsomally restored <i>lpsA</i> Km ^R	This study

Table 2: Oligonucleotides used

Primers	Sequences (5`-3`)	Origin
<i>Mutagenesis</i>		
siaP_1	GTATAACCTGACATCGTACTGC	This study
siaP_2	ATTTAGTGTATGATGGTGTTTTTGAA TTCGCTCCTGTGTGAACTTAT	This study
siaP_3	ATAAGTTCACACAGGAGCGAATTCA AAAACACCATCATACTAAAT	This study
siaP_4	GAACTTTTGCACTTCATAGAATTTTAG GCGTTTAAGGGCACCAATAA	This study
siaP_5	TTATTGGTGCCCTTAAACGCCTAAAA TTCTATGAAGTGCAAAAGTTC	This study
siaP_6	TGGATAAAGCGAAACATCATTAATA	This study

0461_1	ATCATACCAAGCAAATATTCTAAAC	This study
0461_2	TCTTGTGCAATGTAACATCAGAGATTT TTCATTATTTTAGCCATTATCA	This study
0461_3	TGATAATGGCTAAAATAATGAAAAAT CTCTGATGTTACATTGCACAAGA	This study
0461_4	ACCCATTAACCAACATAAACATTGCGT GAAGAAGGTGTTGCTGAC	This study
0461_5	GTCAGCAACACCTTCTTCACGCAATGT TTATGTTGGTTAATGGGT	This study
0461_6	AAAGCGAAAGAAAAACAAGGTAAAA	This study
yrbE_1	ATAAACGAGATATTAATGAATCAAAT	(Roier et al., 2016)
yrbE_2	TGTATGATGGTGTTTTTGAGGTG GATCATTCAAACAATTCCTTCACA	(Roier et al., 2016)
yrbE_3	TGTGAAGGAATTGTTTGAATGAT CCACCTCAAAAACACCATCATACA	(Roier et al., 2016)
yrbE_4	TTGTTTGTCTCATAAAAAAATCCTT AAGGCGTTTAAGGGCACCAATAA	(Roier et al., 2016)
yrbE_5	TTATTGGTGCCCTTAAACGCCTTA AGGATTTTTTTATGAGACAAACAA	(Roier et al., 2016)
yrbE_6	GCAGAGGTTGATTTGTAGTATGA	(Roier et al., 2016)
lpsA_1	AAGCCGACAGTAAAGTCTGGAT	This study
lpsA_2	TGTATGATGGTGTTTTTGAGGTG CATAGAAATTTTAACCGCACTTTT	This study
lpsA_3	AAAAGTGCGGTAAAATTTCTAT GCACCTCAAAAACACCATCATACA	This study
lpsA_4	AAGATTTTTTAACCGCACTTTTGTTA GTTTAAGGGCACCAATAACTGC	This study
lpsA_5	GCAGTTATTGGTGCCCTTAAAC	This study

	TAACAAAAGTGC GGTTAAAAATCTT	
lpsA_6	CAACCGCACTTTGATGTCGTG	This study
<i>Complementation</i>		
lpsA+_1	AAGCCGACAGTAAAGTCTGGAT	This study
lpsA+_2	TCTTGTGCAATGTAACATCAGA ACATAGCCAAGTAAAATTTGAACTT	This study
lpsA+_3	AAGTTCAAATTTTACTTGGCTATGT TCTGATGTTACATTGCACAAGA	This study
lpsA+_4	CACAACAAAAGGCAGTCCAGC TGAAGAAGGTGTTGCTGACTCA	This study
lpsA+_5	TGAGTCAGCAACACCTTCTTCA GCTGGACTGCCTTTTGTGTG	This study
lpsA+_6	GCGATAGAGTGAAAGAAACGCT	This study
<i>MLST analysis¹</i>		
adk_up	GGTGCACCGGGTGCAGGTAA	
adk_down	CCTAAGATTTTATCTAACTC	
atpG_up	ATGGCAGGTGCAAAAGAGAT	
atpG_down	TTGTACAACAGGCTTTTGCG	
frdB_up	CTTATCGTTGGTCTTGCCGT	
frdB_down	TTGGCACTTTCCACTTTTCC	
fucK_up	ACCACTTTCGGCGTGGATGG	
fucK_down	AAGATTTCCAGGTGCCAGA	
mdh_up	TCATTGTATGATATTGCCCC	
mdh_down	ACTTCTGTACCTGCATTTTG	
pgi_up	GGTGAAAAAATCAATCGTAC	
pgi_down	ATTGAAAGACCAATAGCTGA	
recA_up	ATGGCAACTCAAGAAGAAAA	
recA_down	TTACCAAACATCACGCCTAT	

¹according to Meats et al., 2003

Figure 1: **No observable correlation of serum resistance and invasiveness.** Clinical isolates were tested for their resistance to 20% human serum. Invasive isolates (n=46) and non-invasive isolates (n=31) do not show a significant difference in their survival rates ($P > 0.05$, Mann-Whitney U Test). The fluorescence levels are a measure of survival by indicating metabolic activity. The heat-inactivated serum control treated sample readout corresponds to 100% fluorescence signal. Shown is the median with interquartile range, measurement was done once with two technical replicates.

Figure 2: **NTHi23 shows similar serum resistance when compared to the encapsulated strain Hib Eagan.** The survival of the two strains was determined in different amounts of serum. Shown are the mean values and the standard deviation of three independent measurements, normalized to a 0% serum control. To note, no statistical significance can be obtained, e.g. using 2-way ANOVA with Bonferroni posttest for all time points tested.

Figure 3 **Commonly identified serum resistance mechanisms can also be found in NTHi23.** All three mutants (NTHi23 $\Delta siaP$, $\Delta yrbE$ and $\Delta Hi0461$) show decreased survival in 20% human serum, when compared to the NTHi23 wt. Data were normalized to a heat-inactivated control. Median and interquartile range of at least five independent measurements are shown. Single asterisks mark significant differences ($P < 0.05$; Unpaired t Test).

Figure 4: ***In vitro* cultivation leads to the identification of a serum sensitive variant of NTHi23.** The isolated variant strain of NTHi23 shows 1000-fold decreased serum resistance when compared to the original strain in 20% normalized human serum. Data were normalized to a heat-inactivated control. Median and interquartile range is indicated of at least four independent measurements. An asterisk marks significant differences ($P < 0.05$; Mann-Whitney U Test).

Figure 5: NTHi23 $\Delta lpsA$ confirms the role of the $lpsA$ phase-off switch in the serum sensitive phenotype of NTHi23_variant. NTHi23 wt, variant, $\Delta lpsA$ and $\Delta lpsA lpsA^+$ were tested for survival in 20% normal human serum. Both, the $lpsA$ deletion and the $lpsA$ phase-off mutant (NTHi23_variant) show significantly decreased serum resistance when compared to the wt and show comparable survival rates when compared to each other. The complementation strain ($\Delta lpsA lpsA^+$) reaches serum resistance levels as high as the wt strain. Shown is the median and interquartile range of four independent assays. Asterisks mark significant differences ($P < 0.05$; Mann-Whitney U Test).

Figure 6: The NTHi23 wt strain outcompetes $\Delta lpsA$ in the invasion of epithelial cells. NTHi23 wt and $\Delta lpsA$ were tested for adhesion and invasion of human epithelial cells. The wt and the mutant strain show no significant difference in adhesion to A549 cells (A). For the invasion assay, $\Delta lpsA$ shows significant attenuation (B). The assays were performed three times independently, with three technical replicates each. Shown is median and interquartile range. The asterisk marks a significant difference ($P < 0.05$; Mann-Whitney U Test).

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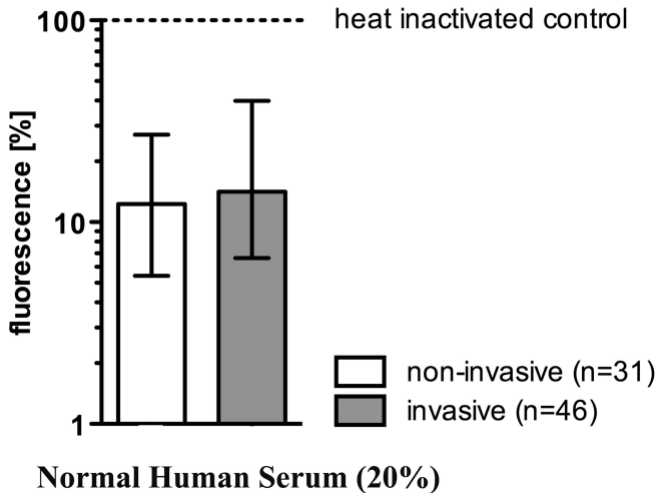
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Fig.1



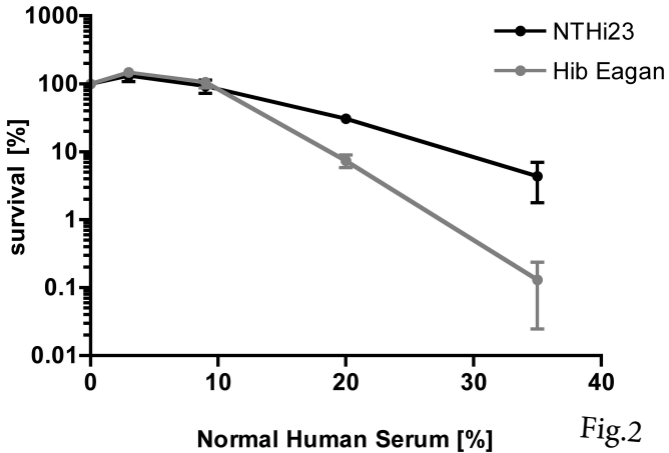


Fig.3

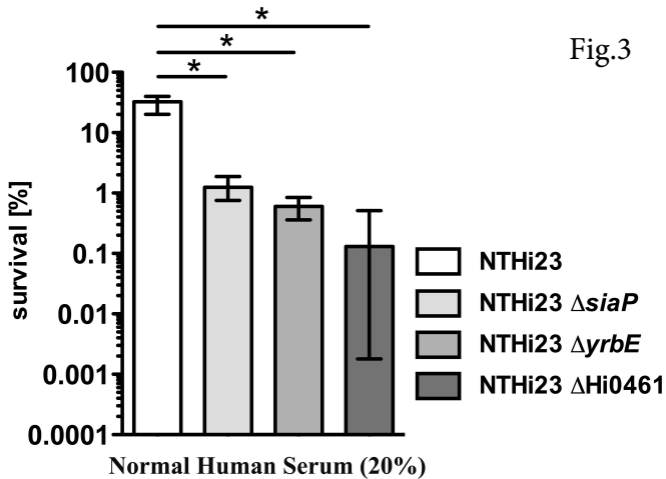


Fig.4

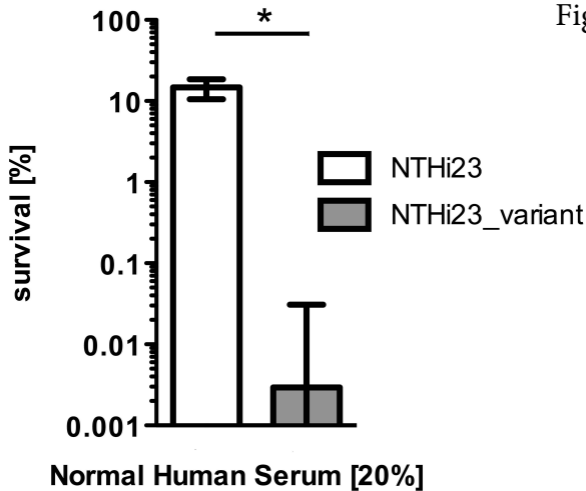
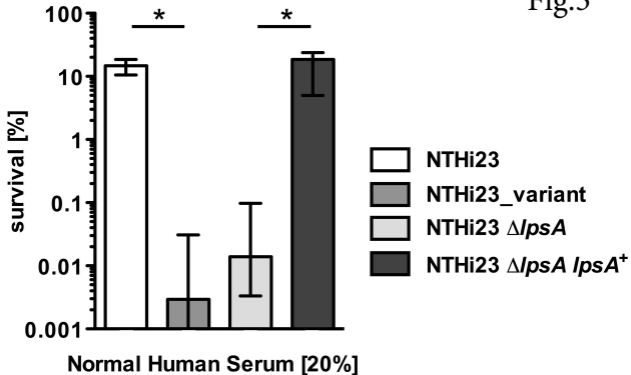


Fig.5



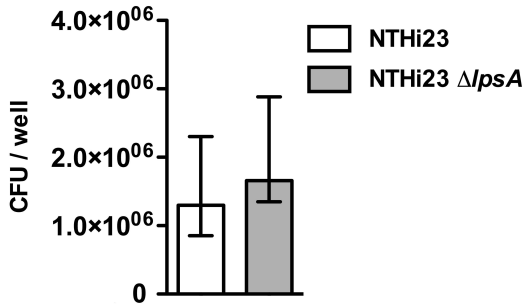
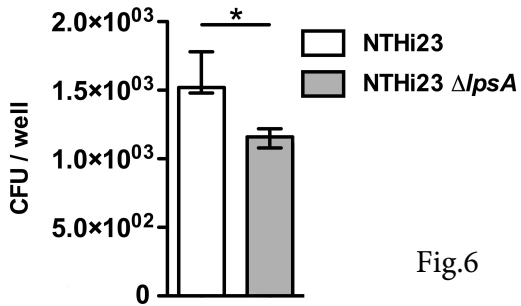
A**B**

Fig.6