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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 N'-terminal region of *lpsA*, leading to a truncated version of the glycosyltransferase (LpsA). We further showed that a NTHi23 *lpsA* 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.

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1. 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

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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 *lic*1 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.

2. Materials and methods

2.1. 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.

2.2. Culturing methods and growth conditions

Clinical NTHi isolates used during this study were obtained from AGES-Graz (Agentur Gesundheit und Ernaehrungssicherheit/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% CO_2.

2.3. 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 (x_1–x_6) were applied with x corresponding to the gene of interest. The upstream fragment was generated using x_1 and x_2, the antibiotic resistance cassette using x_3 and x_4 and the downstream fragment using x_5 and x_6. 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.

2.4. 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.

2.5. 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 Hankís buffer. Normal human serum was applied in concentrations varying from 3 to 35%. After incubation for 45 min at 37 °C with rotation, cells were plated on sBHI to determine viable

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