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A basis for vaccine development: Comparative characterization of *Haemophilus influenzae* outer membrane vesicles

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ABSTRACT

Outer membrane vesicles (OMVs) are spherical and bilayered particles that are naturally released from the outer membrane (OM) of Gram-negative bacteria. They have been proposed to possess several biological roles in pathogenesis and interbacterial interactions. Additionally, OMVs have been suggested as potential vaccine candidates against infections caused by pathogenic bacteria like *Haemophilus influenzae*, a human pathogen of the respiratory tract. Unfortunately, there is still a lack of fundamental knowledge regarding OMV biogenesis, protein sorting into OMVs, OMV size and quantity, as well as OMV composition in *H. influenzae*. Thus, this study comprehensively characterized and compared OMVs and OMs derived from heterologous encapsulated as well as nonencapsulated *H. influenzae* strains. Semiquantitative immunoblot analysis revealed that certain OM proteins are enriched or excluded in OMVs suggesting the presence of regulated protein sorting mechanisms into OMVs as well as interconnected OMV biogenesis mechanisms in *H. influenzae*. Nanoparticle tracking analysis, transmission electron microscopy, as well as protein and lipooligosaccharide quantifications demonstrated that heterologous *H. influenzae* strains differ in their OMV size and quantity. Lipidomic analyses identified palmitic acid as the most abundant fatty acid, while phosphatidylethanolamine was found to be the most dominant phospholipid present in OMVs and the OM of all strains tested. Proteomic analysis confirmed that *H. influenzae* OMVs contain vaccine candidate proteins as well as important virulence factors. These findings contribute to the understanding of OMV biogenesis as well as biological roles of OMVs and, in addition, may be important for the future development of OMV based vaccines against *H. influenzae* infections.

Introduction

Haemophilus influenzae is a small Gram-negative coccobacillus belonging to the *Pasteurellaceae* family of bacteria. Some strains possess a polysaccharide capsule allowing the division into six capsular serotypes (a–f), whereas others are nonencapsulated and thus cannot be serotyped. These strains are designated as nontypeable *H. influenzae*, commonly abbreviated as NTHi. So far, the only known natural habitat of *H. influenzae* is the human respiratory tract. Among the encapsulated strains, serotype b (Hib) is the main human pathogen causing, e.g. meningitis, acute epiglottitis, and sepsis. Although NTHi strains are frequently part of the normal nasopharyngeal flora in most humans, some strains can cause severe infections like otitis media, sinusitis, pneumonia, as well as exacerbations of chronic obstructive pulmonary disease (COPD) (Erwin and Smith, 2007; Johnston and Apicella, 2009; Ulanova and Tsang, 2009; Van Eldere et al., 2014). COPD, in particular, has been a major public health problem and is expected to increase its burden of disease over the next few years to become the third leading cause of death worldwide by 2020 (Soriano and Lamprecht, 2012).

Due to the lack of a conserved capsule as well as highly variable phenotypes and genotypes, there is currently no proven efficient vaccine against NTHi available (Poolman et al., 2000; van Alphen et al., 1997; Van Eldere et al., 2014). However, since studies report increasing invasive NTHi infections as well as the increasing spread of antibiotic-resistant NTHi strains, an efficient vaccine against

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NTHi is highly required (Ladhani et al., 2010; Van Eldere et al., 2014). Therefore, we have recently tested a potential NTHi vaccine candidate in mice and have demonstrated that outer membrane vesicles (OMVs) induce a robust and complex humoral, mucosal, and protective immune response against homologous and heterologous NTHi strains (Roier et al., 2012).

OMVs are naturally released from the outer membrane (OM) of Gram-negative bacteria. These small, spherical, and bilayered particles (approx. 10–300 nm in diameter) have been shown to contain phospholipids, lipopolysaccharides (LPS) or lipooligosaccharides (LOS), outer membrane proteins (OMPs), periplasmic proteins, cell-wall components, proteins of the inner membrane, cytoplasmic proteins, as well as DNA, RNA, ions, metabolites and signalling molecules (Beveridge, 1999; Kulkarni and Jagannadham, 2014; Kulp and Kuehn, 2010; Lee et al., 2008). Because of their energy-consuming production as well as their interesting cargo material, several biological roles of OMVs have been discussed soon after their discovery. Consequently, OMVs have been proposed to play a role in biofilm formation, adherence to host cells, intra- and interspecies delivery of biomolecules, resistance against antibiotics, killing of competing microorganisms, stress response, and modulation of host immune response (Berleman and Auer, 2013; Bonnington and Kuehn, 2014; Ellis and Kuehn, 2010; Haurat et al., 2014; Kulkarni and Jagannadham, 2014; Kulp and Kuehn, 2010).

Although OMV secretion in *H. influenzae* was already discovered in 1982 proposing a role of OMVs in natural competence development (Concino and Goodgal, 1982; Deich and Hoyer, 1982; Kahn et al., 1983), research has only recently regained interest in *H. influenzae* OMVs. For example, it was shown that NTHi OMVs are released within biofilms (Hong et al., 2007), that they contain virulence-associated proteins that can interact with and invade host epithelial cells (Ren et al., 2012; Sharpe et al., 2011), that OMVs derived from β-lactam-resistant NTHi strains contain β-lactamase (Schaar et al., 2014), and that they activate B cells in a T cell-independent manner consequently promoting bacterial survival within the human host (Deknuydt et al., 2014). Unfortunately, there is still a significant lack of fundamental knowledge about *H. influenzae* OMVs. To consider OMVs as therapeutic agents, e.g. as antigen delivery vehicles in vaccine design, future research needs to address the mechanisms of OMV biogenesis. This includes the mechanisms of protein sorting into OMVs, the specific components and composition of OMVs, the OMV size distribution, as well as the quantity of OMVs produced by *H. influenzae*. Therefore, the present study addresses some of these fundamental research topics and tries to shed more light on these very promising vaccine candidates. Here, we report a comprehensive comparative characterization of OMVs and OMs derived from heterologous encapsulated as well as nonencapsulated *H. influenzae* strains in terms of OMP exclusion or enrichment in OMVs, OMV size distributions, OMV quantity, as well as lipidomic and proteomic analyses of OMVs and OMs.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Reference
***E. coli* strain**		
DH5αλpir	F− *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYAargF*) U169, *hsdR17*(rK− mK+), λpirRK6	Hanahan (1983)
***H. influenzae* strains**		
Rd KW20	Unencapsulated variant of a former capsular serotype d strain, obtained from A. Wright	Wilcox and Smith (1975)
Rd *p1*::*cat*	Tn*10d-cat* insertion into *p1* (HI_0401) of Rd-KW20, Cm^r	Kraiss et al. (1998)
Rd Δ*p2*	Deletion of *p2* (Hi_0139) in Rd KW20, Cm^r	Andersen et al. (2003)
Rd Δ*p4*	Deletion of *p4* (Hi_0693) in Rd KW20, also referred to as REI1012, Km^r	Kemmer et al. (2001)
Rd Δ*p5*	Deletion of *p5* (HI_1164) in Rd KW20, Cm^r	Lichtenegger et al. (2014)
Rd Δ*p6*	Deletion of *p6* (HI_0381) in Rd KW20, Cm^r	This study
Hib strain Eagan	Capsular serotype b strain, obtained from A. Wright	Moxon and Murphy (1978)
NTHi 1479-R	Spontaneous Sm^r derivative of NTHi 1479	Roier et al. (2012)
NTHi 2019-R	Spontaneous Sm^r derivative of NTHi 2019	Roier et al. (2012)
NTHi 3198-R	Spontaneous Sm^r derivative of NTHi 3198	Roier et al. (2012)
NTHi 9274-R	Spontaneous Sm^r derivative of NTHi 9274	Roier et al. (2012)
Plasmids		
pMal-c2X-His6	Modified pMal-c2X expression vector (New England Biolabs) carrying a TEV cleavage site and a *N*-terminal His6 tag fused to *malE*, IPTG induceable, Ap^r	Childers et al. (2011)
pMalP1	*p1* (HI_0401) of Rd KW20 in pMal-c2X-His6, Ap^r	This study
pMalP2	*p2* (HI_0139) of Rd KW20 in pMal-c2X-His6, Ap^r	This study
pMalP4	*p4* (HI_0693) of Rd KW20 in pMal-c2X-His6, Ap^r	This study
pMalP5	*p5* (HI_1164) of Rd KW20 in pMal-c2X-His6, Ap^r	This study
pQE-30	Expression vector with *N*-terminal His6 tag, IPTG induceable, Ap^r	Qiagen
pQEP6	*p6* (HI_0381) of Rd KW20 in pQE-30, Ap^r	This study
pAKcat	pACYC177, Tn*10d-cat*, Km^r, Ap^r	Kraiss et al. (1998)

Materials and methods

Ethics statement

Female BALB/c mice (Charles River Laboratories) were used for all immunization experiments in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the national "Bundesgesetzblatt für die Republik Österreich". The corresponding animal protocol (39/53/00 ex 2012/13) has been approved by the Austrian Federal Ministry of Science and Research Ref. II/10b and the Committee on the Ethics of Animal Experiments of the University of Graz. Mice were housed with food and water ad libitum and monitored under the care of full-time staff and in accordance with the rules of the Institute of Molecular Biosciences at the University of Graz.

Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1, oligonucleotide primers are listed in Table S1. Unless stated otherwise, bacteria were grown at 37 °C with aeration in Luria-Bertani (LB) broth or on LB agar in the case of *Escherichia coli* as well as in brain heart infusion (BHI) broth or on BHI agar supplemented with NAD and hemin-solution (stock-solution containing a mixture of hemin, L-histidine, and triethanolamine) in the case of *H. influenzae*. Supplements were used in the following final concentrations: NAD, 10 μg/ml; hemin, 20 μg/ml; L-histidine, 20 μg/ml;