Veterinary Microbiology 167 (2013) 565–572

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

# Outer membrane vesicles reflect environmental cues in *Gallibacterium anatis*

Ragnhild J. Bager [a,1], Gry Persson [a,1], Barbara Nesta [b], Marco Soriani [b], Laura Serino [b], Marina Jeppsson [c], Tine K. Nielsen [c], Anders M. Bojesen [a,*]

[a] Department of Veterinary Disease Biology, Faculty of Health Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark
[b] Novartis Vaccines and Diagnostics, 53100 Siena, Italy
[c] The Novo Nordisk Foundation Center for Protein Research, Faculty of Health Sciences, University of Copenhagen, 2200 Copenhagen N, Denmark

ARTICLE INFO

*Article history:*
Received 30 June 2013
Received in revised form 4 September 2013
Accepted 6 September 2013

*Keywords:*
*Gallibacterium anatis*
Outer membrane vesicles
*tolR* deletion
FhaB
OmpC
OmpA

ABSTRACT

The Gram-negative bacterium *Gallibacterium anatis* is a major cause of salpingitis and peritonitis in egg-laying chickens, leading to decreased egg-production worldwide. Increased knowledge of the pathogenesis and virulence factors is important to better understand and prevent the negative effects of *G. anatis*. To this end outer membrane vesicles (OMVs) are natural secretion products of Gram-negative bacteria, displaying an enormous functional diversity and promising results as vaccine candidates. This is the first study to report that *G. anatis* secretes OMVs during *in vitro* growth. By use of transmission electron microscopy (TEM) and SDS-PAGE, we showed that changes in *in vitro* growth conditions, including incubation time, media composition and temperature, affected the OMV production and protein composition. A large protein band was increased in its concentration after prolonged growth. Analysis by LC–MS/MS indicated that the band contained two proteins; the 320.1 kDa FHA precursor, FhaB, and a 407.8 kDa protein containing a von Willebrand factor type A (vWA) domain. Additional two major outer-membrane (OM) proteins could be identified in all samples; the OmpH-homolog, OmpC, and OmpA. To understand the OMV formation better, a *tolR* deletion mutation (Δ*tolR*) was generated in *G. anatis*. This resulted in a constantly high and growth-phase independent production of OMVs, suggesting that depletion of peptidoglycan linkages plays a role in the OMV formation in *G. anatis*.

In conclusion, our results show that *G. anatis* produce OMVs *in vitro* and the OMV protein profile suggests that the production is an important and well-regulated ability employed by the bacteria, which may be used for vaccine production purposes.



## 1. Introduction

The Gram-negative *Gallibacterium anatis* is a genus in the *Pasteurellaceae* family (Christensen et al., 2003; Bisgaard et al., 2009) commonly associated with poultry (Bisgaard, 1977). Besides constituting a part of the normal microflora of the upper respiratory tract and lower genital tract in chickens (Bojesen et al., 2003a), *G. anatis* has been recognized as a major cause of lesions in the reproductive tract of egg-layers (Mirle et al., 1991; Jordan et al., 2005; Neubauer et al., 2009), causing a drop in egg production and increased mortality (Bojesen et al., 2008). Multiple-drug resistance (Bojesen et al., 2011) and substantial antigenic diversity (Vazquez et al., 2006) hampers the use of traditional antimicrobial agents and vaccines. Very little is known about the pathogenesis of *G. anatis* and to date only a few virulence factors have been identified, including

* Corresponding author. Tel.: +45 35332671; fax: +45 35332757.
*E-mail address:* miki@sund.ku.dk (A.M. Bojesen).
[1] Contributed equally to this work.

0378-1135/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2013.09.005

a new type of RTX toxin (Kristensen et al., 2010), a F17-like fimbriae (Bager et al., 2013), and secreted metalloproteases capable of degrading avian IgG (García-Gómez et al., 2005). Hence, increased knowledge of the pathogenesis and virulence factors is important to understand and prevent the negative effects of *G. anatis*.

Outer-membrane vesicles (OMVs) are naturally secreted by virtually all Gram-negative bacteria (Beveridge, 1999). They are spherical, bilayered, membranous structures between 10 and 300 nm in diameter, produced by budding of the outer membrane (OM). Besides OM components, such as lipids, integral OM proteins and lipid anchored lipoproteins, OMV also contains periplasma-derived soluble proteins and in some cases macromolecules associated with the external surface (MacDonald and Kuehn, 2012). Thus, OMVs are complex structures with an enormous functional diversity that have been proposed to aid the bacteria to thrive and survive in diverse environments (Unal et al., 2011). Their composition of potential antigens and virulence factors make them interesting therapeutic targets, and successful OMV-based vaccines have already been produced against the human pathogen *Neisseria meningitidis* serogroup B (Holst et al., 2009).

To date, no unifying mechanism governing the formation of OMVs has been identified. Changes in the surrounding milieu are however known to affect the level of the OMV production (Kulp and Kuehn, 2010). Additionally, the formation of OMVs is influenced by depletion of the peptidoglycan linkages between the OM and inner membrane (IM), and several studies have demonstrated that impaired production of anchor proteins important for these linkages increases the level of OMV production (MacDonald and Kuehn, 2012). One well-described example is the deletion of the gene encoding the TolR protein, which is part of the Tol-Pal system linking the OM to both the peptidoglycan layer and the IM in *Escherichia coli* (Scorza et al., 2008).

To increase knowledge of the pathogenesis of *G. anatis*, the present study aimed at investigating the production of OMVs from *G. anatis* during different *in vitro* growth conditions.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*G. anatis* bv. *haemolytica* strain 12656-12 Liver (*G. anatis* 12656-12) was used in this study. This strain was originally isolated from the liver of a septicaemic chicken (Bojesen et al., 2003b). Unless otherwise stated, *G. anatis* 12656-12 was incubated at 37 °C on brain heart infusion (BHI) agar plates supplemented with 5% citrated bovine blood in sealed plastic bags. Broth cultures were inoculated by scraping bacteria from plates into sterile BHI broth, and immediately adding this suspension to 60 ml BHI broth to reach an initial optical density at 600 nm ($OD_{600}$) between 0.09 and 0.1. Broth cultures were incubated in a water bath at 37 °C with shaking (160 rpm), and bacterial growth was monitored by measuring the $OD_{600}$ of cultures at serial time points until early stationary growth phase was reached. To investigate the effect of altered growth conditions or media composition, *G. anatis* 12656-12 was incubated until early stationary growth phase in BHI containing 1 mM EDTA (Sigma), in RPMI 1640 GlutaMAX™ HEPES medium (Invitrogen), or in BHI at 41 °C. Concentrations of viable bacteria at harvest-point were quantified by spotting 10 μl aliquots of serial 10-fold dilutions of the broth cultures on blood agar plates. Plates were incubated overnight at 37 °C, colonies were counted, and results were expressed as colony forming units (CFU)/ml. To investigate the effect of prolonged growth, bacterial colonies were inoculated directly into 60 ml of BHI broth and incubated for 14 h in a water bath at 37 °C with shaking (160 rpm).

*E. coli* DH5α (Invitrogen) was used for routine cloning. Cells were incubated in Luria–Bertani (LB) broth and agar at 37 °C with aeration, and the medium was supplemented with 50 μg/ml kanamycin or 100 μg/ml ampicillin when appropriate.

### 2.2. Construction of a tolR deletion mutant in G. anatis 12656-12

A homolog to the *E. coli tolR* gene was identified in the annotated genome of *G. anatis* 12656-12 by use of BLASTp (Altschul et al., 1990). The annotated genomic sequence is accessible online from Wasabi, a web-based annotation system for prokaryotic organisms developed by the Victorian Bioinfomatics Consortium, Monash University, Melbourne, Australia (Bulach et al., 2006; Seeman, 2006).

The *G. anatis tolR* deletion mutant was generated by replacing a part of the *tolR* gene with a kanamycin resistance ($Km^R$) cassette as previously described by Bager et al. (2013). The DNA construct was prepared using chemically competent *E. coli* DH5α (Invitrogen), and the plasmids used are listed in Table 1. The primers used for the construction and verification of the *tolR* mutant are given in Table 2. Transformants were selected on blood agar plates with 5 μg/ml kanamycin, and the size of the deletion site in *tolR* was confirmed using primer pair *tolR*_UF2 and *tolR*_DR2. The PCR product was sequenced by BigDye sequencing to verify the genomic localization of the deletion (Macrogen, Korea). The *tolR* deletion mutant was designated Δ*tolR*.

### 2.3. OMV preparation by ultracentrifugation

OMVs from *G. anatis* 12656-12 were isolated from culture supernatants. Bacterial cells were removed by centrifugation (20 min, 2000 × *g*, 0 °C) and the supernatant was filtered through a 0.45 μm pore-size Minisart® Syringe filter (Sartorius). 100 μl filtered supernatant was plated and incubated overnight at 37 °C to confirm the absence of viable bacteria. Protein degradation was inhibited by the addition of EDTA to a final concentration of 10 mM and protease inhibitor (complete EDTA-free protease inhibitor cocktail, 1 tablet per 50 ml of filtrate; Roche). The filtrate was stored at 4 °C and within a week, OMVs were purified from the filtrate by ultracentrifugation (3 h, 200,000 × *g*, 4 °C) using a Beckmann SW41Ti rotor. The OMVs were resuspended in 100 μl phosphate-buffered saline (PBS)/38 ml ultracentrifuged filtrate.