Conditional growth defect of *Bordetella pertussis* and *Bordetella bronchiseptica* ferric uptake regulator (fur) mutants

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One sentence summary: Bordetella fur mutants display a growth defect in iron-replete media most likely due to iron overload and can be rescued by the heterologous expression of *Escherichia coli* ferritin FtnA.

Editor: Klaus Hantke

**Abstract**

Outer-membrane vesicles (OMVs) are promising tools in the development of novel vaccines against the respiratory pathogens *Bordetella pertussis* and *Bordetella bronchiseptica*. Unfortunately, vesiculation by bordetellae is too low for cost-effective vaccine production. In other bacteria, iron limitation or inactivation of the fur gene has been shown to increase OMV production, presumably by downregulation of the mla genes, which encode machinery for maintenance of lipid asymmetry in the outer membrane. Here, we followed a similar approach in bordetellae. Whereas a fur mutant was readily obtained in *B. bronchiseptica*, a *B. pertussis* fur mutant could only be obtained in iron-deplete conditions, indicating that a fur mutation is conditionally lethal in this bacterium. The fur mutants displayed a growth defect in iron-replete media, presumably because constitutive expression of iron-uptake systems resulted in iron intoxication. Accordingly, expression of the *Escherichia coli* ferritin FtnA to sequester intracellularly accumulated iron rescued the growth of the mutants in these media. The fur mutations led to the constitutive expression of novel vaccine candidates, such as the TonB-dependent receptors FauA for the siderophore alcaligin and BhuR for heme. However, neither inactivation of fur nor growth under iron limitation improved vesiculation, presumably because the expression of the mla genes appeared unaffected.

**Keywords:** Bordetella, iron limitation, outer-membrane vesicles, Fur, FauA, Mla system

**Introduction**

Iron is an essential nutrient metal for most bacteria. During infection, the availability of iron is very low for invading pathogens because the host produces proteins, such as transferrin and lactoferrin, which sequester free iron as a defense mechanism against infection (Hood and Skaar 2012). Bacteria deploy several mechanisms to capture iron from the environment under iron-limiting conditions. In *Bordetella pertussis* and *Bordetella bronchiseptica*, two Gram-negative bacteria that infect the respiratory tract of humans and other mammals, respectively (Mattoo and Cherry 2005), these mechanisms include the production and secretion of the siderophore alcaligin (Moore et al. 1995). After chelating iron in the environment, the ferric complex of the siderophore is taken up by the bacteria in a process that requires for the first step a TonB-dependent receptor in the outer membrane (OM). The receptor for Fe³⁺-alcaligin is FauA (Brickman and Armstrong 1999). Besides alcaligin, bordetellae can also utilize the xeno siderophores enterobactin (Beall and Sanden 1995), ferrichrome, and desferrioxamine B (Beall and Hones 1997), as well as heme as iron sources (Brickman and Armstrong 2009). The uptake of enterobactin and heme requires the TonB-dependent receptors BfaA and BhuR, respectively, whilst the receptors for ferrichrome and desferrioxamine B have not been identified yet. Although iron is essential for growth, its excessive intracellular accumulation is toxic as it may catalyze Fenton chemistry resulting in the production of oxygen radicals (Imlay et al. 1988, Braun 1997). Therefore, the production of iron-acquisition mechanisms needs to be controlled (Braun 1997). The transcription of the genes for these mechanisms is usually repressed under iron-replete conditions by the ferric uptake regulator (Fur) (Braun 1997), as has also been shown to be the case in bordetellae (Brickman et al. 2007, Beall and Sanden 1995b).

Several studies have suggested that outer-membrane vesicles (OMVs) could aid in iron uptake (Schwechheimer and Kuehn 2015, Orench-Rivera and Kuehn 2016). OMVs are released from Gram-negative bacteria as a result of bulging of the OM. The role of OMVs in metal acquisition is supported by the abundance of TonB-dependent receptors in OMVs from *Neisseria meningitidis*, *Porphyromonas gingivalis*, and *B. pertussis* (Lappann et al. 2013, Veith et al. 2014, Gasperini et al. 2017). Indeed, OMVs from *B. pertussis* were reported to be able to load iron and deliver it to bacterial cells (Gasperini et al. 2017). Furthermore, OMV production was found to be increased in *Haemophilus influenzae* under iron-limiting conditions and in fur mutants of *Vibrio cholerae*, *Escherichia coli*, and *H. influenzae* (Roier et al. 2016), indirectly supporting a role for OMVs in iron acquisition. This increased OMV production was related to the downregulation of genes coding for the maintenance of lipid asymmetry (Mia) system (Roier et al. 2016). The OM is an asymmetric bilayer, with phospholipids and lipopolysaccharides being present in the inner and outer leaflet, respectively. The Mia system transports phospholipids that appear in the outer leaflet back to...
the inner membrane, thereby maintaining OM lipid asymmetry (Malinverni and Silhavy 2009). According to the proposed model, downregulation of the Mla system under iron limitation results in the accumulation of phospholipids in the outer leaflet of the OM and, consequently, the OM bulges and OMVs pinch off (Roier et al. 2016). We have previously demonstrated that disruption of the Mla system in B. pertussis resulted in only a slight increase in vesiculation (de Jonge et al., manuscript submitted for publication). However, when also the pldA gene was inactivated, a gene that encodes OM phospholipase A, which degrades phospholipids in the outer leaflet of the OM, OMV production was strongly enhanced.

We are investigating the possibility of using OMVs in the development of novel Bordetella vaccines. Previously developed whole-cell vaccines against B. pertussis appeared to be too reactogenic (Cody et al. 1981), and they were replaced by acellular pertussis (aP) vaccines consisting of one to five purified antigens. However, these aP vaccines induce only short-term immunity and do not protect against colonization and transmission of B. pertussis (Warfel et al. 2014, Wilk et al. 2019). Probably as a consequence, pertussis is resurfacing in the last decades (Esposito et al. 2019). Vaccines against B. bronchiseptica have also been developed but the duration of vaccine-induced immunity remains unclear (Ellis 2015). Thus, novel vaccines against both B. pertussis and B. bronchiseptica need to be developed. OMVs are attractive nanostructures for vaccine development because of their content of a wide variety of OM proteins, their adjuvant properties, and their uptake by antigen-presenting cells (van der Pol et al. 2015). Immunization studies with B. pertussis OMVs in mice demonstrated the induction of a mixed systemic T helper (Th)1/Th2/Th17 response with reduced pro-inflammatory activity compared to immunization with whole cells (Raeven et al. 2016). Efficient bacterial clearance of the lungs and the nasal cavity was achieved after intranasal immunization (Raeven et al. 2020). Together, these data indicate that Bordetella OMVs are promising vaccine candidates. Unfortunately, spontaneous OMV production by Bordetella species is relatively low (Hozbor et al. 1999). In this study, we investigated the effect of iron limitation on OMV production in B. pertussis and B. bronchiseptica. Although we have already reported several methods to increase the production of native OMVs (de Jonge et al. 2021, de Jonge et al., manuscript submitted for publication), the increased production of OMVs under iron limitation could have the additional advantage of the expression of novel relevant antigens. TonB-dependent receptors are considered attractive vaccine candidates, since they are exposed at the cell surface, upregulated in vivo, and essential for successful infection (Wang et al. 2021). Besides, iron limitation also leads to increased expression of other vaccine candidates, such as IRP1-3 and the type III secretion system in Bordetella (Alvarez Hayes et al. 2011, Brickman et al. 2011, Kurushima et al. 2012, Fasciano et al. 2019). Since iron limitation is also expected to restrict bacterial growth, which again could negatively impact OMV yield, we have also constructed fur mutants for their potential application.

**Materials and methods**

**Growth conditions**

Bordetella pertussis and B. bronchiseptica strains were grown on Bordet-Gengou (BG) agar (Di(Cho) plates containing 15% (V/V) defibrinated sheep blood (bioTRADING) or 10 mg/mL of bovine serum albumin (BSA) (Hiramatsu et al. 2019) at 35°C. For liquid cultures, bacteria were scraped from BG plates and grown in Verwey medium (Verwey et al. 1949), which was supplemented with 0.01 g/L of FeSO₄·7H₂O where indicated, or in Stainer-Scholte (SS) medium (Stainer and Scholte 1971) at 35°C while shaking at 175 rpm. For growth of B. pertussis in SS medium, 1 g/L of heptakis(2,6-di-O-methyl)-β-cyclodextrin (Sigma-Aldrich) was added. E. coli strains were grown on lysogeny broth (LB) agar plates at 37°C or in liquid LB while shaking at 200 rpm. For strain selection or plasmid maintenance, 100 µg/mL (for B. pertussis and E. coli) or 200 µg/mL (for B. bronchiseptica) of ampicillin, 10 µg/mL of gentamicin, 300 µg/mL of streptomycin, 50 µg/mL of nalidixic acid, or 5 µg/mL of ceftoxime were added to the medium. To induce gene expression, media were supplemented with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Bacterial growth in liquid cultures was monitored by measuring the optical density at 600 nm (OD₆₀₀) using a Novaspec III + spectrophotometer (Biochrom).

**Construction of mutants and plasmids**

Details of genetic constructions are provided in the Supplementary Materials, where all strains, primers, and plasmids used are listed in Supplementary Tables S1, S2, and S3, respectively.

**Siderophore production**

Production of siderophores was determined with the chrome azurol S (CAS) assay (Schwyn and Neilands 1987). Bacterial cells were pelleted from cultures by low-speed centrifugation, and 0.5 mL of supernatant, not normalized to OD₆₀₀, was mixed with 0.5 mL of CAS solution. After incubation for 1 h at room temperature, absorbance at 630 nm (A₆₃₀) was measured.

**OMV isolation and quantification**

Bacterial strains were pre-grown for 24 h in Verwey medium. Cultures were then diluted to an OD₆₀₀ of 0.05 in 50 mL Verwey medium, supplemented with FeSO₄·7H₂O where indicated, and grown in 250-mL baffled flasks. After 48 h of growth, OMVs were isolated as described (de Jonge et al. 2021) and resuspended in phosphate-buffered saline. OMV yield was determined based on protein content using the Lowry DC protein assay (Bio-Rad) following the instructions of the manufacturer.

**SDS-PAGE and Western blotting**

SDS-PAGE and Western blotting were performed as described (de Jonge et al. 2021). Primary antibodies used were a rabbit anti-FauA antiserum (de Jonge et al. 2021) and horseradish peroxidase-conjugated anti-FLAG-tag monoclonal antibodies (Sigma Aldrich).

**RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)**

Bacterial strains were grown in Verwey medium, and cells were harvested during exponential growth. Harvested cells were stored at -80°C in RNAprotect Bacteria Reagent (Qiagen) until RNA isolation. RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel), and residual DNA was removed with the TURBO DNA-free kit (Ambion). Next, cDNA was generated from 1 µg of RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). RT-qPCR was performed using the SYBR Green master mix (ThermoFisher) and a ViiA 7 Real-Time PCR System (Applied Biosystems) with primers listed in Supplementary Table S2 with three technical replicates per sample. Data was analyzed with QuantStudio v1.3, and relative gene expression was determined using the 2⁻ΔΔCt method with rpoB for normalization. Gene expression was related to one sample of the wild-type strains per gene.
Results

Isolation of *B. pertussis* and *B. bronchiseptica fur* mutants

To investigate the influence of iron availability on OMV production in *Bordetella*, we first attempted to construct fur mutants of *B. pertussis* and *B. bronchiseptica* via allelic exchange. However, whilst a fur mutant was readily obtained in *B. bronchiseptica*, the same selection procedure failed for *B. pertussis*, suggesting that fur is an essential gene in the latter species. The selection procedure was performed on BG agar plates supplemented with blood, which is rich in iron resources. We considered the possibility that the anticipated constitutive expression of iron-uptake systems in a fur mutant could lead to excessive iron uptake, which could potentially be toxic for the cells. To get around this potential problem, mutant selection was next performed on BG plates containing BSA instead of blood. Following this strategy, we succeeded to also obtain a *B. pertussis fur* mutant.

Growth characteristics of the fur mutants

To test the effect of the fur mutations on growth, wild-type *B. pertussis* and *B. bronchiseptica* and their fur mutant derivatives were grown in two liquid media routinely used for growth of *B. pertussis*, Verwey medium and SS medium. We previously reported that Verwey medium is limited in iron and that growth of wild-type *Bordetella* in this medium results in the induction of the synthesis of the alcaligin receptor FauA (de Jonge et al. 2021). In contrast, standard SS medium contains iron sulfate as an iron source. Compared to the wild types, growth of both fur mutants was reduced in both media, but growth restriction was most severe in the SS medium, where particularly the *B. pertussis* fur mutant failed to grow at all (Fig. 1A). As the composition of the media used also differs in other respects besides the iron content, we next wanted to determine directly the influence of iron availability. To this end, growth was also studied in Verwey medium supplemented with iron sulfate. Indeed, whilst iron supplementation, as expected, improved the growth of the wild-type strains considerably, it impacted the growth of both mutants in this medium (Fig. 1A). The residual growth of the *B. pertussis* fur mutant in iron-supplemented Verwey medium could be due to the presence of starch in this medium, which has the capacity to bind iron (Thomas et al. 1976). Large variability was noticeable in the final growth yield of the *B. bronchiseptica fur* mutant in Verwey medium supplemented with iron sulfate (Fig. 1A). This could be the result of suppressor mutations arising in the cultures, allowing for a better growth of the fur mutant in iron-rich conditions. Accordingly, whilst colonies of the wild-type *B. bronchiseptica* strain streaked on blood-agar plates were rather uniform in size, the size of the fur mutant colonies was heterogeneous with large colonies appearing in a background of small colonies, indicating the selection of suppressor mutants with improved growth on these plates (Supplementary Fig. S1). Together, these results indicate a growth defect of the fur mutants in iron-replete conditions, presumably because of an excessive uptake of iron.

Expression of *E. coli* FtnA rescues *Bordetella fur* mutants from iron toxicity

To further substantiate the idea that the growth defect of the *Bordetella fur* mutants under iron-replete conditions is due to the toxic accumulation of iron within the cells, we considered the possibility of sequestering intracellular iron by expressing an iron-storage protein. In *E. coli*, excessive intracellular iron can be stored in ferritin (FtnA) and bacterioferritin (Bfr). Of these two, FtnA seems to play the major role in iron storage (Abdul-Tehrani et al. 1999). Bordetella pertussis and *B. bronchiseptica* do contain homologues of Bfr (locus tags BPF0174 and BB4918 in reference strains Tohama I and RB50, respectively), but not of FtnA. Thus, we expressed codon-optimized ftnA of *E. coli* in the fur mutant strains of *B. pertussis* and *B. bronchiseptica*, and expression was confirmed by Western blotting targeting the engineered C-terminal FLAG tag on the protein (shown for *B. pertussis* in Supplementary Fig. S2). Subsequently, growth was assessed in iron-replete SS medium. The growth curves indeed showed that expression of ftnA drastically improved the growth of the fur mutants in this medium (Fig. 1B). Thus, we conclude that the growth defect of the fur mutants is due to the toxic intracellular accumulation of iron.

Constitutive synthesis of siderophores and TonB-dependent receptors in fur mutants

To confirm the expected phenotype of the constructed fur mutants, the biosynthesis of the alcaligin receptor FauA, which is repressed by Fur under iron-replete conditions (Brickman and Armstrong 2009), was assessed by Western blot analysis of whole-cell lysates grown in iron-poor (Verwey) or iron-replete (Verwey medium supplemented with iron and SS) media. As expected, FauA was detected after growth of the wild-type strains in the iron-deficient Verwey medium but not after growth in the iron-replete media (Fig. 2). In contrast, FauA was detected in the fur mutants, independently of the culture conditions used. We also assessed the production of the siderophore alcaligin in iron-rich SS medium using the CAS assay. As expected, siderophore production was drastically increased in the *B. bronchiseptica fur* mutant relative to the wild type (Supplementary Fig. S3). However, such increase was not observed in the *B. pertussis fur* mutant. This is probably due to the very poor growth of the *B. pertussis fur* mutant in SS medium (Fig. 1A). Thus, these data show the constitutive production of FauA in the fur mutants of *B. pertussis* and *B. bronchiseptica*, independent of the growth medium used.

The expression of fauA was further evaluated by RT-qPCR, a method, which also allowed us to assess the expression of genes for other antigens for which no antisera were available. For this purpose, the heme receptor BhuR and another TonB-dependent receptor, BfrD, which functions as a receptor for catecholamines (Brickman et al. 2015), were elected. The synthesis of both of them was expected to be regulated by iron availability in a Fur-dependent manner, since a putative Fur-binding site is present in the promoter regions of their genes (Brickman and Armstrong 1999, Passerini de Rossi et al. 2003, 2009). Wild-type *B. pertussis* and *B. bronchiseptica* and their fur mutant derivatives were grown in Verwey medium as SS medium could not be used due to poor growth of the fur mutants. Verwey is an iron-poor medium, and the limited iron sources available are consumed during growth. RNA was isolated at time points early in the growth when FauA was still absent in the wild-type strains, that is before the limited iron resources available were depleted (Supplementary Fig. S4). Expression of fauA and bhuR was higher in the fur mutants compared to the wild types, although the difference for fauA was not significant in *B. pertussis* (*P = 0.07*) (Fig. 3A and B). Remarkably, a large variation in bhuR expression was noticeable between the biological replicates of wild-type *B. pertussis*, where high and low expression correlated with a relatively high and low OD<sub>600</sub> (0.81 ± 0.01 versus 0.39 ± 0.05), respectively, at the time of harvest. Presumably, reaching a higher OD implicates that the lim-
Figure 1. Influence of fur inactivation on growth. (A) Wild types (WT) and fur mutants of B. pertussis (Bp) and B. bronchiseptica (Bb) were grown in Verwey medium, either supplemented with iron (+Fe) or not, and in SS medium, and growth was monitored by measuring the OD600. (B) The fur mutants, either containing pFtnA or not, as indicated, were grown in SS medium. The pFtnA-containing strains were grown either with, or where indicated (w/o IPTG), without IPTG, and these cultures were inoculated from precultures on BG blood agar plates containing IPTG. Growth was monitored by measuring the OD600. (A,B) Graphs show mean values with standard deviations of three independent experiments, except for the growth of the B. bronchiseptica fur mutant in Verwey medium supplemented with iron, where each individual result of four replicates is depicted and for the B. pertussis fur mutant containing pFtnA grown in the absence of IPTG (n = 1).

Figure 2. FauA production in fur mutants. Wild-type (WT) B. pertussis (Bp) and B. bronchiseptica (Bb) and their fur mutants were grown in Verwey medium, either supplemented with iron (+Fe) or not, or in SS medium. Whole-cell lysates from equal amounts of cells (based on OD600) were analyzed by Western blotting using antiserum directed against FauA. Only the relevant part of the blot is shown. Molecular weight markers are indicated on the left.

The influence of iron limitation on OMV production

To determine if iron availability affects OMV production, as has been reported in several other Gram-negative bacteria (Roier et al. 2016), OMV production by wild-type B. bronchiseptica and B. pertussis grown in either Verwey medium or Verwey medium supplemented with iron and by the fur mutants was evaluated. To verify iron limitation of the wild types grown in Verwey medium, FauA and siderophore production in the cultures used for OMV isolation was confirmed (Supplementary Fig. S5). Quantification of isolated OMVs based on protein content indicated no significant effect of iron limitation or the fur mutations on OMV production (Fig. 4A).

Increased OMV production by the fur mutants of V. cholerae, H. influenzae, and E. coli was related to downregulation of the synthesis of the Mla system, which leads to an accumulation of phospholipids in the outer leaflet of the OM and bleb formation (Roier et al. 2016). Therefore, we analyzed whether the fur mutations affected expression of the mla operon in Bordetella. RT-qPCR analysis indicated an increased, rather than the anticipated decreased mlaF
expression in the *B. pertussis* *fur* mutant, although this difference was not significant (*P* = 0.17) (Fig. 4B). Inactivation of *fur* did not change *mlaF* expression in *B. bronchiseptica*.

**Discussion**

To study the effect of iron limitation on OMV production in *B. pertussis* and *B. bronchiseptica*, we chose to inactivate the *fur* gene to mimic iron limitation independent of iron availability. Inactivation of *fur* has been successful in many other Gram-negative bacteria (Roier et al. 2016, Lee et al. 2017). Also for *B. bronchiseptica*, *fur* mutants have been described before (Brickman and Armstrong 1995), and such mutants were readily obtained in the present study. Inactivation of *fur* in *B. pertussis*, however, proved to be more challenging and, to our knowledge, such mutants have not been described before. Indeed, *fur* has previously been classified as an essential gene in *B. pertussis* based on high-throughput transposon sequencing (Tn-seq) experiments both in vitro and in vivo in an intranasal murine infection model in which *fur* mutants were not obtained (Gonyar et al. 2019, Belcher et al. 2020). Nevertheless, we could isolate *B. pertussis* *fur* mutants on plates containing BSA instead of blood, presumably because *fur* mutants are not viable on blood agar due to excessive iron uptake. Accordingly, growth of the *fur* mutants of both *B. pertussis* and *B. bronchiseptica* was severely restricted in iron-rich liquid media. In a few other species, Fur has been described to be essential, including *Pseudomonas aeruginosa* and *Chromobacterium violaceum* (Pasqua et al. 2017, Santos et al. 2020). However, in *C. violaceum*, essentiality of Fur appeared to be conditional (Santos et al. 2020), as, like in *B. pertussis*, *fur* mutants could eventually be obtained in iron-deplete conditions. Growth of the *fur* mutants in iron-rich medium could be rescued by the expression of iron-storage protein FtnA of *E. coli*, demonstrating that the growth defect is due to intracellular iron accumulation. Although *B. pertussis* and *B. bronchiseptica* contain a homologue of another iron-storage protein, Bfr, sequestration of iron by this protein in these conditions is apparently not sufficient to sustain growth. Expression of *bfr* in *B. pertussis* is induced under iron-rich conditions (Brickman et al. 2011) and is presumably positively regulated by Fur, as has been described in *E. coli* (Massé and Gottesman 2002). Consequently, *bfr* expression in the *Bordetella* *fur* mutants is probably low and insufficient to avoid iron intoxication.
Inactivation of fur was expected to result in the constitutive production of iron-limitation-inducible receptors in the OM, which are potentially relevant vaccine antigens (Wang et al. 2021). So far, 12 and 16 putative Tonb-dependent receptors have been identified in B. pertussis and B. bronchiseptica, respectively (Brickman et al. 2007). At least four of these receptors, i.e. FauA, BfeA, BhuR and BfrD, are involved in iron acquisition (Brickman et al. 2007, Brickman et al. 2015). We examined expression of the fauA, bhuR, and bfrD genes. Expression of fauA and bhuR was indeed up-regulated in the fur mutants, but expression of bfrD did not increase, even though a putative Fur box was identified upstream of bfrD (Passerini de Rossi et al. 2003). Previous studies gave conflicting results in this respect and showed either an increase (Passerini de Rossi et al. 2003) or a decrease (Brickman et al. 2015) of bfrD expression during iron limitation. BfrD was previously selected as a promising vaccine candidate, and proteomic analysis demonstrated the presence of BfrD in OMVs (Gasperini et al. 2016). Inactivation of fur mutants under iron-replete conditions, which is needed for utilization of exogenous ferric enterobactin. Microbiology 1995a; 141:3193–205.

As these OMVs were isolated from cultures grown in iron-rich SS medium, BfrD synthesis is apparently already high under those conditions.

We expected that fur inactivation and iron limitation would increase OMV production in bordetellae as has been reported in other bacteria (Roier et al. 2016). However, such an increase was not observed. The improved OMV production in E. coli, V. cholerae, and H. influenzae in the absence of Fur was reported to be related to the downregulation of the synthesis of the Mla system (Roier et al. 2016). In the absence of the Mla system, phospholipids accumulate in the outer leaflet of the OM which results in bulging of the OM and subsequent OMV formation (Roier et al. 2016). Fur-binding sites were predicted upstream of the mla operons in E. coli, V. cholerae, and H. influenzae, indicating a role for Fur in positive regulation of mla transcription (Roier et al. 2016). We have previously shown that OMV production is increased in an Mla-deficient B. pertussis mutant also lacking the OM phospholipase A (de Jonge et al., manuscript submitted for publication), consistent with the proposed model of OMV biogenesis (Roier et al. 2016). However, expression of mlaF was not significantly decreased in the Bordetella fur mutants (Fig 4B), which is in agreement with data of a previous transcriptomics study that did not show altered expression of the B. pertussis mla genes in iron-limiting conditions (Brickman et al. 2011). Accordingly, we could not identify an obvious Fur-binding site in the promoter region of the mla operon. Thus, a different regulation of the expression of the mla genes could explain why iron limitation and fur mutations do not increase OM vesiculation in Bordetella.

OMVs are promising tools in the development of new vaccines for B. pertussis and B. bronchiseptica (Raenve et al. 2016, 2018, 2020). Since spontaneous OMV production by bordetellae is low, we are studying ways to increase OMV release. Unfortunately, neither iron limitation nor inactivation of fur increased OMV production. However, since genes encoding relevant antigens, such as FauA and BhuR, are upregulated in the fur mutant strains, these strains could still be useful in the development of OMV-based vaccines. OMV production by the fur mutants could then be increased, e.g. by applying a heat shock which was previously demonstrated to increase vesiculation (de Jonge et al. 2021). Either expression of E. coli FtnA or suppressor mutations could improve the growth of fur mutants under iron-replete conditions, which is needed for cost-effective vaccine production.

**Supplementary data**

Supplementary data are available at FEMSLE online.

**Acknowledgments**

We thank Liz Fransman, Jianjun Wu, and Leon Bekedam for their contributions in initial experiments.

**Funding**

This work was supported by the domain Applied and Engineering Sciences (TTW) of The Netherlands Organization for Scientific Research (NWO) (TTW Perspectief Grant number 14921), which received financial contributions for this grant from GlaxoSmithKline Biologicals SA and PULIKE Biological Engineering Inc.

**Conflict of interest statement.** None declared.

**References**

Abdul-Tehrani H, Hudson AJ, Chang YS et al. Ferritin mutants of Escherichia coli are iron deficient and growth impaired, and fur mutants are iron deficient. J Bacteriol 1999;181:1415–28.

Alvarez Hayes J, Erben E, Lamberti Y et al. Identification of a new protective antigen of Bordetella pertussis. Vaccine 2011;29:8731–9.

Beall B, Hones T. An iron-regulated outer-membrane protein specific to Bordetella bronchiseptica and homologous to ferric siderophore receptors. Microbiology 1997;143:135–45.

Beall B, Sanden GN. A bordetella pertussis fepA homologue required for utilization of exogenous ferric enterobactin. Microbiology 1995a; 141:3193–205.

Beall BW, Sanden GN. Cloning and initial characterization of the Bordetella pertussis fur gene. Curr Microbiol 1995b;30:223–6.

Belcher T, MacArthur I, King JD et al. Fundamental differences in physiology of Bordetella pertussis dependent on the two-component system bvg revealed by gene essentiality studies. Micr Genomics 2020;6:mgn000496.

Braun V. Avoidance of iron toxicity through regulation of bacterial iron transport. Biol Chem 1997;378:779–86.

Brickman TJ, Anderson MT, Armstrong SK. Bordetella iron transport and virulence. Biometals 2007; 20:303–22.

Brickman TJ, Armstrong SK. Bordetella pertussis fur gene restores iron resupplyability of siderophore and protein expression to deregulated Bordetella bronchiseptica mutants. J Bacteriol 1995;177:268–70.

Brickman TJ, Armstrong SK. Essential role of the iron-regulated outer membrane receptor FauA in alcaligin siderophore-mediated iron uptake in Bordetella species. J Bacteriol 1999;181:5958–66.

Brickman TJ, Armstrong SK. Temporal signaling and differential expression of Bordetella iron transport systems: the role of ferromones and positive regulators. Biometals 2009;22:33–41.

Brickman TJ, Cummings CA, Liew S-Y et al. Transcriptional profiling of the iron starvation response in Bordetella pertussis provides new insights into siderophore utilization and virulence gene expression. J Bacteriol 2011;193:4798–812.

Brickman TJ, Suhadolc RJ, Armstrong SK. Interspecies variations in Bordetella catecholamine receptor gene regulation and function. Infect Immun 2015;83:4639–52.

Cody CL, Baraff LJ, Cherry JD et al. Nature and rates of adverse reactions associated with DTP and DT immunizations in infants and children. Pediatrics 1981;68:650–60.

de Jonge EF, Balhuizen MD, van Boxtel R et al. Heat shock enhances outer-membrane vesicle release in Bordetella spp. Curr Res Microb Sci 2021;2:100009.

Ellis JA. How well do vaccines for Bordetella bronchiseptica work in dogs? A critical review of the literature 1977–2014. Vet J 2015;204:5–16.
Esposito S, Stefanelli P, Fry NK et al. Pertussis prevention: reasons for resurgence, and differences in the current acellular pertussis vaccines. Front Immunol 2019;10:1344.

Fasciano AC, Shaban L, Mecasía J. Promises and challenges of the type three secretion system injectisome as an anti-virulence target. EcoSal Plus 2019;8. DOI: 10.1128/ecosalplus-esp-0032-2018.

Gasperini G, Arato M, Pizza M et al. Physiopathological roles of spontaneously released outer membrane vesicles of Bordetella pertussis. Future Microbiol 2017;12:1247–59.

Gasperini G, Biagini M, Arato V et al. Outer membrane vesicles (OMV)-based and proteomics-driven antigen selection identifies novel factors contributing to Bordetella pertussis adhesion to epithelial cells. Mol Cell Proteomics 2018;17:205–15.

Gonyar LA, Gelbach PE, McDuffie DG et al. In vivo gene essentiality and metabolism in Bordetella pertussis. Msphere 2019;4:e00694–18.

Hiramatsu Y, Osada-Oka M, Horiguchi Y. Bordet-Gengou agar medium supplemented with albumin-containing biologics for cultivation of bordetellae. Microbiol Immunol 2019;63:513–6.

Hood MI, Skaa EP. Nutritional immunity: transition metals at the pathogen-host interface. Nat Rev Microbiol 2012;10:525–37.

Hozbor D, Rodriguez ME, Fernández J et al. Release of outer membrane vesicles from Bordetella pertussis. Curr Microbiol 1999;38:273–8.

Imlay JA, Chin SM, Linn S. Toxic DNA damage by hydrogen peroxide through the fenton reaction in vivo and in vitro. Science 1988;240:640–2.

Kurushima J, Kuwae A, Abe A. Iron starvation regulates the type III secretion system in Bordetella bronchiseptica. Microbiol Immunol 2012;56:356–62.

Lappann M, Otto A, Becher D et al. Comparative proteome analysis of spontaneous outer membrane vesicles and purified outer membranes of Neisseria meningitidis. J Bacteriol 2013;195:4425–35.

Lee AY, Kao CY, Wang YK et al. Inactivation of ferric uptake regulator (Fur) attenuates Helicobacter pylori J99 motility by disturbing the flagellar motor switch and autoinducer-2 production. Helicobacter 2017;22:e12388.

Malinverni JC, Silhavy TJ. An ABC transport system that maintains lipid asymmetry in the Gram-negative outer membrane. Proc Natl Acad Sci 2009;106:8009–14.

Massé E, Gottesman S. A small RNA regulates the expression of genes involved in iron metabolism in Escherichia coli. Proc Natl Acad Sci 2002;99:4620–5.

Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev 2005;18:326–82.

Moore CH, Foster LA, Gerbig DG et al. Identification of algalin as the siderophore produced by Bordetella pertussis and B. bronchiseptica. J Bacteriol 1995;177:1116–8.

Orench-Rivera N, Kuehn MJ. Environmentally controlled bacterial vesicle-mediated export. Cell Microbiol 2016;18:1525–36.

Pasqua M, Visaggio D, Lo Scuoto A et al. Ferric uptake regulator fur is conditionally essential in Pseudomonas aeruginosa. J Bacteriol 2017;199:e00472–17.

Passerinide Rossi BN, Friedman LE, Belzoni CB et al. virR90, a virulence-activated gene coding for a Bordetella pertussis iron-regulated outer membrane protein. Res Microbiol 2003;154:443–50.

Raeven RHM, Brummelman J, Pennings JLA et al. Bordetella pertussis outer membrane vesicle vaccine confers equal efficacy in mice with milder inflammatory responses compared to a whole-cell vaccine. Sci Rep 2016;6:38240.

Raeven RHM, Brummelman J, Pennings JLA et al. Molecular and cellular signatures underlying superior immunity against Bordetella pertussis upon pulmonary vaccination. Mucosal Immunol 2018;11:979–93.

Raeven RHM, Rockx-Brouwer D, Kanojia G et al. Intrasalinal immunization with outer membrane vesicle pertussis vaccine confers broad protection through mucosal IgA and Th17 responses. Sci Rep 2020;10:7396.

Roier S, Zingl FG, Cakar F et al. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. Nat Commun 2016;7:10515.

Santos RERS, Batista BB, da Silva Neto JF. Ferric uptake regulator fur coordinates siderophore production and defense against iron toxicity and oxidative stress and contributes to virulence in Chromobacterium violaceum. Appl Environ Microbiol 2020;86:e01620–20.

Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol 2015;13:605–19.

Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem 1987;160:47–56.

Stainer DW, Scholte MJ. A simple chemically defined medium for the production of phase I Bordetella pertussis. J Gen Microbiol 1971;63:211–20.

Thomas FB, Falko JM, Zuckerman K. Inhibition of intestinal iron absorption by laundry starch. Gastroenterology 1976;71:1028–32.

van der Pol L, Stork M, van der Ley P. Outer membrane vesicles as platform vaccine technology. Biotechnol J 2015;10:1689–706.

Veith PD, Chen YY, Gorasia DG et al. Porphyromonas gingivalis outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. J Proteome Res 2014;13:2420–32.

Verwey WJ, Thiele EH, Sage DN et al. A simplified liquid culture medium for the growth of Helminthobacter pertussis. J Bacteriol 1949;58:127–34.

Wang J, Xiong K, Pan Q et al. Application of tonb-dependent transporters in vaccine development of Gram-negative bacteria. Front Cell Infect Microbiol 2021;10:589115.

Warrfel JM, Zimmerman LJ, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. Proc Natl Acad Sci 2014;111:787–92.

Wilk MM, Borkner L, Misiak A et al. Immunization with whole cell but not acellular pertussis vaccines primes CD4 T(Th) cells that sustain protective immunity against nasal colonization with Bordetella pertussis. Emerg Microbes Infect 2019;8:169–85.