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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.
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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 Fe^{3+}-alcaligin is FauA (Brickman and Armstrong 1999). Besides alcaligin, bordetellae can also utilize the xenosiderophores enterobactin (Beall and Sanden 1995a), ferrichrome, and desferrioxamine B (Beall and Hoenes 1997), as well as heme as iron sources (Brickman and Armstrong 2009). The uptake of enterobactin and heme requires the TonB-dependent receptors BfeA 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 (Mla) 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 Mla system transports phospholipids that appear in the outer leaflet back to

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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 resurging 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 (Difco) 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 $FeSO_4{\cdot}7H_2O$ 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 cefotaxime 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 (OD_{600}) 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 OD_{600}, was mixed with 0.5 mL of CAS solution. After incubation for 1 h at room temperature, absorbance at 630 nm (A_{630}) was measured.

OMV isolation and quantification

Bacterial strains were pre-grown for 24 h in Verwey medium. Cultures were then diluted to an OD_{600} of 0.05 in 50 mL Verwey medium, supplemented with $FeSO_4{\cdot}7H_2O$ 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 $2^{-\Delta\Delta Ct}$ 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 BP0174 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_{600} (0.81 ± 0.01 versus 0.39 ± 0.05), respectively, at the time of harvest. Presumably, reaching a higher OD implicates that the lim-

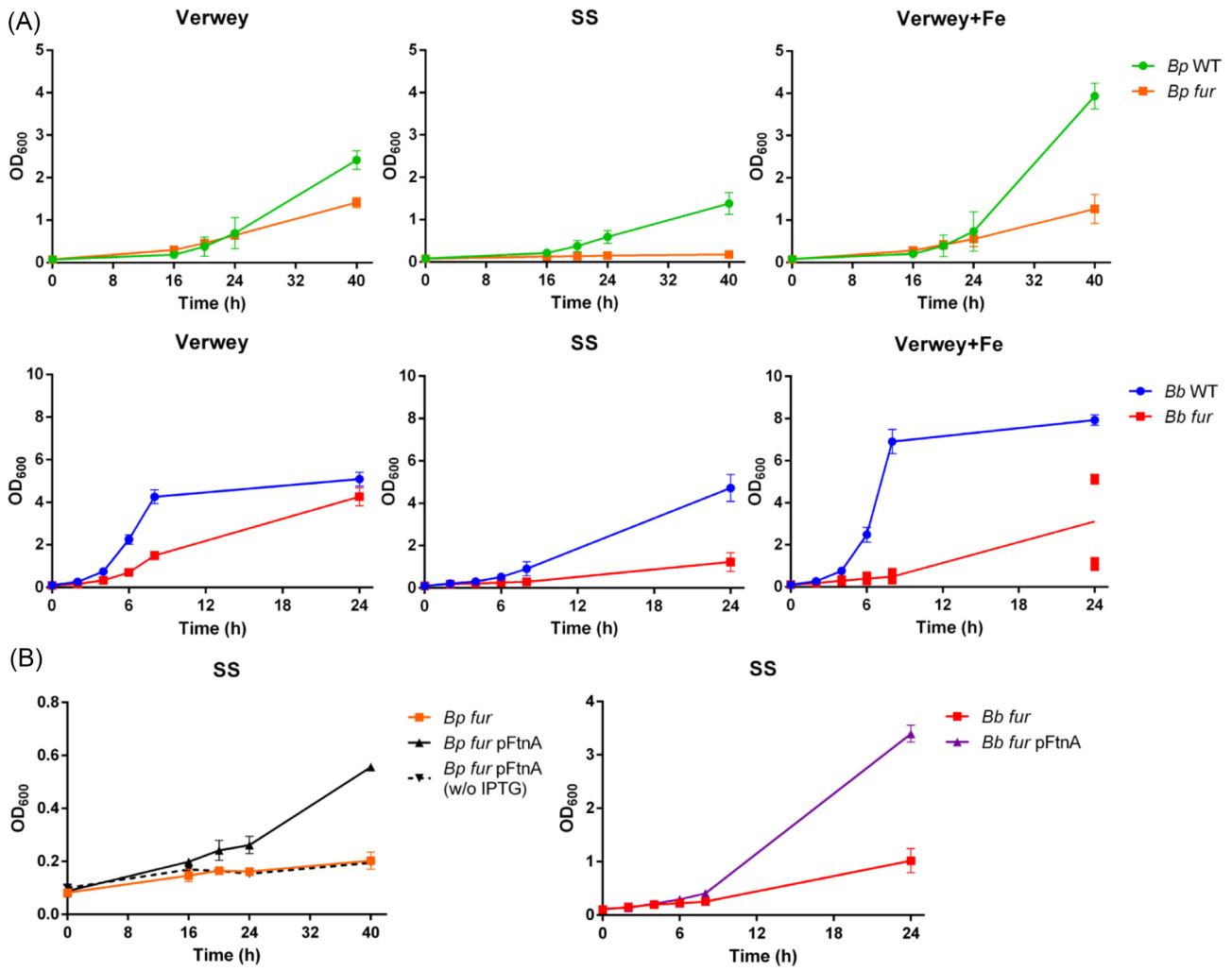

Figure 1. Influence of *fur* inactivation on growth. **(A)** Wild types (WT) and *fur* mutants of *B. pertussis* (*Bp*) and *B. bronchiseptica* (*Bb*) were grown in Verwey medium, either supplemented with iron (+Fe) or not, and in SS medium, and growth was monitored by measuring the OD_{600}. **(B)** 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 OD_{600}. **(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).

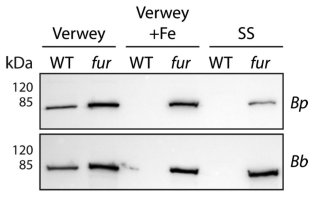

Figure 2. FauA production in *fur* mutants. Wild-type (WT) *B. pertussis* (*Bp*) and *B. bronchiseptica* (*Bb*) and their *fur* mutants were grown in Verwey medium, either supplemented with iron (+Fe) or not, or in SS medium. Whole-cell lysates from equal amounts of cells (based on OD_{600}) were analyzed 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.

ited iron resources available were more exhausted than in the cultures with lower OD. Expression of *bfrD* was not upregulated in the *fur* mutants or perhaps even downregulated in the *fur* mutant of *B. bronchiseptica*, although the difference was not significant ($P = 0.17$) (Fig. 3A and B).

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*

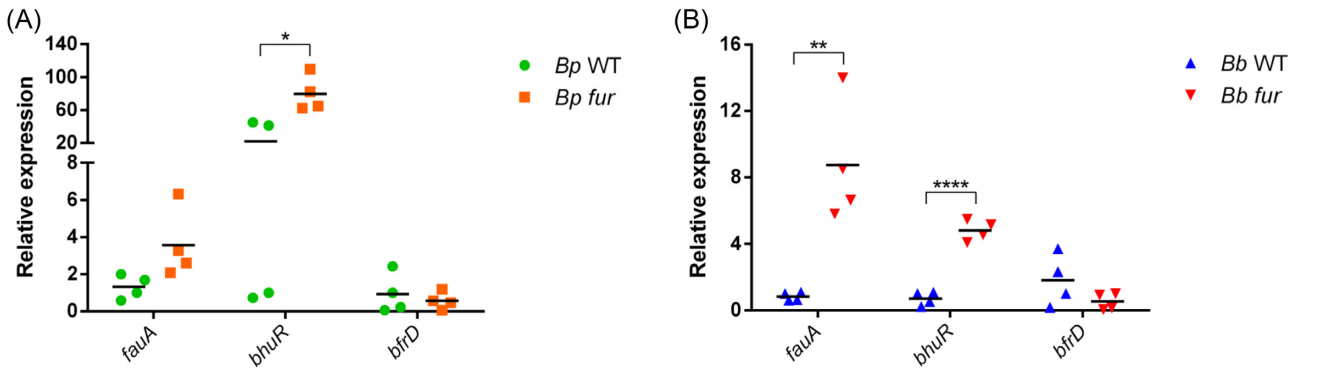

Figure 3. Expression of genes for relevant antigens in *fur* mutants. Relative gene expression of selected vaccine candidates was determined by RT-qPCR in wild-type (WT) *B. pertussis* (*Bp*) **(A)** and *B. bronchiseptica* (*Bb*) **(B)** and their *fur* mutant derivatives. The mean value of each data set of four biological replicates is depicted with a horizontal line. Significant differences were determined with multiple *t* tests using GraphPad Prism 6 and are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

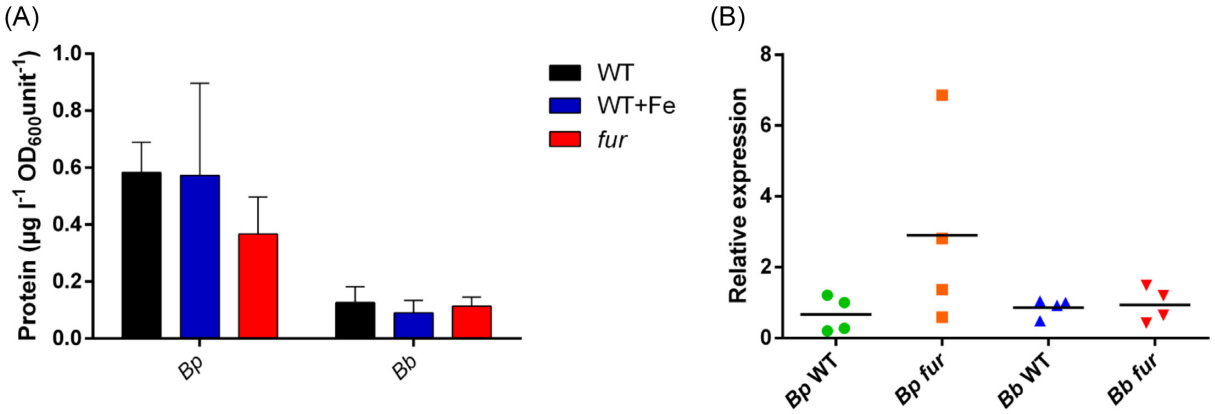

Figure 4. Effect of iron limitation on OMV production and *mlaF* expression. Wild-type (WT) *B. pertussis* (*Bp*) and *B. bronchiseptica* (*Bb*) and their *fur* mutant derivatives were grown in Verwey medium, either supplemented with iron (+Fe) or not. **(A)** OMVs released were isolated and quantified based on protein content using a Lowry assay. The yield is expressed as the amount of protein per liter of bacterial culture per OD_{600} unit. Graph shows mean values with standard deviations of three independent experiments. Significant differences were determined using one-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism 6. No statistically significant differences were found. **(B)** Relative expression of *mlaF* was determined using RT-qPCR. The mean value of each data set of four biological replicates is depicted with a horizontal line. Significant differences between the wild types and their *fur* mutant derivates were determined with unpaired *t* tests using GraphPad Prism 6. No statistically significant differences were found.

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 upregulated 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.* 2017, 2018). 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* (Raeven *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 the *fur* mutants under iron-replete conditions, which is needed for cost-effective vaccine production.

Supplementary data

Supplementary data are available at FEMSLE online.

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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, Hoenes 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. *Microb Genomics* 2020;**6**:mgen000496.

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 represibility 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 ferrimones 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, Mecsas 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 V, 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, Skaar 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 alcaligin 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 Sciuto A *et al.* Ferric uptake regulator fur is conditionally essential in *Pseudomonas aeruginosa*. *J Bacteriol* 2017;**199**:e00472–17.

Passerini de Rossi BN, Friedman LE, Belzoni CB *et al. vir90*, 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.* Intranasal 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 WF, Thiele EH, Sage DN *et al.* A simplified liquid culture medium for the growth of *Hemophilus 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.

Warfel JM, Zimmerman LI, 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_{RM} cells that sustain protective immunity against nasal colonization with *Bordetella pertussis*. *Emerg Microbes Infect* 2019;**8**:169–85.