Outer membrane protein OmpQ of *Bordetella bronchiseptica* is required for mature biofilm formation

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*Bordetella bronchiseptica*, an aerobic Gram-negative bacterium, is capable of colonizing the respiratory tract of diverse animals and chronically persists inside the hosts by forming biofilm. Most known virulence factors in *Bordetella* species are regulated by the BvgAS two-component transduction system. The Bvg-activated proteins play a critical role during host infection. OmpQ is an outer membrane porin protein which is expressed under BvgAS control. Here, we studied the contribution of OmpQ to the biofilm formation process by *B. bronchiseptica*. We found that the lack of expression of OmpQ did not affect the growth kinetics and final biomass of *B. bronchiseptica* under planktonic growth conditions. The ΔompQ mutant strain displayed no differences in attachment level and in early steps of biofilm formation. However, deletion of the *ompQ* gene attenuated the ability of *B. bronchiseptica* to form a mature biofilm. Analysis of *ompQ* gene expression during the biofilm formation process by *B. bronchiseptica* showed a dynamic expression pattern, with an increase of biofilm culture at 48 h. Moreover, we demonstrated that the addition of serum anti-OmpQ had the potential to reduce the biofilm biomass formation in a dose-dependent manner. In conclusion, we showed for the first time, to the best of our knowledge, evidence of the contribution of OmpQ to a process of importance for *B. bronchiseptica* pathobiology. Our results indicate that OmpQ plays a role during the biofilm development process, particularly at later stages of development, and that this porin could be a potential target for strategies of biofilm formation inhibition.

INTRODUCTION

*Bordetella* species are aerobic, Gram-negative bacteria that colonize the respiratory tract of humans and animals. These bacteria have the ability to infect the mammalian respiratory tract and eventually persist inside their hosts (de Gouw *et al*., 2011; Mattoo & Cherry, 2005). *Bordetella pertussis*, a strictly human pathogen, causes whooping cough or pertussis, an infectious disease indicated to be re-emergent worldwide (Chiappini *et al*., 2013; Weir, 2002). *Bordetella bronchiseptica* mainly causes chronic respiratory infections in animals, although it has sporadically been isolated from humans, demonstrating a zoonotic transmission (Woolfrey & Moody, 1991).

Whilst the strategies used by *Bordetella* species to adapt and persistently colonize their respective hosts are not well understood, multiple lines of evidence indicate that enduring microbial infections are caused by bacteria growing as biofilms (Costerton *et al*., 1999). In this regard, it has been reported that *B. bronchiseptica* can produce recalcitrant infections for as long as 40 days, hosted within the mouse nasal cavity by forming biofilm (Sloan *et al*., 2007). Additionally, we and others have previously reported the fitness of *B. pertussis* to grow attached to biotic and abiotic surfaces (Conover *et al*., 2010; Mishra *et al*., 2005; Serra *et al*., 2007, 2008, 2011; Sloan *et al*., 2007). Biofilms are surface-associated microbial communities, whose member cells are enclosed by self-produced extracellular matrix components and exhibit significantly distinct phenotypic features with respect to cells grown...
The restriction sites on the respective primers are underlined.

Table 1. Strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Description or sequence</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>B. bronchiseptica</em> RB50</td>
<td>WT genotype, Sm&lt;sup&gt;r&lt;/sup&gt;</td>
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<td><em>B. bronchiseptica</em> ΔompQ</td>
<td>Rb50 derivative, <em>ompQ</em> mutant strain</td>
<td>This study</td>
</tr>
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<td><em>E. coli</em> CC118&lt;sup&gt;pir&lt;/sup&gt;</td>
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freely suspended in liquid culture (planktonic cells) (Costerton et al., 1995). Biofilm growth allows pathogenic bacteria to escape the immune response, and to endure the effects of antibiotics and different nutrient stresses (Amato & Brynildsen, 2014; Donlan & Costerton, 2002; Høiby et al., 2014; Mah & O’Toole, 2001; Missineo et al., 2014; Mulcahy & Lewenza, 2011; Stewart & Costerton, 2001). This ability of growing attached to a surface represents an efficient way of persistence and of spreading bacteria between hosts (Hall-Stoodley & Stoodley, 2005).

The process of biofilm formation in *Bordetella* species is regulated by the BvgAS two-component system (Irie et al., 2004; Mishra et al., 2005). This signal transduction system controls the expression of virulence factors necessary for colonization and infection, in response to environmental stimuli (Cotter & Jones, 2003). Studies attempting to dissect the role of individual BvgAS-regulated factors in biofilm development by *Bordetella* species have only concentrated on a particularly small group of virulence factors, such as the adhesins filamentous haemagglutinin and fimbia (Fim). In addition, it was reported that flagella, whose expression is negatively regulated by BvgAS, are involved during the first attachment step of *B. bronchiseptica*, whilst their presence represents a deleterious effect on biofilm maturation (Nicholson et al., 2012b). The role of other virulence determinants has not yet been analysed and as a consequence we still ignore their functions in biofilm physiology. Components of the extracellular matrix of *B. bronchiseptica* and *B. pertussis* have also been characterized, i.e. Bps is a polysaccharide present in the matrix, with a composition similar to poly-<i>1,6</i>-<i>N</i>-acetyl-<i>D</i>-glucosamine, whose production is Bvg-independent and negatively regulated by the repressor BpsR (Conover et al., 2010; Parise et al., 2007).

OmpQ is an outer membrane porin protein in *Bordetella* species, whose expression is regulated by the BvgAS system. OmpQ is well conserved among *B. bronchiseptica*, *B. pertussis* and *Bordetella* para-pertussis, and it has been proposed to act as a general transporter allowing access to an essential nutrient (Finn et al., 1995). In the first studies of OmpQ, Finn et al. (1995) reported that the absence of this protein did not affect the ability of *B. pertussis* 18323 to survive in the mouse respiratory tract. However, more recently, several reports have recognized OmpQ as an immunodominant antigen and, moreover, as a potential novel protective antigen (de Gouw et al., 2014; Brynildsen, 2014; Donlan & Costerton, 2002; Høiby et al., 2014; Mulcahy & Lewenza, 2011; Stewart & Costerton, 2001).
et al., 2014; Packard et al., 2004; Tefon et al., 2011; Williamson et al., 2012; Zhu et al., 2010). In addition, it has been proposed that OmpQ should play a role in the pathogenesis of Bordetella, perhaps during the first steps of infection or in the establishment of a carrier state (Tefon et al., 2011). Given that OmpQ expression is under the control of the BvgAS system, it seems logical to envisage a role for this porin during the bacterial infection cycle. The objective of this work was to explore the role of OmpQ in biofilm formation by *B. bronchiseptica*. To this aim, we constructed an isogenic strain derivative of the *B. bronchiseptica* RB50 WT strain, harbouring an in-frame non-polar deletion in the *ompQ* gene. Surprisingly, we found that the absence of OmpQ did not affect the behaviour of *B. bronchiseptica* cells in planktonic cultures, even under diverse growth conditions. Also, the growth of the RB50 WT strain and the OmpQ-deficient mutant on abiotic surfaces was not significantly different at early time points. However, this changed at later stages, when the OmpQ-deficient mutant exhibited defects when forming mature biofilm structures. Consistent with this, real-time PCR analysis revealed a sharp increase of *ompQ* expression in 48 h biofilm cells, supporting a role for the porin in the maturation of the biofilm. Moreover, we observed that the addition of serum against OmpQ to a *B. bronchiseptica* culture rendered the bacteria impaired to grow attached to surfaces, resembling the effect of knocking out *ompQ*.

**METHODS**

**Bacterial strains, plasmids and oligonucleotides.** Strains, plasmids and primers used in this work are listed in Table 1.

**Growth conditions.** *B. bronchiseptica* strains were cultured and maintained on Bordet–Gengou agar (BGA; Difco) medium supplemented with 15 % (w/v) defibrinated sheep blood (Instituto Biológico, La Plata, Argentina). For planktonic and biofilm cultures, *B. bronchiseptica* was grown in Stainer–Scholte (SS) broth. *Escherichia coli* strains used for genetic manipulation were cultured and/or maintained on Luria–Bertani (LB) broth or agar media. When necessary, growth medium was supplemented with appropriate antibiotics at the following concentrations: streptomycin, 50 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; chloramphenicol, 50 μg ml⁻¹; and ampicillin, 50 μg ml⁻¹.

**Deletion of the *ompQ* gene.** An in-frame, non-polar deletion of the *ompQ* gene was constructed by allelic exchange following the methodology described previously (Parise et al., 2007). Briefly, a 613 bp DNA fragment containing the upstream region and the first 36 codons at the N terminus of the *ompQ* gene was amplified by PCR using *B. bronchiseptica* RB50 genomic DNA as template and 5A-BB3993/3B-BB3993 primers (Table 1). The PCR product was double digested using endonucleases XbaI and KpnI, ligated to the XbaI/KpnI-digested host vector pBBR1MCS, and transformed into *E. coli* DH5α chemically competent cells. An *E. coli* clone harbouring the pBBR1MCS plasmid with the correct inserted sequence of the promoter and coding region of *ompQ* (pBBR ompQ) was selected and used as donor strain in triparental mating experiments, including *B. bronchiseptica* ΔompQ as recipient strain and an *E. coli* strain containing the helper vector pSS1827.

**Biofilm formation in microtitre plates.** *B. bronchiseptica* biofilm formation was evaluated using a microtitre dish assay following the methodology described previously (O’Toole et al., 1999). For each strain, 100 μl bacterial suspension adjusted to OD₆₅₀ 0.05 was inoculated into the corresponding wells of a microtitre plate. Plates were incubated statically at 37 °C for 24 or 48 h. For 48 h cultures, the medium was entirely replaced with fresh medium after 24 h of biofilm growth. To quantify biofilm formation, the liquid medium containing planktonic bacteria was first removed from each well. The remaining adhered biomass in every well was gently washed twice with PBS and subsequently stained for 20 min with a 0.1 % (w/v) crystal violet solution. After staining, the crystal violet solution was removed and every well was washed twice with distilled water. Finally, the crystal violet that remained associated with the biofilm biomass was solubilized with 200 μl ethanol/acetone solution (80 : 20, v/v) and A₅₉₀ of the resulting solution was determined. Three independent experiments with four replicates each were performed for each strain.

**Fluorescence microscopy.** Bacterial adhesion to borosilicate surface was examined by fluorescence microscopy. A Lab-Tek culture chamber with a 1 mm borosilicate coverglass at the base (Nunc; Thermo Fisher Scientific) was used as a system to examine the attachment of *B. bronchiseptica* RB50 and ΔompQ strains. To visualize bacteria by fluorescence microscopy, cells of both strains were transformed with a medium copy number vector harbouring the gene coding for GFP under the control of the constitutive tac promoter (pTAC-GFP). For each strain, three chamber wells were inoculated with a 1.5 ml suspension of GFP-expressing bacteria adjusted to OD₆₅₀ 0.05. After incubation for 2 h at 37 °C, bacterial suspension was removed and each chamber well was washed twice with PBS. Bacteria that remained attached to the borosilicate base of the chamber well were visualized at a magnification of ×1000 using a Leica DMLB fluorescence microscope equipped with a standard blue, green and red filter set (excitation 400/20, 495/15 and 570/50 nm, respectively), and with a charge-coupled device digital camera. At least three independent adhesion experiments were performed for each strain. Quantification of adhered cells was performed by counting attached GFP-expressing bacteria using ImageJ (Schneider et al., 2012) and the ITCN plug-in (Byun et al., 2006).

**Confocal laser scanning microscopy (CLSM).** Biofilm formation was also examined by CLSM using static and continuous flow culture.
Briefly, biofilm cultures were grown on glass Petri dishes, inoculating cultures and from planktonic cells at the exponential growth phase. Biofilms were visualized with a Leica TCS SP5 microscope. CLSM images were analysed with COMSTAT 2 software (Heydorn et al., 2000) in order to acquire quantitative data. Triplicate experiments were performed for each strain and culture system.

Gene expression level quantification. Real-time PCR assays were performed in order to evaluate ompQ gene expression during B. bronchiseptica RB50 biofilm formation. RNA samples were obtained from sessile cells harvested from 24, 48 and 72 h biofilm cultures and from planktonic cells at the exponential growth phase. Briefly, biofilm cultures were grown on glass Petri dishes, inoculating with a bacterial suspension adjusted to OD_{650} 0.05. When needed, the medium was replaced every 24 h. At each time point, the planktonic cells were removed from the dish, and the remaining biofilm was washed two times with sterile PBS and suspended in 1 ml RNAlater solution (Invitrogen). For planktonic cultures, 30 ml SS medium was inoculated with an overnight culture, adjusting to OD_{650} 0.2 and incubated until OD_{650} 1 was reached. Aliquots of 1 ml were taken and centrifuged; the cell pellet was suspended in 1 ml RNAlater solution. Samples were then incubated for 24 h at 4 °C and stored at −20°C until use. RNA extraction was carried out with a Purelink RNA Mini kit (Invitrogen) following the manufacturer’s instructions. For cDNA synthesis, 1 μg each RNA sample was treated with DNase I (Promega) for 30 min at 37°C. The DNA-free RNA was then used as template to synthesize the cDNA with M-MLV Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. Real-time PCR assays were performed with SYBR Premix (Thermo Fisher Scientific) and primers detailed in Table 1. Reactions were carried out on triplicate biological samples and technical duplicates. The level of ompQ mRNA was calculated with normalization to 16S rRNA, using the ΔΔC_{t} method, as described by Conover et al. (2012).

ompQ gene molecular cloning for recombinant protein production. A DNA fragment including the coding region of the ompQ gene without its signal sequence was amplified by PCR using RB50 genomic DNA as template and FBB399/exp/RBB399/exp primers (Table 1). The PCR product was double digested with BamHI and HindIII restriction enzymes, and ligated with the equally digested pET28a expression vector such that six His residues were added at the N-terminal region of the OmpQ primary sequence. The ligation was transformed into chemically competent BL21-CodonPlus cells, and a bacterial clone harbouring the pET28a construct was cultured in LB medium supplemented with kanamycin and chloramphenicol, and incubated at 37°C under shaking conditions (160 r.p.m.). At the point where the culture reached OD_{560} 0.4, the expression of rOmpQ was induced by the addition of IPTG to a final concentration of 0.5 mM. At 4 h after the initial induction, cells were harvested by centrifugation for 15 min at 5000 r.p.m. (4°C). The bacterial pellet was recovered and suspended in a lysis buffer containing 50 mM Na_{2}PO_{4}, 300 mM NaCl, 10 mM imidazole and 8 M urea (pH 7.4), and incubated overnight at room temperature under slight shaking. Afterwards, the cell lysate was centrifuged for 20 min at 10,000 g and the supernatant containing rOmpQ was recovered. The purification of rOmpQ was carried out using a cobalt-nitritolriatic acid-agarose affinity resin under denaturing conditions, as specified by the supplier (Pierce Biotechnology). After applying the supernatant to the column, the resin containing bound rOmpQ was washed with 30 mM imidazole buffer. rOmpQ was eluted from the column by the addition of 150 mM imidazole. The presence of proteins in the eluted fractions was determined by Coomassie blue staining in a 10 % SDS-polyacrylamide gel. The purified rOmpQ was then dialysed, freeze-dried and resuspended in PBS. The quantification of the recovered rOmpQ was done using the Bradford method.

Antibody production. To obtain antibodies against OmpQ, three female BALB/c mice (2 weeks old) were immunized intraperitoneally with 10 μg purified rOmpQ emulsified with complete Freund’s adjuvant. Booster doses (10 μg rOmpQ emulsified with incomplete Freund’s adjuvant) were applied at days 17 and 33 post-immunization. The immunological response was followed by ELISA using rOmpQ. The mice were bled at day 55 post-immunization, and the sera were separated by centrifugation and stored at −20°C.

Western blotting. Analysis of whole-cell protein samples and purified rOmpQ was done by Western blotting. Protein samples were prepared using 2 × Laemmlı buffer and separated by 10 % SDS-PAGE. After separation, proteins were transferred to PVDF membranes (Immobilon; Millipore), and then subjected to immunochemical detection using anti-rOmpQ sera and goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (Jackson Immuno Research).

Biofilm inhibition assay. The inhibitory action of pre-immune and anti-OmpQ sera on biofilm formation was assessed by using the microtitre plate assay described earlier. Bacterial suspensions were incubated in SS medium with the addition of three different serum dilutions (1 : 100, 1 : 200 and 1 : 400) and incubated at 37°C in microtitre plate wells for 48 h under static conditions. Biofilm formation was assessed after 48 h of growth by using the crystal violet staining technique described earlier.

Bioinformatic analysis and protein modelling. We used the sequence of OmpQ to search for putative homologous proteins in the National Center for Biotechnology Information nr (non-redundant) database using BLAST. Retrieved sequences were aligned using CLUSTAL V (Higgins et al., 1992). The aligned proteins were used to reconstruct a phylogenetic tree using the program PhyML (Guindon et al., 2009) using the JTT+I model with a gamma distribution to simulate rate heterogeneity amongst sites. To obtain node support, a non-parametric bootstrap was performed with 500 resamplings. Fold assignment methods were run in order to estimate putative templates for OmpQ and OmpP proteins. HHpred (Söding et al., 2006) and FFAS03 (Jaroszewski et al., 2005) were used, and the putative templates obtained were used with MODeller (Webb & Sali, 2014) to obtain 100 3D models for each protein. Best models according to the MODeller objective function were further evaluated with ProSali (Wiederstein & Sippl, 2007). Macromolecular electrostatics calculations were done with APBS and PDB2PQR (Dolinsky et al., 2004).
RESULTS

Deletion of ompQ does not affect planktonic growth of B. bronchiseptica under varied conditions

Porins primarily play physiological roles that in many cases decisively influence the ability of a bacterium to thrive in a certain environment. We, therefore, began our study by assessing how OmpQ impacts the ability of B. bronchiseptica RB50 to grow in vitro under regular liquid culture conditions. To this aim, we generated a B. bronchiseptica mutant strain deficient in OmpQ. In this RB50 derivative strain the chromosomal copy of the ompQ gene was deleted by allelic exchange (ΔompQ). We then compared its growth kinetics in defined SS liquid medium with those of the RB50 WT strain. Both strains exhibited almost the same growth curve, reaching similar levels of biomass at the end of the culture, indicating that the absence of OmpQ did not affect the ability of B. bronchiseptica to grow under planktonic conditions (Fig. S1, available in the online Supplementary Material). In addition, we comparatively studied the effect of nutritional and stress conditions on the planktonic growth of both RB50 WT and ΔompQ strains. We evaluated different culture media considering those conditions in which porins from other organisms were shown to be involved, such as hyperosmotic stress, nutrient and oxygen limitation, and antibiotic resistance (Aunkham et al., 2014; Finelli et al., 2003; Kojima & Nikaido, 2014; Liu & Ferenci, 2001; Yoon et al., 2002). Surprisingly, under all of the different conditions assayed, the ΔompQ mutant strain showed similar growth to the RB50 WT strain (Fig. S2, Table S1).

OmpQ-deficient mutant is impaired in biofilm formation

OmpQ expression is regulated by the BvgAS two-component system. As this system has been shown to control biofilm formation in Bordetella species, we reasoned that OmpQ may be involved in this process. To test this, we first examined the growth of the RB50 WT and ΔompQ mutant on microtiter plate wells. Plates were inoculated and incubated statically for 24 or 48 h at 37 °C. The biofilm biomass adhered to the wells was quantified by crystal violet staining, whereas the suspended biomass in the liquid phase was determined by measuring OD650. At 24 h, both strains formed virtually the same amount of biofilm. At 48 h, however, whilst the ΔompQ strain exhibited almost the same level of biofilm biomass as shown at 24 h, the RB50 WT strain doubled its amount, suggesting that OmpQ becomes important at an advanced stage of biofilm growth, possibly when structural maturation takes place (Fig. 1). This difference was not attributed to defects in growth of the ΔompQ mutant, as both strains showed similar planktonic growth in the wells at both time points (data not shown), which was also consistent with the results described earlier. To further confirm that the defect of the ΔompQ in forming biofilms was precisely due to deletion of ompQ, we then examined biofilm growth of the ΔompQ strain expressing theompQ gene in trans (ΔompQ pBBR/ompQ). The results showed that the complemented ΔompQ strain indeed formed more biofilm than the ΔompQ strain harbouring the empty vector, although it still produced less biofilm than RB50 WT (Fig. 1).

Additionally, we proposed to gain more insights into the adherence and biofilm growth of the ΔompQ strain by using CLSM and fluorescence microscopy. To this aim, the RB50 WT and mutant strain were transformed with a plasmid harbouring the egfp gene under control of a constitutive promoter. GFP-tagged bacteria of both strains were then inoculated in individual wells of Lab-Tek culture chambers, whose bases consisted of a 1 mm borosilicate coverglass. After 2 h of incubation at 37 °C, bacteria adhered to the borosilicate glass base were visualized by fluorescence microscopy (Fig. 2a, b) and quantified by using a cell counting tool operated via ImageJ. A mean of 5.2 and 5.7 cells per 10 μm² were detected for the ΔompQ and RB50 WT strains, respectively, which demonstrated no defects in attachment due to the absence of OmpQ.

Similarly, biofilms allowed to form for 48 h on the borosilicate glass base of the chambers were then examined by CLSM. z-Stack collections of images were analysed using COMSTAT2 software. As shown in Fig. 3(a, b), the RB50 WT strain developed irregularly inter-spaced microcolonies that were relatively wide (in some cases up to 60 μm).
and thick (up to 42 µm). In contrast, the ΔompQ strain formed significantly smaller microcolonies, with a reduction in biomass, maximum thickness and mean thickness (Table 2).

We also studied the effect of OmpQ absence during biofilm formation under continuous flow conditions by monitoring growth of the same culture after 24 and 48 h. After 24 h of culture, no differences were evident, whereas at 48 h, the mutant strain presented lower values of biomass, maximum thickness and mean thickness (Fig. 3c, d, Table 2). The biofilm-related features of the RB50 WT strain were consistent with those reported previously (Conover et al., 2011). In addition, at 48 h of culture, the biofilm formed by the ΔompQ strain presented a larger number and size of water channels. This feature may be a strategy of this mutant to facilitate nutrient access inside the biofilm, in a way to compensate for the absence of the transporter. Overall, the microscopic analysis also supported a role

Table 2. Quantitative (mean ± SD) analysis of biofilm structural characteristics determined by COMSTAT2 software

<table>
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<tr>
<th>Culture system</th>
<th>Time point (h)</th>
<th>Strain</th>
<th>Biomass (µm³ µm⁻²)</th>
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<th>Mean thickness (µm)</th>
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<td>24</td>
<td>RB50 WT</td>
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<td>ΔompQ</td>
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<td></td>
<td>48</td>
<td>RB50 WT</td>
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<td></td>
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<td>1.55 ± 0.70</td>
<td>27.47 ± 7.73</td>
<td>6.16 ± 2.76</td>
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<td>48</td>
<td>RB50 WT</td>
<td>12.93 ± 2.62</td>
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<td>ΔompQ</td>
<td>8.04 ± 3.07</td>
<td>24.25 ± 2.69</td>
<td>13.45 ± 3.59</td>
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Fig. 2. Analysis of adhesion to coverglasses by B. bronchiseptica RB50 WT and the ΔompQ strain. (a) Fluorescence micrographs of adhered bacteria after 2 h of incubation at 37 °C observed at ×1000 original magnification (RB50 WT, left panel; ΔompQ, right panel). (b) Quantification of adhered cells observed by microscopy. The number of cells was determined with the ITCN counting tool run with ImageJ software.
for OmpQ in biofilm formation by *B. bronchiseptica*, particularly at the stage when the biofilm developed its mature structure.

**ompQ is highly expressed in mature biofilms**

The fact that OmpQ becomes relevant at the time when the biofilm needs to consolidate its structure (i.e. ~48 h after inoculation) led us to speculate whether the expression of the porin is particularly altered at this stage. To test this, we evaluated the expression level of *ompQ* in RB50 cells grown in biofilm for 24, 48 and 72 h by using real-time PCR. These expression levels were then compared with those of planktonic RB50 cells collected in the exponential growth phase. Whereas 24 h biofilm cells showed slightly increased *ompQ* expression with respect to exponentially growing planktonic cells, 48 h biofilm cells exhibited levels of *ompQ* expression that were more than twofold higher than those of the planktonic population (Fig. 4). Strikingly, the expression of *ompQ* dropped significantly in 72 h biofilm cells. The biofilm formation defects of the ∆ompQ strain observed at 48 h were extended over time (at least until 72 h, when the WT strain showed a biomass level as determined by the $A_{590}$ of crystal violet solution of $3.14 \pm 0.32$, whilst the ∆ompQ strain showed a value of $1.73 \pm 0.19$); therefore these results suggest that the sharp increase of *ompQ* mRNA levels, and consequently the OmpQ protein levels, at 48 h could be necessary to promote development and possibly maintenance of the mature biofilm structure.
Immune serum against OmpQ inhibits biofilm formation

On the basis that OmpQ is relevant for *B. bronchiseptica* biofilm maturation and that it is an outer membrane protein, we reasoned that OmpQ could be a good candidate for antibody targeting and, thus, a target for blocking biofilm formation. To investigate this idea, we first cloned and overexpressed a recombinant OmpQ protein in *E. coli* cells (rOmpQ). rOmpQ was then purified and used to produce an anti-rOmpQ serum in mice. The collected serum was evaluated for recognition of OmpQ by Western blotting. As shown in Fig. 5(a), in a whole-cell protein extract of *B. bronchiseptica* RB50 only a band of the same size as rOmpQ was detected by the serum, which demonstrated its specificity. Additionally, the serum also showed reactivity on the rOmpQ sample to bands with different molecular mass. This might be the result of cross-reaction with unspecific proteins from the *E. coli* induction lysate or degraded rOmpQ present in the purified protein sample used to inoculate mice. Nevertheless, the serum showed specific reactivity to only one protein, i.e. OmpQ, in *B. bronchiseptica* RB50 whole-cell lysate.

To test whether the anti-rOmpQ serum interfered with biofilm formation by *B. bronchiseptica*, bacterial suspensions of RB50 were supplemented with three different dilutions of anti-rOmpQ serum (1:100, 1:200 and 1:400). As shown in Fig. 5(b), the anti-rOmpQ serum significantly reduced the biofilm formation by RB50. The control (normal serum) did not show any inhibitory effect.

### Fig. 4. Relative *ompQ* mRNA expression during the biofilm formation process. The relative *ompQ* expression level was assessed by real-time PCR in RB50 biofilms cultured at 24, 48 and 72 h (*ompQ* level expression in planktonic culture is set to 1). 16S mRNA was used as standardization control. Data represent mean ± SEM from at least three biological and technical replicates. The fold change was calculated using the ΔΔCt method. Statistical significance of relative expression changes was analysed by Student’s t-test; *P<0.05.

### Fig. 5. Biofilm inhibition assay with serum against rOmpQ. (a) Recognition of OmpQ by the anti-rOmpQ serum. Whole-cell lysate from RB50 WT (lane 1) and purified rOmpQ (lane 2) were subjected to SDS-PAGE (10%) and transferred to PVDF membrane. Immunoblot analysis was performed with polyclonal mouse antiserum obtained against rOmpQ. The gel was loaded with 5 μg protein of each sample. (b) Biofilm inhibition assay. Several dilutions of anti (α)-rOmpQ serum were added to the biofilm growth medium. Normal serum (NS) was used as negative control at the same dilutions assayed for the anti-rOmpQ serum. The experiment was conducted on microtitre dishes for 48 h and the biofilms were quantified by the crystal violet method. Data represent mean ± SD of solubilized crystal violet. Statistical significance was assessed by Student’s t-test; *P<0.05. (c) Planktonic growth was evaluated after 48 h of culture by measuring mean ± SD OD650 of the recovered medium.
1:400), inoculated in microtitre plate wells and allowed to grow as biofilm for 48 h at 37 °C. Similarly, the pre-immune serum (normal serum, NS), diluted in the same manner, was used as negative control. Biofilms were quantified after 48 h of growth by crystal violet staining. In support of our hypothesis, we found that anti-rOmpQ serum reduced RB50 biofilm formation significantly (Fig. 5b). This reduction was proportional to the amount of serum present in the medium, which ranged from 14 to 28 % of the biofilm biomass, when compared with conditions in which similar dilutions of the pre-immune serum were used. No significant differences were found in the planktonic growth fraction (Fig. 5c). These results resembled to a great extent the effect of knocking out ompQ and supported the role of OmpQ in the formation of mature biofilms by *B. bronchiseptica*.

**Bioinformatic and protein structural modelling analysis**

In order to approximate a structural characterization of OmpQ that could allow us understand its possible function, we performed a bioinformatic analysis of OmpQ. As found previously, fold assignment methods predict that OmpQ belongs to the family of porin protein channels (Nikaido, 2003). Several porin structures [Protein Data Bank (PDB) IDs: 2FGQ, 2VY8, 1E54 and 4AUI, from different organisms] were found with statistically significant values by either the HHpred or FFAS03 method. Using these PDB templates, we obtained structural models for OmpQ and also for the constitutive porin OmpP. It was previously reported that both porins show 61 % similarity and 39 % identity over the entire length of the two proteins (Finn *et al.*, 1995); for this reason, in order to compare both proteins we included OmpP in the structural modelling analysis. Best models were found with an adequate quality after ProsalII evaluation. All the structural models obtained showed the typical antiparallel β-barrel built of amphipathic β-strands (Zeth *et al.*, 2000), as shown in Fig. 6(a).

Using electrostatic evaluation, we found a differential charge distribution in OmpQ with reference to OmpP and other well-characterized porins such as the anion-selective porin from *Comamonas acidovorans* (PDB ID: 1E45) or the PorB porin from *Neisseria gonorrhoeae* (PDB ID: 4AUI) included in the set of close homologous proteins retrieved from similarity searches (Fig. 6b). As the type of electrostatic field in the channel defines the general types of molecules transported through
portains, the differences observed between OmpQ and OmpP proteins could indicate a functional differentiation. In fact, in the phylogenetic tree, OmpQ from *B. bronchiseptica* forms a separate cluster from the majority of porins, including crystallized and well-characterized proteins (Fig. S3). In this cluster, OmpQ can be found along with other orthologues of other *Bordetella* species (such as *B. pertussis*, *Bordetella trematum*, *Bordetella homensis*, *Bordetella hinzii* and *Bordetella bronchiseptica* isolates), and a few predicted proteins from non-related micro-organisms (such as *Alcaligenes faecalis*, *Achromobacter xylosoxidans*, *Achromobacter insuavis*, and *Kerstersia gyiorum*; none of them previously studied). This result reinforces the idea that a functional adaptation of the channel selectivity and/or protein function happened during the evolution of OmpQ.

**DISCUSSION**

Porins are outer membrane proteins assembled as oligomers that allow the transport through the membrane of hydrophilic solutes, such as ions, amino acids, nucleosides and sugars, and, in some cases, may exclude the entrance of antibiotics or other inhibitors, contributing to bacterial resistance to antimicrobial agents (Koebnik et al., 2000; Schirmer, 1998; Schulz, 1996). OmpQ is one the two major porins identified in the genus *Bordetella*. The other major porin, OmpP, is a 40 kDa general porin with constitutive expression and selectivity for anionic substrates (Armstrong et al., 1986). The few reports on the role of OmpQ are controversial. On the one hand, Finn et al. (1995) showed that the absence of OmpQ does not affect the capacity of *B. pertussis* to colonize mouse lung tissue, discarding the possibility that this protein may play a role in the survival of the bacterium inside the host. On the other hand, recent proteomic studies proposed OmpQ as a novel protective antigen candidate (de Gouw et al., 2014; Packard et al., 2004; Tefon et al., 2011; Williamson et al., 2012; Zhu et al., 2010) and even suggested that OmpQ could play a role in the pathogenesis of *Bordetella* (Tefon et al., 2011).

Two main lines of evidence prompted us to investigate the role of OmpQ in *B. bronchiseptica* biofilm formation. First, being outer membrane proteins, porins can play a role in the pathogenesis of bacteria (e.g. interaction with host epithelial tissues) beyond their transporter function. In this regard, some reports have associated the presence of particular porins with bacterial virulence and ability to form biofilms; such is the case for OmpF and OmpF, a substrate-specific and a general porin, respectively, which contribute to biofilm formation by *Pseudomonas aeruginosa* (Finelli et al., 2003; Fito-Boncompte et al., 2011). Second, OmpQ expression is positively regulated by BvgAS, a two-component system that controls the expression of most virulence genes in *Bordetella* species, and that was shown to be essential for biofilm formation *in vitro* and *in vivo* (Irie et al., 2004; Mishra et al., 2005). *Bordetella* species utilize the BvgAS system to sense the environment and regulate gene expression amongst at least three phenotypic phases: a virulent phase (Bvg+), an intermediate phase (Bvg0) and an avirulent phase (Bvg-). OmpQ is expressed in the Bvg+ phase ( Cotter & Miller, 1994; Deora et al., 2001; Hot et al., 2003; Kinnear et al., 2001), which is the phenotypic phase necessary and sufficient to initiate an infectious cycle (Nicholson et al., 2012a), and is also the phase that *Bordetella* transits when grown in defined SS medium at 37 °C, as was the case in this study.

As porins primarily play physiological roles that in many cases determine the ability of a bacterium to survive in a certain environment, we first evaluated whether the absence of OmpQ affected the ability of *B. bronchiseptica* to grow in defined SS medium. Under such culture conditions OmpQ does not seem to play a significant physiological role that impacts on *B. bronchiseptica* growth. This result is perhaps not surprising in view of the fact that OmpQ is a BvgAS-regulated factor and therefore *B. bronchiseptica* would not require the expression of this porin when the bacterium is in an avirulent Bvg-phase. Being a major porin in the outer membrane, however, it is somewhat surprising that OmpQ does not significantly contribute to coping with any of the frequent environmental stresses that the bacterium has to face, such as osmotic upshifts, nutrient and oxygen limitation, and even the presence of antibiotics.

Biofilm formation in *B. bronchiseptica* is regulated by the BvgAS two-component system, with a Bvg+ phase being necessary in order to grow as a biofilm (Irie et al., 2004; Mishra et al., 2005). It has been proposed that a biofilm lifestyle could allow *B. pertussis* and *B. bronchiseptica* to establish a carrier state in their hosts, and in this sense, we and other authors have demonstrated the ability to form biofilms *in vitro* and in a mouse model for both species (Conover et al., 2010; Parise et al., 2007; Serra et al., 2011; Sloan et al., 2007). Very few virulence factors have been analysed in the context of biofilm production in *Bordetella*. In the case of OmpQ, no function has been associated with this protein that could explain why it is a Bvg-regulated gene. Thus, we decided to study the effect of the absence of OmpQ in the biofilm produced by *B. bronchiseptica*.

We found that the *B. bronchiseptica* ΔompQ mutant strain showed an impaired capacity to form mature biofilms when compared with the *B. bronchiseptica* RB50 WT strain, growing under similar experimental conditions. Although the WT and mutant strains showed similar biomass until 24 h of biofilm growth, a reduction of biofilm mature biomass was evident for the mutant strain at 48 h of incubation. At this time of growth, *B. bronchiseptica’s* biofilm reached a more organized and mature structure, such as was reported previously (Conover et al., 2011; Mishra et al., 2005). In addition, by using microscopic structural analysis, it was shown that, whilst the lack of OmpQ did not affect the ability of the mutant strain to
adhere to abiotic surfaces, the ΔompQ strain was unable to produce highly organized biofilm structures after 48 h of growth in a batch-type culture. Moreover, when cultured under continuous flow conditions, the mutant strain displayed larger water channels than the WT strain. This structural characteristic may be relevant to overcoming the absence of OmpQ by increasing the biofilm area in contact with nutrients. Regarding other structural parameters calculated by COMSTAT2 under flow conditions, we still observed a reduction in biomass, maximum thickness and mean thickness, although the decrease was not as noticeable as in static culture. Nevertheless, it is possible to speculate that growth under static conditions could represent a higher limitation of nutrients than under continuous flow conditions. These microscopy results indicate the necessity of OmpQ to achieve a mature biofilm structure in nutrient-limited conditions. When a biofilm is formed, bacterial growth becomes organized in a complex structure, represented by microcolonies. The maturation of a biofilm depends on the diffusion of nutrients—a process that is affected not only by the complexity of the biofilm structure, but also by the nature of both the nutrient and the matrix (Stewart, 2003). In this scenario, bacteria within a biofilm may detect a reduction of the availability of some nutrients and therefore could exploit particular nutrient transporters to facilitate the entrance of a specific substrate. In our case we speculate that bacteria could compensate for the lack of OmpQ by forming a biofilm with more pronounced channels.

The importance of OmpQ in biofilm maturation is supported by the temporal increase in the expression of the ompQ gene observed in WT sessile cells at 48 h by realtime PCR analysis. The pattern of expression detected in our experimental conditions correlates with that reported by Nicholson et al. (2012b), in which a transcriptomic approach was conducted during the biofilm formation process of B. bronchiseptica from 6 to 48 h, observing an increase in the expression of ompQ at 48 h of biofilm culture, under similar experimental conditions. Taking these results together, we can conclude that this protein is involved in the development of B. bronchiseptica mature biofilm, although its specific mechanism remains to be determined.

Several studies have shown the effect of the application of antibodies against surface components of bacteria in biofilm formation as a first approach to reduce biofilm formation in vitro (Shahrooei et al., 2009; Tashiro et al., 2008). Here, we found that the presence of polyclonal serum against OmpQ in the growth medium significantly reduced WT biofilm formation, similar to that observed in the B. bronchiseptica ΔompQ strain.

Finally, we analysed structural features of OmpQ by protein modelling. The electrostatic distribution in OmpQ was opposite to that presented in OmpP. This feature implies a differentiation in both porins, which could involve opposite functions in molecule transport across the membrane. A predominant negative charge distribution could indicate a preference for positively charged substrates. Moreover, when we studied the phylogeny of OmpQ with regard to porins with a high score after BLAST analysis, we observed that this OmpQ forms a separate cluster from the majority of porins. This finding reinforces the idea of a differentiation in structural features and consequently a divergence of OmpQ from other porins. This aspect would be better understood after crystallographic structural determination of OmpQ, which would also provide information about the possible types of molecules that are transported by OmpQ.

Overall, our results suggest that OmpQ plays a role during the maturation of B. bronchiseptica biofilms and that it is a potential candidate to study for prevention of biofilm formation in vivo.

In conclusion, we have provided several lines of evidence indicating that OmpQ participates in mature biofilm formation of B. bronchiseptica. We found that OmpQ absence is associated with a reduction of biofilm biomass at 48 h and an under-structured biofilm. We demonstrated a variable level of expression during biofilm formation, and that the timing of the increase in level of expression of ompQ and of the requirement of a functional ompQ gene for biofilm are coincident. Moreover, the negative effect of antibodies against OmpQ on B. bronchiseptica biofilm suggests that, in terms of pathogen control, this protein could be a potential target. Certainly, further studies are needed in order to elucidate the molecular mechanism of OmpQ in Bordetella pathogenesis, including biofilm formation in a mouse infection model.

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REFERENCES


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