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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 Δ*ompQ* 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

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Abbreviations: PDB, Protein Data Bank; r, recombinant.

One supplementary table and three supplementary figures are available with the online Supplementary Material.

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

The restriction sites on the respective primers are underlined.

Strain, plasmid or primer	Description or sequence	Reference
Strains		
B. bronchiseptica RB50	WT genotype, Sm^r	Cotter & Miller (1994)
B. bronchiseptica Δ*ompQ*	RB50 derivative, *ompQ* mutant strain	This study
E. coli CC118*λpir*	Host strain for *pir*-dependent plasmids	de Lorenzo *et al.* (1990)
E. coli DH5α	*E. coli* laboratory strain	Stratagene
E. coli BL21-CodonPlus BL21(DE3)	*E. coli* strain for recombinant protein expression	Novagen
Plasmids		
pRE112	Allelic exchange vector, Cm^r	Edwards *et al.* (1998)
pRE112/AB3993	pRE112 derivative, *ompQ* deletion plasmid	This study
pRK2013	Mobilization helper plasmid, Kan^r	Figurski & Helinski (1979)
pBBR1MCS	Broad-host-range plasmid, Kan^r	Kovach *et al.* (1994)
pBBR/ompQ	*ompQ* complementation plasmid, pBBR1MCS derivative plasmid containing the *ompQ* locus	This study
pSS1827	Mobilization helper plasmid, Amp^r	Novagen
pET28a	Recombinant protein expression plasmid, Kan^r	Novagen
pET28a/ompQ	*ompQ* expression plasmid, pET28a derivative	This study
pTac-GFP	Constitutive GFP expression plasmid, Cm^r	Sloan *et al.* (2007)
Primers		
5A-BB3993	5′-TGCTCTAGATCTGCCTGGATTTCTCG-3′	This study
5B-BB3993	5′-CCCAAGCTTCACCACCCCATACAACTC-3′	This study
3A-BB3993	5′-CCCAAGCTTAGCCTTTATGCCTATGG-3′	This study
3B-BB3993	5′-CGGGGTACCAGGAAGACGAACAGC-3′	This study
FBB3993exp	5′-GCGGATCCTCGAGTTGTATGGGGTGG-3′	This study
RBB3993exp	5′-CCCAAGCTTTGATTGTAGTTGACGACG-3′	This study
QFBB3993	5′-CACCAGCCTTTATGCCTATG-3′	This study
QRBB3993	5′-GTCATTCCCACGCCAAAC-3′	This study
QF16S	5′-TCAGCATGTCGCGGTGAAT-3′	Nicholson *et al.* (2012a)
QR16S	5′-TGTGACGGGCGGTGTGTA-3′	Nicholson *et al.* (2012a)

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.*, 2010; 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 fimbria (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-1,6-*β*-*N*-acetyl-D-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 amongst *B. bronchiseptica*, *B. pertussis* and *Bordetella parapertussis*, 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; 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 % (v/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-BB3993/3A-BB3993 primers (Table 1), which include an *Xba*I or *Hin*dIII restriction site at the 5′ end, respectively. Similarly, a 548 bp DNA fragment containing the last 37 codons and a downstream region of the *ompQ* gene was amplified by PCR using 5B-BB3993/3B-BB3993 primers (Table 1), which include a *Hin*dIII or *Kpn*I restriction site at the 5′ end, respectively. Each DNA fragment was double digested accordingly with either *Xba*I/*Hin*dIII or *Hin*dIII/*Kpn*I restriction enzymes and ligated together with the *Xba*I/*Kpn*I-digested pRE112 vector. An *Escherichia coli* clone harbouring pRE112 with the correct inserted sequences of both fragments (pRE112-AB3993) was selected and used as donor strain in triparental mating experiments, including *B. bronchiseptica* RB50 as recipient strain and an *E. coli* strain harbouring the helper plasmid pRK2013. Exconjugants were selected on BGA plates containing streptomycin and chloramphenicol. Bacterial clones that underwent first recombination events and had the WT and the truncated version of *ompQ* (Δ*ompQ*) in their genomes were confirmed by PCR. These clones were cultured overnight in SS broth and counter-selected for second recombination events on LB agar plates supplemented with 7.5 % (w/v) sucrose. Clones that contained only the truncated version of *ompQ* were confirmed by PCR and DNA sequencing of the PCR product.

Genetic complementation of the *ompQ* gene. A 2024 bp DNA fragment including the *ompQ* gene coding region and its promoter region was amplified by PCR using genomic DNA from *B. bronchiseptica* RB50 as template and 5A-BB3993/3B-BB3993 primers (Table 1). The PCR product was double digested using endonucleases *Xba*I and *Kpn*I, ligated to the *Xba*I/*Kpn*I-digested broad-host-range 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* Δ*ompQ* 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 OD_{650} 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 A_{590} 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 Δ*ompQ* 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 OD_{650} 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

systems. As described earlier, a Lab-Tek culture chamber was used as a static system to examine the biofilms formed by *B. bronchiseptica* RB50 and Δ*ompQ* strains harbouring the pTac-GFP plasmid. For each strain, three chamber wells were inoculated with a 1.5 ml suspension of GFP-expressing bacteria adjusted to OD_{650} 0.05. The SS medium was supplemented with chloramphenicol and changed every 24 h. After 24 and 48 h of incubation at 37 °C, each chamber well was washed twice with PBS before microscopic visualization. In addition, a continuous flow system was used to grow the biofilms. Flow chambers were inoculated with an exponential culture with an OD_{650} adjusted to 0.2 and incubated statically for 2 h at 37 °C. Then, fresh SS medium supplemented with chloramphenicol was added at a flow rate of 0.1 ml min^{-1}. Biofilms were evaluated at 24 and 48 h.

Biofilms were visualized with a Leica TCS SP5 microscope. CLSM images were analysed with COMSTAT2 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 final 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 by normalization with 16S rRNA, using the $\Delta\Delta C_t$ 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 FBB3993exp/RBB3993exp primers (Table 1). The PCR product was double digested with *Bam*HI and *Hin*dIII restriction enzymes, and ligated with the equally *Bam*HI/*Hin*dIII-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 with the correct inserted sequence was selected and used in further experiments on OmpQ overexpression and purification. Identity of the cloned DNA sequences was confirmed by sequencing and bioinformatics analysis.

Recombinant OmpQ expression and purification. Overexpression and purification of the recombinant His-tagged OmpQ (rOmpQ) were performed following the protocol described previously by Alvarez Hayes *et al.* (2011). Briefly, *E. coli* BL21-CodonPlus cells harbouring the pET28a/ompQ construct were 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_{600} 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_3PO_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-nitrilotriacetic 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× Laemmli 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 assayed 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 + F 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 ProsaII (Wiederstein & Sippl, 2007). Macromolecular electrostatics calculations were done with APBS and PDB2PQR (Dolinsky *et al.*, 2004).

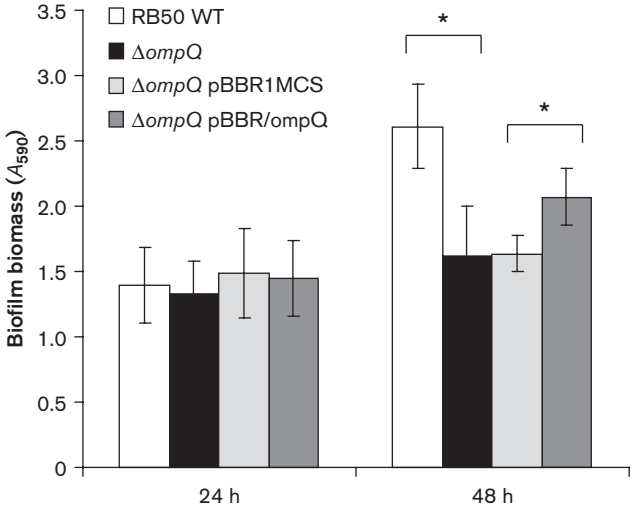

Fig. 1. Biofilm quantification. Biofilm biomass quantities were assessed for *B. bronchiseptica* RB50 WT, Δ*ompQ*, Δ*ompQ* pBBR1MCS (complementation control) and Δ*ompQ* pBBR/ompQ (complemented) strains. Data represent the mean ± SD A_{590} of solubilized crystal violet of three independent assays. Statistical significance was analysed by Student's *t*-test; **P*<0.05.

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 (Δ*ompQ*). 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 Δ*ompQ* 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 Δ*ompQ* 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 Δ*ompQ* mutant on microtitre 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 OD_{650}. At 24 h, both strains formed virtually the same amount of biofilm. At 48 h, however, whilst the Δ*ompQ* 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 Δ*ompQ* 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 Δ*ompQ* in forming biofilms was precisely due to deletion of *ompQ*, we then examined biofilm growth of the Δ*ompQ* strain expressing the *ompQ* gene *in trans* (Δ*ompQ* pBBR/ompQ). The results showed that the complemented Δ*ompQ* strain indeed formed more biofilm than the Δ*ompQ* 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 Δ*ompQ* 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. GPF-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 μm^2 were detected for the Δ*ompQ* 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)

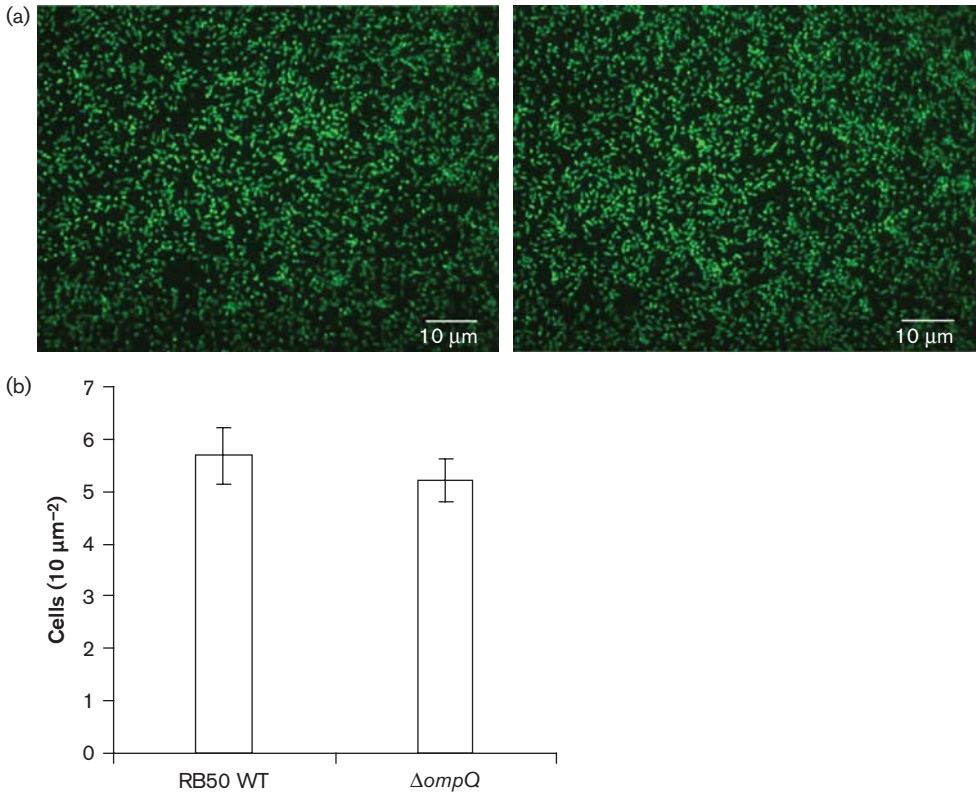

Fig. 2. Analysis of adhesion to coverglasses by *B. bronchiseptica* RB50 WT and the Δ*ompQ* strain. (a) Fluorescence micrographs of adhered bacteria after 2 h of incubation at 37 °C observed at ×1000 original magnification (RB50 WT, left panel; Δ*ompQ*, right panel). (b) Quantification of adhered cells observed by microscopy. The number of cells was determined with the ITCN counting tool run with ImageJ software.

and thick (up to 42 µm). In contrast, the Δ*ompQ* 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 Δ*ompQ* 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

Culture system	Time point (h)	Strain	Biomass ($\mu m^3\ \mu m^{-2}$)	Maximum thickness (µm)	Mean thickness (µm)
Static	24	RB50 WT	2.244 ± 0.153	31.977 ± 1.918	1.326 ± 0.079
		Δ*ompQ*	1.839 ± 0.473	30.879 ± 0.447	1.273 ± 0.202
	48	RB50 WT	2.493 ± 1.46	42.33 ± 14.16	13.17 ± 5.17
		Δ*ompQ*	1.55 ± 0.70	27.47 ± 7.73	6.16 ± 2.76
Continuous flow	24	RB50 WT	3.123 ± 1.490	19.101 ± 1.509	8.154 ± 1.285
		Δ*ompQ*	2.973 ± 0.795	16.655 ± 3.490	6.750 ± 1.707
	48	RB50 WT	12.93 ± 2.62	28.19 ± 3.19	17.69 ± 2.56
		Δ*ompQ*	8.04 ± 3.07	24.25 ± 2.69	13.45 ± 3.59

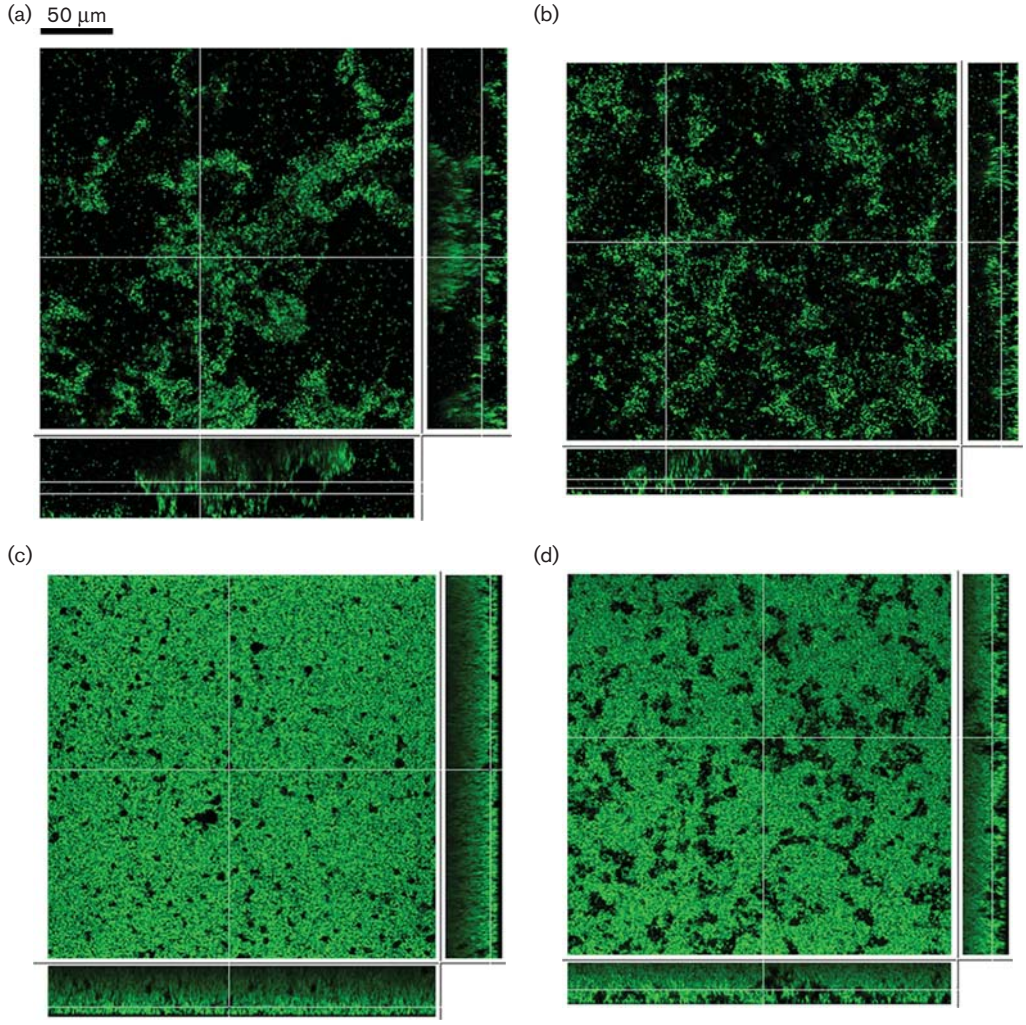

Fig. 3. Microscopy analysis of biofilms formed by *B. bronchiseptica* RB50 WT and the Δ*ompQ* strains. (a–d) *B. bronchiseptica* RB50 (a, c) and Δ*ompQ* (b, d) GFP-tagged strains were cultured on chambered coverslides (a, b) and in a continuous flow system (c, d), as described in Methods. In all cases, confocal laser micrographs of 48 h biofilms are shown with their respective *x*–*y*, *x*–*z* and *y*–*z* focal planes. A representative CLSM image for each sample is presented.

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 Δ*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 ± 0.32, whilst the Δ*ompQ* strain showed a value of 1.73 ± 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.

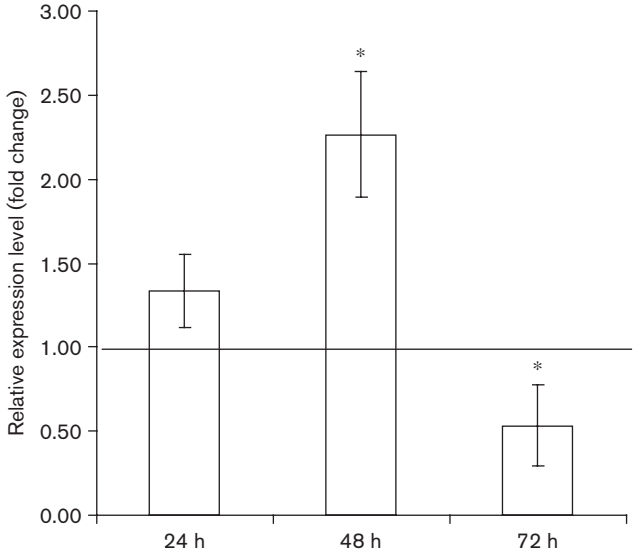

Fig. 4. Relative *ompQ* mRNA expression during the biofilm formation process. The relative *ompQ* expression level was assessed by real-time PCR in RB50 biofilms cultured at 24, 48 and 72 h (*ompQ* level expression in planktonic culture is set to 1). 16S mRNA was used as standardization control. Data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

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

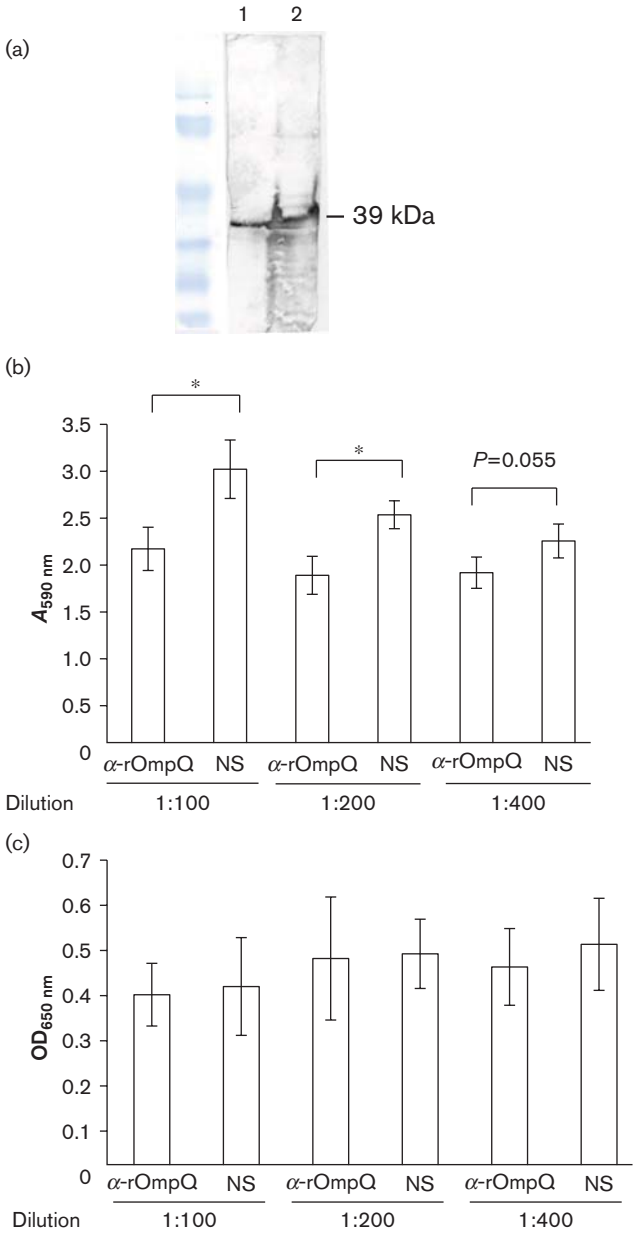

Fig. 5. Biofilm inhibition assay with serum against rOmpQ. (a) Recognition of OmpQ by the anti-rOmoQ serum. Whole-cell lysate from RB50 WT (lane 1) and purified rOmpQ (lane 2) were subjected to SDS-PAGE (10 %) and transferred to PVDF membrane. Immunoblot analysis was performed with polyclonal mouse antiserum 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 A_{590} 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 OD_{650} of the recovered medium.

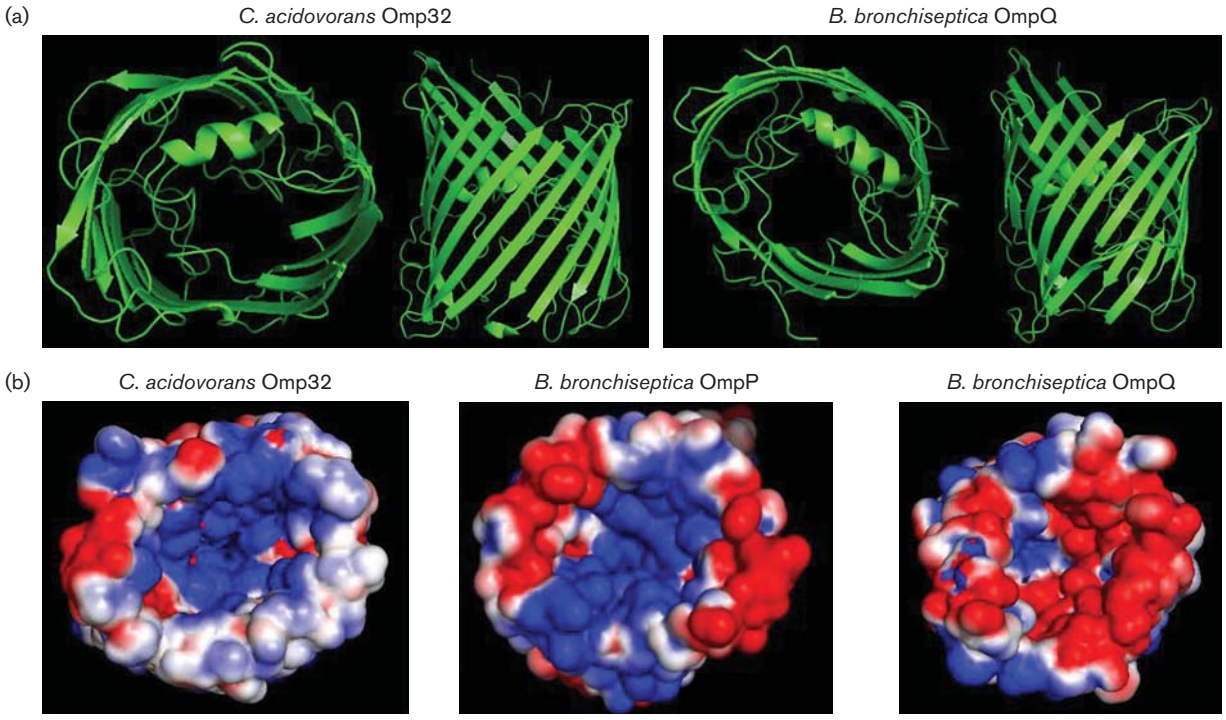

Fig. 6. Bioinformatic analysis of OmpQ structural features. (a) Cartoon representation of anion-selective porin Omp32 from *C. acidovorans* (PDB ID: 1E45) (bottom and side view, left panels) and structural model for OmpQ (bottom and side view, right panels). (b) Surface representation showing the electrical potential as derived from APBS of the anion-selective porin from *C. acidovorans* (PDB ID: 1E45), the structural model for *B. bronchiseptica* OmpP and the structural model for *B. bronchiseptica* OmpQ. The orientation of the molecules is from the periplasmic face (bottom face). It is possible to see that the channel in *C. acidovorans* and OmpP is mostly positive, whilst it is mostly negative in *B. bronchiseptica* OmpQ.

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 ProsaII 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

porins, 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 orthologue proteins of other *Bordetella* species (such as *B. pertussis*, *Bordetella trematum*, *Bordetella homesii*, *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 OpdF and OprF, 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 (Bvg^i) 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* Δ*ompQ* 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 Δ*ompQ* 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 real-time 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* Δ*ompQ* 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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