



Acid tolerance response of *Bordetella bronchiseptica* in avirulent phase



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ABSTRACT

Bordetella bronchiseptica is a Gram-negative bacterium responsible for respiratory diseases in many mammalian hosts, including humans. This pathogen has been shown as able to persist inside the host cells, even in the phagosomes that are acidified to pH 4.5–5.0 after bacterial infection. Here we evaluated the resistance of *B. bronchiseptica* to survive under acidic conditions. In particular we analyzed the bacterial capacity to develop the mechanism known as acid tolerance response (ATR). Our studies were mainly focused on the avirulent phase of the bacteria since this phenotypic phase was reported to be more resistant to environmental stress conditions than the virulent phase. Results from *B. bronchiseptica* in virulent phase were also included for comparison purposes. In fact, for *B. bronchiseptica* 9.73 bacteria in virulent phase we observed that the viability of bacteria does not decrease significantly when grown at pH as low as 4.5, but it is affected when the pH of the medium was equal to or less than 4.0. After acid-adaptation at pH 5.5 for several hours, the survival rate of *B. bronchiseptica* 9.73 at lethal pH 4.0 for 6 h was increased. Interestingly, the avirulent phase mediated by the two-component BvgAS system conferred further resistance to lethal acid challenge and a marked increase in the magnitude of the expressed ATR. The ATR for this avirulent phase seems to be associated with changes in LPS and surface protein profiles. 2D-gel electrophoresis revealed at least 25 polypeptides differentially expressed, 17 of which were only expressed or over-expressed under acid conditions. Using MALDI-TOF mass spectrometry, 10 of these differentially expressed polypeptides were identified.

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1. Introduction

Bordetella bronchiseptica is a widespread respiratory pathogen causing infections in several mammalian species such as canines, felines, equines, swine, and occasionally, humans (Gerlach et al., 2001; Mattoo and Cherry, 2005). Like other *Bordetellae*, *B. bronchiseptica* is able to synthesize a set of infection-related molecules called virulence factors that include adhesion or colonization factors, such as the filamentous haemagglutinin, fimbriae and autotransporter proteins; and also several toxins, like tracheal cytotoxin, dermonecrotic toxin and adenylate cyclase (Ehrmann et al., 1992; Gerlach et al., 2001; Hewlett et al., 1989; Parton, 1999). The expression of these virulence factors with the exception of traqueal cytotoxin is coordinately regulated via a highly conserved two-

component system encoded by the *bvgAS* locus (Arico et al., 1989; Cotter and DiRita, 2000; Weiss et al., 1983).

Under certain environmental conditions, BvgAS simultaneously activates the genes coding for the virulence factors (*vag* genes for virulence-activated genes), and represses the so-called *vrg* genes (virulence repressed genes). The bacterial phenotype associated with this state is called virulent phase. Interestingly, the *bvgR* gene is a *vag* gene that appears to encode a repressor protein (BvgR) involved in regulation of *vrg* genes (Beattie et al., 1993; Merkel et al., 1998). The *vrg* genes are only expressed in a bacterial phenotypic phase known as avirulent phase. In this last phenotypic phase, the virulence genes are repressed. In fact by controlling the intracellular concentration of phosphorylated BvgA, the two components system mediates a progressive transition between the two extreme phenotypic phases, avirulent and virulent phases. The virulent phase occurs at 37 °C and is necessary for *B. bronchiseptica* to cause respiratory infections. Under laboratory conditions, negative signals such as nicotinic acid, MgSO₄ or low temperature trigger modulation to the avirulent phase.

Although the function of virulent phase for infection is well defined, the role of the factors expressed during the avirulent phase is not completely clear. It is known that *B. bronchiseptica*

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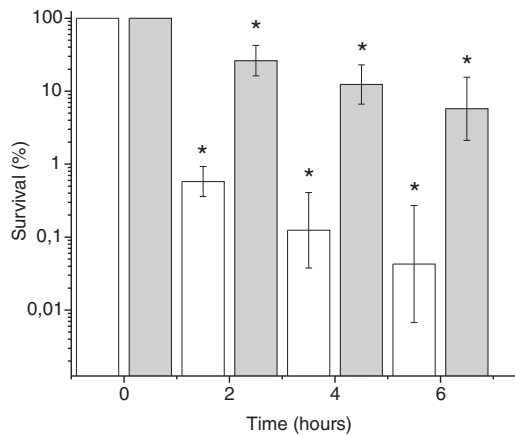


Fig. 1. Effects of acid-adaptation on acid resistance of avirulent phase-locked mutant *BbBvgA*⁻. *BbBvgA*⁻ bacteria were incubated both in adapting conditions pH 5.5 (grey bars) and neutral pH 7.2 (white bars), for at least four consecutive doubling times. Bacterial suspensions of the latter cultures were exposed to an acid challenge at pH 3.8 for 6 h. Viable bacterial counts at each time point were expressed as a proportion of initial values (survival%). Four independent experiments were performed. Standard deviation bars were represented and significant differences were indicated with an asterisk (Student's *t*-test, $p < 0.001$).

bacteria in avirulent phase grow faster, and are less sensitive to antibiotics. Moreover, *B. bronchiseptica* in avirulent phase exhibits a greater potential to survive in extreme acidic pH after engulfment by phagocytes (Banemann et al., 1998; Banemann and Gross, 1997; Cotter and Miller, 1997; Ishikawa et al., 1988; Savelkoul et al., 1993; Schipper et al., 1994). Strengthening acid tolerance seems to be critical to survival of *B. bronchiseptica*. Pre-exposure to sublethal pH has been shown to increase the viability of several bacteria during subsequent exposure to the lethal pH. This phenomenon is known as the acid tolerance response (ATR) (Foster and Hall, 1990, 1991). There are many studies of ATR in different microorganisms, but it has not yet been described for *B. bronchiseptica*.

Undoubtedly further work is needed to increase our knowledge regarding avirulent phase and *Bordetella* ecology under suboptimal environmental conditions. In this context, we evaluated the resistance of *B. bronchiseptica* in avirulent phase to survive under acidic conditions. In particular we evaluated the bacterial capacity to develop an ATR. For comparison purposes bacteria in virulent phase were also used in this study.

2. Materials and methods

2.1. Strains and growth conditions

B. bronchiseptica CIP 9.73 strain (CIP for Collection de l'Institute Pasteur) and its derivative avirulent phase-locked mutant, strain *BbBvgA* (Fernandez et al., 2005) were used throughout this study. The *B. bronchiseptica* strains were grown on Bordet Gengou (BG) agar medium (Difco, Houston, US) supplemented with 10% (v/v) defibrinated sheep's blood (BGA plates). Bacteria grown at 37 °C for 48 h were restreaked in the same medium for 24 h. For liquid broth cultures, the synthetic Stainer Scholte medium (SS) was used (Stainer and Scholte, 1970). To maintain the pH values constant during bacterial growth, the concentration of Tris-(hydroxymethyl) aminomethane buffer (Tris-HCl buffer) in the SS medium was elevated from 12.5 mM to 20 mM. To diminish the pH values of SS medium below 6.0, the Tris-HCl buffer was replaced by MES (2-(*N*-morpholino) ethane-sulfonic acid (20 mM). All cultures were incubated at 37 °C under agitation. The proportions of virulent and

avirulent bacteria were determined based on colony morphology and presence of hemolytic halo in BGA.

2.2. ATR assessment assays

ATR assays were performed as described (Foster and Hall, 1990, 1991), with minor modifications. Briefly, two bacterial cultures were grown in parallel, one at pH 5.5 (hereafter named adapting conditions) and the other at pH 7.2 (hereafter named non-adapting conditions). The growth rate of both the wild type and mutant strains under these two pre-conditioning pH's conditions are shown in Fig. S1 to better compare what this preconditioning does to the different strains.

Both SS cultures at different pHs started at the same optical density at 650 nm (OD_{650 nm}): 0.2 were incubated at 37 °C and with 160 rpm for at least two doubling times (final OD_{650 nm}: 0.8–1.0 approximately 1×10^9 CFU/ml). Cultures were then centrifuged for 10 min at 10,000 × g, resuspended in fresh SS media to a final OD_{650 nm}: 0.2 and incubated under the same conditions for a second two doubling times period (37 °C, 160 rpm). When cultures reached the same OD_{650 nm} value they were centrifuged for 10 min at 10,000 × g and resuspended in lethal acid challenge media at OD_{650 nm}: 0.2. The lethal acid challenge media used for ATR assessment in wild type CIP 9.73 strain and *BbBvgA* mutant were the SS media at pH 4.00 and 3.80, respectively. Bacteria were exposed to the lethal acid condition for a 6 h period at 37 °C and 160 rpm agitation, and samples were taken at 0, 2, 4 and 6 h. These samples were serially diluted in sterile phosphate saline buffer (PBS) and 20 μl aliquots were plated in BGA. Viable colonies recovered after an incubation of 48 h at 37 °C were classified as virulent or avirulent depending on the presence of the beta-hemolytic halo and the morphology of the colonies: small doomed beta-hemolytic colonies are considered virulent bacteria, while large and flat non-hemolytic colonies were considered avirulent bacteria. At least four independent ATR assays were performed for each strain. Statistical differences in log-transformed viable colony recovery levels between conditions were analyzed by Student's *t*-test ($p < 0.05$). Graphical representations of surviving bacteria were done with the Origin 8.0® software package (OriginLab corp). Where results are presented as percentage of survival, this was calculated as viable cell counts after acid challenge expressed as a percentage of viable cell counts immediately prior to treatment. Bacterial counts at the beginning of the acid challenge were similar in every assay (approximately 1×10^9 CFU/ml).

2.3. Protein samples preparation and proteomics

Protein samples were obtained from bacterial cultures grown under adapting or non-adapting conditions. At the end of the second culturing period, bacteria were collected by centrifugation (10,000 × g, 10 min at 4 °C) and washed two times with low salting buffer (KCl 3 mM, NaCl 68 mM, KH₂PO₄ 1.5 mM and NaH₂PO₄ 9 mM). Cell pellets were resuspended up to OD_{650 nm} = 10 in Tris-HCl 10 mM, pH 8.40 supplemented with 0.25 mM Phenylmethylsulfonyl Fluoride (PMSF) for serine protease activity inhibition. Total cellular proteins were extracted using a 150 Digital Sonifier®, Branson sonicator. The sonication was carried out on ice and the cell suspension was pulsed 6 times (12 μm amplitude) for 60 s with 30 s intervals between pulses.

Cell debris was recovered by centrifugation at 100,000 × g for 1 h. The pellet obtained was resuspended in Urea 7M, thiourea 2M, isopropanol 10%, triton X100 10% (UTIT buffer). The resuspended proteins were precipitated with ice-cold acetone. The pellet obtained was resuspended in urea 7M, thiourea 2M, 4% chaps (UTC buffer) with 0.5% v/v of an ampholytes solution and 1% v/v of DeStreak® reducing agent (GE-Healthcare). This final protein

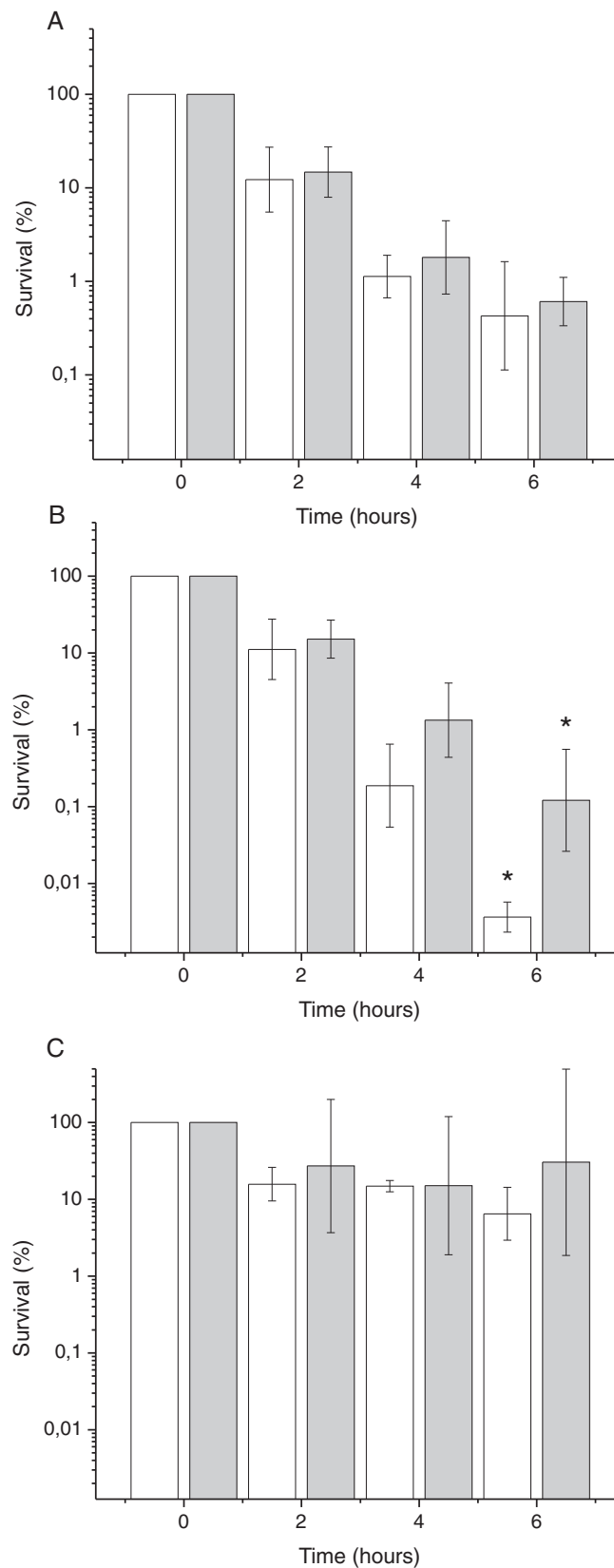


Fig. 2. Effects of acid-adaptation on acid resistance of wild type *B. bronchiseptica* CIP 9.73.

B. bronchiseptica CIP 9.73 strain was incubated in both in adapting conditions pH 5.5 (grey bars) or neutral pH 7.2 (white bars), for at least four consecutive doubling times. Bacterial suspensions of the latter cultures were exposed to an acid challenge at pH 4.0 for 6 h. Samples were taken every 2 h and dilutions were plated on BGAb media. Total viable bacterial counts at each time point were expressed as a proportion of initial values (Panel A). Virulent and avirulent bacterial counts were plotted separately: virulent (Panel B) and avirulent (Panel C). Four independent experiments were performed. Standard deviation bars are represented and significant differences detected are indicated in the plot with an asterisk (Student's *t*-test, $p < 0.05$).

extract was considered as fraction enriched in surface proteins (FSP). The protein contents in FSP extracts were quantified with the commercial kit 2D Quant kit® (Amersham Biosciences).

The supernatant obtained after the first centrifugation at $100,000 \times g$ for 1 h was collected, treated with DNase I and RNAse A and precipitated with cold acetone. The protein-enriched pellet was washed and resuspended in urea 7 M, thiourea 0.5 M and 4% chaps (UTC modified buffer). This sample was referred to as fraction enriched in cytosolic proteins (FCP). Protein content in this extract was quantified by the Bradford technique (Bradford, 1976).

For two-dimensional gel electrophoresis (2D-PAGE), the first dimension gel was a precast Immobiline DryStrip (Pharmacia) with two different resolutive pH ranges: pH 4.0–7.0 and pH 6.0–11.0. For the second dimension sodium dodecylsulfate-polyacrylamide gel electrophoresis SDS-PAGE 12.5% w/v was used. Gels were stained following the colloidal coomassie blue technique (Candiano et al., 2004) and preserved in 1% v/v acetic acid solution until image digitalization. Stained gels' images were scanned with an office scanner Samsung® L-DE 50. Image analysis was performed with Image Master 6.0 Platinum® software (GE-Healthcare).

Gel images from FSP and FCP from each set of culture samples and processed under the same 2D-PAGE were grouped, each group containing images of at least three independent samples. Individual spots were matched and assigned an identification number, taking one of the gels inside each group as reference. Spots from groups composed by samples resolved under the same 2D-PAGE conditions but derived from bacteria cultured under each different pH condition, were matched against each other (interclass matching). Spot quantification was performed by means of its spot volume, a variable obtained by the software as the product of maximum spot intensity and the area of the spot (in pixels) with an intensity of at least 70% of the maximal value.

For statistical comparison of differences in protein levels a parametric test was used (Meunier et al., 2005). The original spot volume value for each spot in each gel was logarithmically transformed and normalized, according to total staining intensity in each gel (corrected volume, V_{corr}), in order to obtain a parametric variable for comparison (Hunt et al., 2005; Meunier et al., 2005). The effect of this methodology in spot volume frequency distribution normalization was confirmed using the Statistica 7.0 software (Stat soft Inc.). Relative expression differences were analyzed only for spots present in every single gel from each group and class of gels. Differences in expression were considered with a potential biological effect when mean spot volume value was doubled between conditions.

A total of 3 FSP and 4 FCP independent sample extracts from bacteria cultured under each condition were compared. Statistical differences between V_{corr} values between classes were evaluated by the Student's-*t* test, considering $\alpha < 0.05$. A simultaneous cut-off level power of at least 80% was considered in order to decrease the number of false positive differences (Hunt et al., 2005).

Those spots present in every group of gels corresponding to one growth condition but completely absent in gels from the other growth condition were considered absolute expression differences.

Differential proteins were identified by mass spectrometry associated with 2D electrophoresis as previously reported (Perkins et al., 1999; Bottero et al., 2007; Bottero et al., 2012).

2.4. Lipopolysaccharide (LPS) extraction and SDS-PAGE.

LPS from *B. bronchiseptica* were extracted as described (Valverde et al., 1997). Electrophoresis was performed at room temperature and constant voltage. The LPS was visualized by the BioRad silver-staining technique.

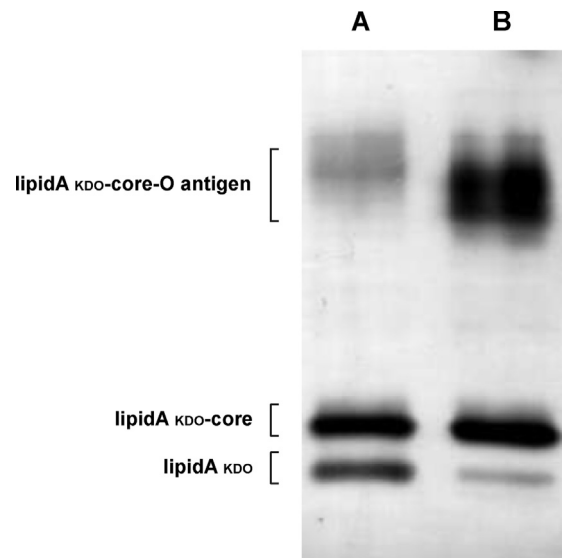


Fig. 3. SDS-PAGE 15% w/v of *B. bronchiseptica* LPS. LPS were isolated from *BbBvgA* avirulent phase-locked mutant grown in pH 5.5 or 7.2 media (lanes A and B, respectively). LPS was visualized by the BioRad silver-staining technique.

3. Results

3.1. ATR assessment in *B. bronchiseptica* avirulent phase-locked mutant

Since the avirulent phenotype is advantageous for *B. bronchiseptica* when exposed to stressful environmental conditions such as acid stress, we decided to evaluate the ability of *B. bronchiseptica* in avirulent phase to develop the ATR mechanism. As first step of our work we characterized the behavior of the *B. bronchiseptica* 9.73 mutant strain locked in avirulent phase (*BbBvgA*⁻ from our laboratory, Fernandez et al., 2005) in different acid culture condition to define an adequate adaptive pH condition. We estimated and compared the bacterial specific growth rate (μ) when grown under different acidic (Fig. S1). Since at pH 5.5 the estimated μ was almost 75% of the one observed at neutral pH values, this pH was selected as the adaptive condition. On the other hand, pH 7.2 was selected as the non-adaptive pH condition for ATR assessment. To determine the challenge pH useful to detect the effect of acid-adaptation for *BbBvgA*⁻ mutant strain, bacteria were first grown in regular Stainer Scholte liquid broth (Stainer and Scholte, 1970) up to mid-exponential phase and then exposed for a 6 h period to different extreme acidic conditions, ranging from pH 3.5 to pH 4.7. In these experiments we observed that bacteria were rapidly killed at pH values below 4.0, in fact at pH 3.8 viable bacterial counts were reduced by four orders of magnitude. Hence, pH 3.8 was selected as the lethal acid condition.

Having established adapting (pH 5.5), non-adapting (pH 7.2) and lethal (pH 3.8) conditions, we then assessed for the presence of an ATR response in the *BbBvgA*⁻ strain. As shown in Fig. 1, the *BbBvgA*⁻ mutant was clearly able to express a functional ATR response. The survival rate of the cells subjected to pre-incubation at pH 5.5 was maintained at a high level after acid challenge at pH 3.5 for 2 h. However, pre-incubation treatment at pH 7.2 was ineffective at protecting cells against a lethal acid challenge. After the first 2 h of challenge, viable bacterial counts were approximately 45 times higher for the cells pre-incubated at pH 5.5 than those became from non-adapted cultures. This difference continued to grow in time, becoming 135 times higher at the end of the challenge period (6 h).

Our results thus show that *B. bronchiseptica* is able to express an ATR, at least in association with avirulent phase expression.

As far as we know, ATR response has been assessed only once in *B. bronchiseptica*, with negative results (Schneider et al., 2000). These authors, in contrast to our work, have assayed predominantly virulent cultures of a different wild type *B. bronchiseptica* strain. For comparison purposes with this previous report, we repeated the above-described ATR experiments employing predominantly virulent cultures from *B. bronchiseptica* CIP 9.73 in virulent phase (the parental strain of *BbBvgA*), under the same adapting and non-adapting pH conditions (Table S2). When we performed the experiments to determine the challenge pH useful to detect the effect of acid-adaptation for *B. bronchiseptica* CIP 9.73 virulent strain, we observed that the bacteria were rapidly killed at pH below to pH 4.1. In fact at pH 4.0 viable bacterial counts were reduced by three orders of magnitude (Table S1). Hence, pH 4.0 was chosen as the challenge pH to detect the effect of acid-adaptation. Following the same procedure employed with the *BbBvgA*⁻ mutant strain, we then evaluated the survival rate of the parental strain at the previously determined challenge pH from cultures pre-incubated at pH 7.2 and pH 5.5. As shown in Fig. 2A, when total viable bacteria were taken in consideration we observed that the pre-incubation at pH 5.5 did not seem to confer protection to lethal acid challenge. However, when viable bacteria counts were discriminated in virulent and avirulent bacteria based on their morphological characteristics on BGA solid medium, we observed that the proportion of virulent bacteria changed over the challenge period. At the beginning of the experiment most of the bacteria were in virulent phase while at the end they were mostly

in avirulent phase. Though it is expected that phase variation (virulent to avirulent phase) occurs *in vitro* at a frequency dependent on the strain, the behavior observed here under acidic condition is novel. Interestingly, when survival analysis was performed considering viable virulent and avirulent colonies separately, a differential behavior among bacteria in these two phases was observed (Fig. 2B and C). While the number of virulent CFU was strongly affected by the pH challenge, avirulent bacteria survival was significantly less affected. Nevertheless, survival for virulent bacteria in adapted cultures was almost 30-fold higher than in non-adapted cultures ($p < 0.05$) (Fig. 2B). This result suggests the presence of an ATR associated with virulent phase expression. However, the level of protection conferred by this response is significantly lower than the resistance associated with the avirulent phase. As we previously observed, avirulent bacteria survival was much less affected, the survival level for this bacteria at the end of the acid challenge being almost 1800 and 60 fold greater than for non-adapted and adapted virulent bacteria, respectively ($p < 0.01$) (Fig. 2C).

All these results showed that *B. bronchiseptica* CIP 9.73 is able to develop an ATR response, which is significantly higher for the avirulent phase of the bacteria.

3.2. The ATR of avirulent phase-locked mutant *BbBvgA*⁻ is associated with modifications in bacterial composition

To investigate whether acid adaptation occurred in association to changes in bacterial surface components of *BbBvgA*⁻ mutant strain, we first analysed the LPS electrophoretic profile, since this ubiquitous component of the outer membrane is in close contact

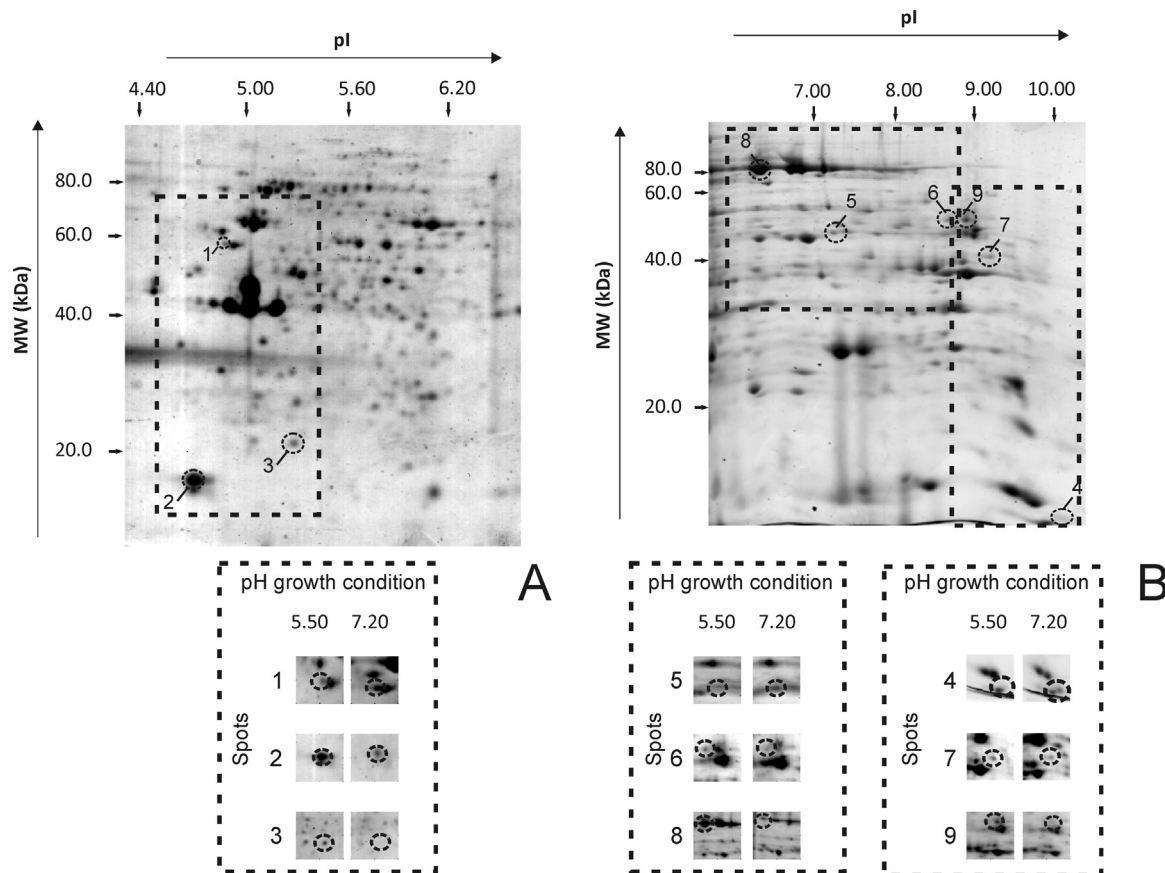


Fig. 4. 2D-PAGE electrophoresis of FSP extracts.

Proteins samples were separated by IEF at pH 4–7 (panel A) or pH 6–11 (Panel B) in the first dimension and then by 12.5% SDS-PAGE in the second dimension. Protein spots were visualized by colloidal Coomassie staining. Each image is the consensus gel image of at least three independent 2D-PAGE experiments. Below each gel image a representative differential spot from each culturing condition is shown, for visual effect but not for analytical purposes.

Table 1
Spots detection and analysis of results from 2D-PAGE gel images.

	N° of analyzedspots	N° of differentialsspots	Ratio ^a (%)
FSP pH 4.0–7.0	51	3	5.88
FSP pH 6.0–11.0	93	6	6.45
FCP pH 4.0–7.0	470	16	3.40
Total	614	25	4.07

^a Ratio: proportion (%) of differential spots over analyzed spots, for each category displayed on the first column.

with the environment. LPS were therefore extracted from avirulent phase-locked *BbBvgA*⁻ bacterial cultures performed either under adapting and non-adapting conditions. Fig. 3 shows that the LPS extracted from both culture conditions displayed the 3 expected bands: a diffuse band (lipid A KDO-core-O antigen) containing the O-antigen (a single sugar polymer consisting of 2,3-dideoxy-di-N-acetylgalactosaminuronic acid) and the two other faster migrating bands, i.e., band A (lipid A KDO-core) and band B (lipid A KDO) (Preston et al., 2006). However, some differences were detected between the LPS extracted from both culturing conditions. Bacteria cultured under adapting conditions exhibited a higher proportion of B band in comparison with that from non-adapting conditions. Furthermore, a low amount of O band with a delay in its electrophoretic mobility was also observed, indicating a higher mass/charge ratio. These differences between the LPS profiles were observed at least in four independent experiments.

Effects of acid adaptation on bacterial protein profiles were also assessed. Protein extracts were obtained from both adapted and non-adapted late mid-exponential phase cultures, and then analysed by 2D-PAGE. Starting materials for these studies were obtained under the same conditions as for LPS analysis, e.g., two parallel cultures of the *BbBvgA*⁻ strain under both acidic conditions, with at least two doubling times duration. A fraction enriched in surface proteins (FSP) and one enriched in cytosolic proteins (FCP) were obtained from each bacterial culture. Extracts were then resolved by 2D-PAGE, employing a separation range in the isoelectrofocusing (IEF) step of pH 4.0–7.0 for FCP, and both pH 4.0–7.0 and pH 6.0–11.0 for FSP. The reason to include pH 6.0–11.0 for FSP was that at pH 4.0–7.0 range the FSP samples but not FCP samples exhibited high amounts of proteins unfocused at pH equal or higher than pH 7.0. Representative protein extracts resolution profiles obtained experimentally are shown in Figs. 4 and 5.

Visual inspection of 2D-PAGE gels revealed differences between samples extracted from adapted and non-adapted cultures of the *BbBvgA*⁻ strain. Consensus gels for each class of 2D-PAGE gels were constructed by software-assisted quantitative proteomic expression analysis. A total of 470 spots for pI 4.0–7.0 FCP gels, 51 spots for pI 4.0–7.0 for FSP gels, and 93 spots for pI 6.0–11.0 FSP gels were assigned (Table 1). Differences in expression between culturing conditions detected were classified according to two categories: absolute and relative (Table 1). Any spot present in every gel replica from one culturing condition and absent in every gel replica from the other culturing condition was considered an absolute difference. Relative differences were based upon the comparison of a variable generated by the software that is associated with the polypeptide quantity contained on each spot, namely the *spot volume*.

As Table 1 shows, 16 proteins were differentially expressed over the 470 spots present in FCP samples. Six of these differences between both culturing conditions were absolute, with 5 spots absent and 1 spot present under the non-adapting condition. The other 10 differential spots were categorized as relative spots (4 spots increased and 6 decreased under the same condition).

FSP sample analysis included a total of 144 spots, 51 of them from pI range 4.0–7.0 and 93 from pI range 6.0 to 11.0. Considering

Table 2
Differentially expressed spots identified by mass spectrometry.

ID ^a	Extract/IEF range	ORF	Description
2	FSP/pH 4.0–7.0	BB4918	Bacterioferritin (Bfr)
8	FSP/pH 6.0–11.0	BB1844	Aconitase A (AcnA)
9	FSP/pH 6.0–11.0	BB0111	Possible dehydrogenase
16	FCP/pH 4.0–7.0	BB1884	Probable solute transporter
18	FCP/pH 4.0–7.0	BB1986	Arginino-succinate synthase (ArgG)
19	FCP/pH 4.0–7.0	BB0026	Elongation factor G (EF-G)
20	FCP/pH 4.0–7.0	BB3932	Zn-protease
21	FCP/pH 4.0–7.0	BB3749	Serine protease (MucD)
22	FCP/pH 4.0–7.0	BB3594	Hypothetical protein
23	FCP/pH 4.0–7.0	BB2534	TCS regulatory component (TtrR)

^a ID (Identity): the number depicted in this column refers to the identification number assigned for differential spots displayed in Figs. 4 and 5.

these 144 spots as a whole group, 6 spots were over-expressed at pH 5.5 and 3 at pH 7.2. Among these, only 1 was absolutely increased under each pH condition.

The observed predominance of protein induction under acidic conditions is in line with previous observations in other bacteria, such as *Salmonella typhimurium* and *Listeria monocytogenes* (Carrondo, 2003; Foster and Hall, 1990, 1991; Foster, 1993). Furthermore, there was also a bias toward the induction of proteins associated with membranes: the proportion of differential spots in FSP samples almost doubles its equivalent in FCP samples, 6.3% over 144 spots vs. 3.2% over 464 spots, respectively.

3.3. Identification of proteins differentially expressed under adapting conditions.

Several spots from the different 2D-PAGE gels were excised and 98 of them could be identified by UV-MALDI-ToF mass spectrometry (Supplementary Tables S3 and S4, Figs. S2 and S3). *In silico* prediction of cellular localization of the identified proteins (Psorb 3.0 software, <http://www.psorb.org/psorb/>) validated the effectiveness of our enrichment approach (Supplementary Tables S5 and S6). From a total of 25 spots differentially expressed between both culturing conditions of *BbBvgA*⁻ mutant strain, 9 could be identified by mass spectrometry, all of them corresponding to proteins increased under adapting conditions (Table 2).

As shown in Table 2, at least two of the proteins over-expressed in acid media are related to iron bioavailability and metabolism. These proteins are Bacterioferritin (Bfr, BB4918), which is frequently associated with protection against deleterious effects of free intracellular iron (Carrondo, 2003; Wandersman and Delepelaire, 2004), and aconitase hydratase (AcnA, BB1844), an enzyme that catalyzes the reversible transformation of citrate to isocitrate and senses the intracellular Fe(II) availability (Parkhill et al., 2003; Tang and Guest, 1999). Regarding this last enzyme, the genome of *B. bronchiseptica* contains three sequences that code for three different AcnA (BB1844, BB3687, and BB1342) and one ORF that codes for a related aconitase (AcnB, BB1850) (Parkhill et al., 2003; Thalen et al., 1999). BB1844, BB3687 and AcnB could be detected by mass spectrometry in the 2D-PAGE gels; however, BB1844 expression was the only one affected by the acidic growth conditions (Supplementary Tables S3 and S4). This result suggests a separate regulation for BB1844, independent from that of the two other aconitases.

The proteins Elongation factor G, (EF-G, BB3932), Arginino-succinate synthase (ArgG, BB1986), BB3594, BB3749, and BB0111 were also increased under adapting conditions. According to NCBI Conserved Domain Databases (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>), BB3932 protein contains two types of PqqL domains (COG0612) that confer it a Zn-dependent protease activity. The presence of a signal peptide in its sequence (Psorb 3.0b), suggests a final location of the mature protein in the external

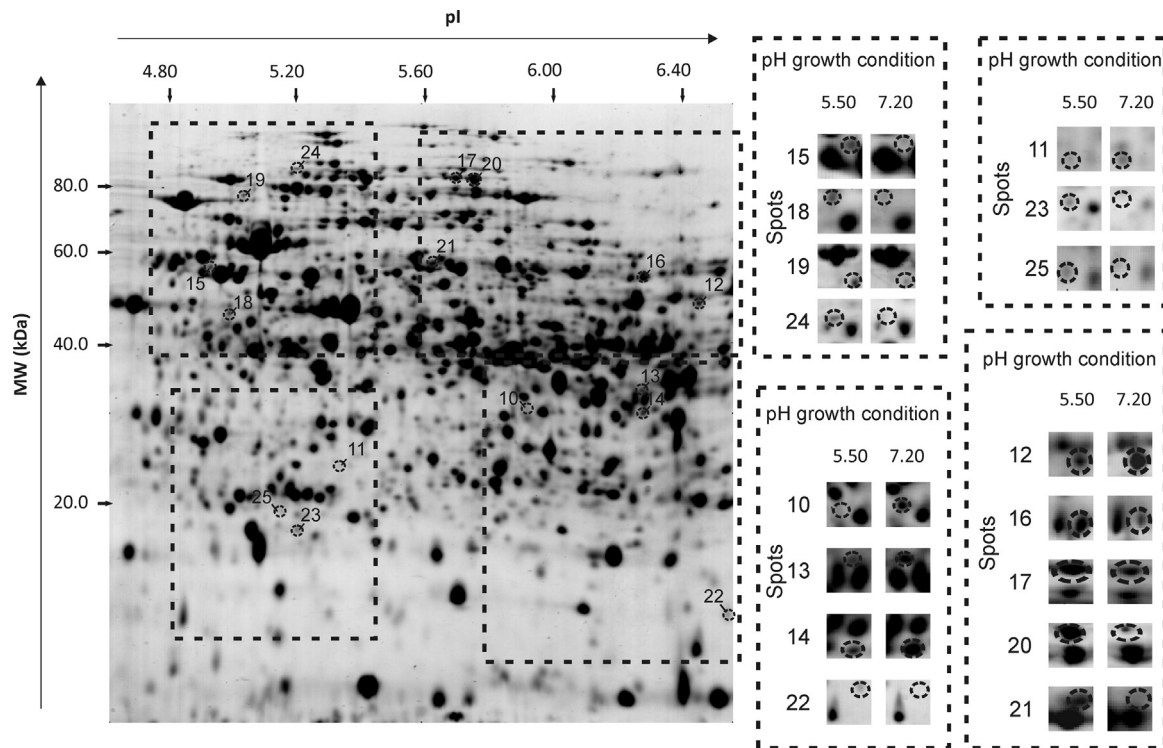


Fig. 5. 2D-PAGE electrophoresis of FCP extracts.

Proteins samples were separated by IEF at pH 4–7 in the first dimension and then by 12.5% SDS-PAGE in the second dimension. Protein spots were visualized by colloidal Coomassie staining. Each image is the consensus gel image of four separations obtained under the same conditions of resolution. On the right side of the 2D-PAGE gel image a representative differential spot from each culturing condition is shown, for visual effect but not for analytical purposes. Differential spot detection and evaluation were based on specific image-analysis software and statistic procedures.

membrane. Arginino-succinate sintase (ArgG, BB1986) catalyzes the synthesis of arginino-succinate from citrulline, ATP and aspartate, in two successive steps. The up-regulation in ArgG levels could reflect the need for an increment in *de novo* synthesis of the amino acid arginine, due to a high rate of consumption. BB3594, whose product was exclusively expressed in FCP under acidic growth conditions, shows high homology to a group of proteins involved in forming and accumulating the polymeric component poly- β -hydroxybutyrate or PHB (<http://www.genome.jp/kegg/ssdb/>). PHB synthesis has been reported in *Bordetella* spp. under non-restricted carbon or nitrogen source conditions. The proteins DegP or MucD (BB3749) are periplasmic serine proteases, with chaperone activity, closely related to HtrA (Pallen and Wren, 1997). BB0111 product shows a high sequence homology with a membrane-associated quinone-protein that catalyzes the oxidative transformation of choline into betaine-aldehyde (Gadda and McAllister-Wilkins, 2003). This is expected to be a final metabolic product in *B. bronchiseptica* (<http://www.genome.jp/dbget-bin/www.bget?bbr:BB0111>), possibly acting as an osmo-protecting substance (Lin et al., 1995; Waditee et al., 2003). Finally, the expression of the regulatory subunit of one of the 16 two-component systems present in *B. bronchiseptica* genome (TtrR, BB2534) was also increased under adapting conditions. As far as we know, the role of this regulatory system has not been studied yet in this bacterium.

4. Discussion

The results presented here demonstrate that *B. bronchiseptica* CIP 9.73 is able to develop an acid tolerance response (ATR) when previously exposed to sublethal acid conditions. Thus, previous culturing under intermediately acidic conditions provoked changes on this bacterium that induced its resistance to exposure to oth-

erwise lethal extremely acidic conditions. The magnitude of this adaptation appeared to be maximized when occurred concomitant with the expression of a full avirulent phenotype. Previous reports from Schneider et al. (Schneider et al., 2000) suggested the absence of ATR in *B. bronchiseptica*. Although at first glance this report is contradictory to our results, it should be considered that induction of ATR strongly depends on strain and culturing conditions, and differences in any of those variables might justify the discrepancies (Foster and Hall, 1991; Kobayashi et al., 1986).

Our results are in agreement with the ability of *B. bronchiseptica* to survive even inside professional phagocytic cells for long periods, without interfering with phagosomal maturation (Banemann and Gross, 1997; Schneider et al., 2000). Mature phagolysosomal vesicles containing the pathogen reach a final value near pH 5.1 (Schneider et al., 2000). These latter aspects drive the attention toward a potential connection between the pathogen's ability to survive intracellularly and its avirulent phase to withstand or even adapt to acid stress. Therefore these results point out the possible relevance of the avirulent phase to *B. bronchiseptica* survival in phagocytes or in mammalian hosts.

Though the potential role of the avirulent phase of *Bordetella* species *in vivo* is still a matter of debate, it is generally accepted the knowledge that while the virulent phases of *B. bronchiseptica* and *Bordetella pertussis* (a closely related pathogen) appear to be adapted to respiratory tract colonization, the avirulent phase of *B. bronchiseptica* promotes survival under nutrient-limiting conditions (Akerley et al., 1995; Cotter and Miller, 1994; Martinez de Tejada et al., 1998; Porter et al., 1991). In fact a high number of Bvg-repressed (and thus avirulent phase related loci) metabolic, motility, transport, and chemotaxis loci were described in *B. bronchiseptica*, suggesting a role for this avirulent phase in optimizing nutrient scavenging and survival *ex vivo*. Since these gene classes

have been proven important for *in vivo* survival in other systems a role for the avirulent phase inside the host cannot be ruled out.

The ATR detected here correlated with changes in surface bacterial components. This correlation was also observed in other microorganisms including *Escherichia coli*, *S. typhimurium* and *Streptococcus faecalis* (Foster and Hall, 1991; Guo et al., 1997; Kobayashi et al., 1986). In *S. typhimurium*, changes observed under acidic conditions involve, for example, the replacement of phosphate groups by positive charged monosaccharides in the lipid A of the LPS (Guo et al., 1997). Here we also detected changes in the LPS of *B. bronchiseptica*. LPS extracted from avirulent phase-locked mutant bacteria, cultured under adapting conditions exhibited a higher proportion of B band and lower amount of O band with a delay in its electrophoretic mobility. Nevertheless, the nature of these changes at the molecular level remains to be characterized.

2D-PAGE analysis of ATR associated proteins also revealed changes on 25 polypeptides over 614 spots analyzed, which represented differences in 0.5% of the 4994 ORF from the sequenced *B. bronchiseptica* strain RB50 *in silico* proteome. Other mucosal pathogens that also display ATR express similar levels of changes relative to their proteomes. Growth under adaptive acidic conditions showed differences in 0.4% and 1.4% from *in silico* proteomes of *S. typhimurium* (Foster, 1993; Foster and Hall, 1991; McClelland et al., 2001) and *L. monocytogenes* (Nelson et al., 2004), respectively. Also, similarly to *S. typhimurium* (Foster and Hall, 1991), the effects of acid exposure in *B. bronchiseptica* here described had, proportionally, a deeper effect on membrane-associated proteins (Table 1). The denaturing effects of the acid stress over this cellular compartment might be reflected in the induction detected of the periplasmic chaperone DegP under acidic conditions (Clausen et al., 2002; Varghese et al., 2003).

Two proteins involved in bacterial iron metabolism were also induced in acidic growth conditions of *BbBvgA*⁻ mutant strain: Aconitase A (AcnA) and Bacterioferritin (Bfr). Since the induction of both proteins has been seen associated with iron availability (Brickman and Armstrong, 2012), the enhanced levels detected in the acid condition could be a consequence of an increase in iron availability under such condition. In fact, Fe(II) concentration in the medium assayed has been shown to be significantly increased at pH values below neutrality (Brickman and Armstrong, 2012). It is worth mentioning that one of the main transcriptional regulators of iron-induced effectors in bacteria is the protein Fur (*ferric uptake regulator*). Although Fur induction depends upon the increase in the level of bacterial Fe(II) in the cytosol, it is independently regulated by acid and has an important role on ATR expression in other bacteria (Gancz et al., 2006; Hall and Foster, 1996). Thus, considering that *B. bronchiseptica* has an active version of Fur (Burgos et al., 2010), it could be important to study the relationship between the observed differences on iron effectors levels and ATR in this bacterium and Fur levels (Gancz et al., 2006; Hall and Foster, 1996; Tsolis et al., 1995).

The fact that the proteins ArgG (BB1986) and EG-G (BB0026) were induced under adapting conditions could be related to an association between ATR expression and *de novo* synthesis of proteins involved in aminoacid metabolism. Though it is not clear enough the role of these proteins in ATR, it is expected that aminoacid metabolism became affected during low pH exposure, in part related to cytoplasmic buffering processes. In fact aminoacid decarboxylation represents one important mechanism employed by bacteria to maintain intracellular pH homeostasis since is associated with proton consumption (Sachs et al., 2005). Moreover, arginine decarboxylases have been associated to ATR expression in many different bacteria (Alvarez-Ordóñez et al., 2010; Lin et al., 1995; Seputiene et al., 2006).

Taken together, these results depict a beneficial effect of avirulent phenotype expression under acid stress conditions. Fur-

thermore, acid exposure induces changes at the molecular level with consequences on bacterial metabolism and external membrane composition associated with a marked increase in bacterial resistance to stress conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.micres.2015.09.001>.

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