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Heterologous protective immunization elicited in mice by *Pasteurella multocida fur ompH*

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Summary. Different strategies have been developed to produce vaccines against *Pasteurella multocida.* The approach described herein involves overexpression on the bacterial cell surface of Fur-regulated IROMPs (iron-regulated outer-membrane proteins). Accordingly, the ability of *fur* mutants to promote heterologous protection was examined in a Swiss mouse animal model. Two *fur* mutants derived from *P. multocida* were isolated, one of which was also defective in the OmpH protein. In mice challenged with virulent *P. multocida*, outer-membrane protein (OMP) extracts of *fur* cells conferred the same protection as obtained with wild-type cells grown in iron-depleted medium. Total protection was achieved with 40 μg of OMP extract from the *fur ompH* mutant. Mice administered heat-inactivated *fur ompH* cells were 60% cross-protected. The presence of a *galE* mutation in these cells did not further increase the protection level. Additionally, cell disruption by sonication provoked a higher level of protection than conferred by heat-treated cells. Taken together, the results showed that *P. multocida fur ompH* cells offer a simple and suitable approach for cross-protecting animals against infection with *P. multocida*. [**Int Microbiol** 2008; 11(1):17-24]

Key words: *Pasteurella multocida* · vaccine · gene *fur* · gene *ompH* · iron-regulated outer membrane proteins (IROMPs)

Introduction

Iron is a critical element required by almost all living cells. The outer-membrane proteins (OMPs) synthesized by many gram-negative bacterial pathogens, such as *Haemophilus influenzae* and *Neisseria meningitidis*, bind iron-binding molecules, including transferrin, lactoferrin, hemoglobin, heme, and ferritin, present in the blood and mucosa of their host organisms [1,30]. The expression of nearly all of these OMPs is under the control of the Fur (ferric uptake regulator) protein [34], which binds iron in the cytoplasm of bacterial cells. In this reaction, Fur forms a complex with Fe(II) and then binds to a specific sequence, known as the Fur box [3,16,17], in the promoter region of iron-regulated genes, thereby blocking their transcription. Many iron-regulated outer-membrane proteins (IROMPs) are powerful antigens as well as essential virulence factors during the infection process of numerous pathogens [30]. For this reason, protection against these pathogens may be conferred either by vaccines based on the purified IROMP receptor [13] or by anti-IROMP antisera [33].

Pasteurella multocida is a gram-negative bacterial pathogen that causes several infectious diseases, including fowl cholera, bovine pneumonia, hemorrhagic septicemia, and swine atrophic rhinitis, in animals used in the production of food. In veterinary medicine, a vaccine against *P. multocida*, mainly based on inactivated *P. multocida* cells, known as bacterins, or on live attenuated bacteria is available; however, bacterins only confer homologous protection. Although cur-

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rent live vaccines promote heterologous and homologous protection, they carry unknown attenuation markers and, in some cases, have even been associated with disease outbreaks [28]. Homologous protection has been obtained using OMPs from *P. multocida* grown without iron deprivation [4]. In addition, several well-defined live attenuated and *thy* mutant strains that are promising vaccine candidates have been recently described [5,14,21].

Cross-protection has been achieved with bacterins [19] as well as with OMP extracts [2,31] from *P. multocida* grown in iron-depleted medium. The basis of this heterologous protection seems to be the overexpression of *P. multocida* IROMPs, induced by the non-availability of iron in the environment and, consequently, inside the cells [Kennett et al. 1993. ACIAR Proceedings no. 43, pp 144-148]. However, bacteria grow very poorly in the presence of divalent-cation chelators, such as 2,2′-dipyridyl (DPD), which is an important limitation for obtaining vaccines in large quantities [26].

Two hemoglobin-binding receptors of *P. multocida* were initially characterized [7,15], and it was subsequently shown that this bacterium has at least six other hemin- and/or hemoglobin-binding proteins, all of which are immunogenic [8,29]. Nonetheless, when inoculated individually, none of these proteins conferred protection against heterologous challenge [7,8; and B. Adler, personal communication]. The recent isolation of the *P. multocida fur* gene has allowed us to construct *fur* mutants of *P. multocida* [9]. Given that Fur regulates many bacterial iron-uptake proteins, such as hemin- and/or hemoglobin-binding proteins, we postulated that overexpression of IROMPs by *P. multocida fur* mutants would mimic the response of *P. multocida* wild-type cells grown in iron-depleted medium. Therefore, the aim of the present study was to examine heterologous protection conferred by *P. multocida fur* cells.

Materials and methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. All *P. multocida* strains were grown in liquid medium, either in buffered peptone water (BPW, Merck, Darmstadt, Germany) or brain-heart infusion (BHI, Oxoid, Madrid, Spain), and on sheep-blood agar (SBA, Biomérieux, Madrid, Spain) or BHI plates. When necessary, antibiotics were added at the stated concentrations [11]. The concentration of the divalent-cation chelator DPD (Sigma, Madrid, Spain) used in the experiments was 150 μM.

Genetic methods. The *P. multocida* insertional *fur* mutant was obtained from plasmid pUA891, which also contains a gene encoding resistance to streptomycin. Plasmid pUA1089 was used to construct the double-mutant *fur galE*. pUA1089 is a derivative of pKO3, a gene-replacement vector containing a temperature-sensitive replication protein [23], and harbors the 2-kb *mob* region of plasmid pUA826. This region was isolated by restriction of pUA826 with *Bam*HI and then cloned into the *Bam*HI site of pKO3. *P. multocida* mutants were obtained by triparental mating, using pRK2013 as the mobilizing plasmid, as reported [11].

To determine the stability of the *fur* mutation, *fur* mutants were consecutively passaged 20 times on SBA plates in the absence of antibiotics. The concentration of viable bacteria was determined at 5, 15, and 20 passages by plating adequate dilutions of a cell suspension (10^9 colony-forming units, CFU/ml) on SBA plates with and without streptomycin. Percent stability was calculated as the number of colonies obtained on plates supplemented with antibiotic relative to the number on plates without antibiotic. Accordingly, the *fur* mutation was found to be maintained at 100% stability in cells after 20 passages in the absence of selective pressure (data not shown).

Biochemical methods, DNA and RNA techniques. DNA procedures and computerized sequence analysis were carried out as previously described [11]. Nucleotide sequences were determined by the dideoxy method on an ALF Sequencer (Pharmacia Biotech, Cerdanyola del Valles, Barcelona). Total RNA extraction and reverse transcriptase PCR (RT-PCR) analysis were done as reported [6]. In all cases, the absence of DNA in RNA samples was tested by PCR without the addition of reverse transcriptase. OMPs from *P. multocida* were extracted and analyzed as previously reported [9]. Protein concentration was measured as described [24].

Protection studies against *P. multocida*. Groups of five 3-week-old female Swiss mice (Harlan Ibérica, Barcelona, Spain) were intraperitoneally injected with 10 or 40 μg OMP extract/animal. The extracts were prepared from several *P. multocida* strains grown under different culture conditions. In all cases, the inoculated volume of extract was 100 μl and was given in two doses 2 weeks apart. Control mice were inoculated with 100 μl of phosphate-buffered saline (PBS). Heterologous challenge was carried out 3 weeks later by intraperitoneal inoculation with a virulent strain of *P. multocida* (PM1002).

The same methodology was used to study protection conferred by heat inactivation or sonication disruption of *P. multocida* strains PM1094 and PM1096. Thermal inactivation was achieved by overnight incubation at 45°C of a culture previously grown in BHI at 30°C until a density of 7×10^8 CFU/ml was reached. Sonication-disrupted cells were prepared as follows: The cells (7×10^8 CFU/ml) were resuspended in BPW, placed in an ice bath, and then sonicated five times for 5 min at 80% duty cycle with an output of 40 W by using a Braun LabsonicU (Braun Biotech, Bethlehem, PA). The absence of viable cells was tested by plating on SBA plates. In all cases, the inoculated volume of extracts from inactivated cells was 100 μl, given as two doses 2 weeks apart. Negative control and heterologous challenge were done as described before.

Results and Discussion

Construction of a *P. multocida fur* mutant. A 394-bp internal fragment of the *P. multocida fur* gene was obtained by PCR amplification with Fur1 and Fur2 oligonucleotide primers. This fragment was cloned into the suicide plasmid pUA826, yielding plasmid pUA891. After introduction of pUA891 into *P. multocida* by triparental mating, several putative *fur* mutants were isolated after plating the bacteria onto suitable selective plates. PCR analysis of the bacterial chromosomal DNA confirmed that the wild-type *fur* gene had been interrupted by integration of pUA891 in two clones (Fig. 1). The electrophoretic profiles of the outer-membrane fractions of these two *fur* mutants were subse-

Table 1. Bacterial strains and plasmids used in this work

Organism and plasmid	Relevant features	Source or reference
Escherichia coli		
DH5α	F′/*supE4 ΔlacU169* (Ø80 *lacZ*ΔM15)	Clontech
	hsdR17 recA1 endA1 gyrA96 thi-1 relA1	
MC1061(λ*pir*)	*hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74*	Our laboratory
	gal1 galK rpsL thi lysogenized with l*pir* bacteriophage	
Pasteurella multocida		
PM25	Wild-type, serogroup D	Isolated from a rabbit nasal secretion
PM108	Wild-type, serogroup A	Isolated from an ovine pneumonia outbreak
PM1002	PM25 Rif^R Spc^R	Our laboratory
PM1011	PM108 Rif^R Spc^R	Our laboratory
PM1094	PM1011 *fur ompH*	This work
PM1095	PM1011 *fur*	This work
PM1096	PM1094 *galE*	This work
Plasmid		
pGEM-T	PCR cloning vector Ap^R	Promega
pRK2013	*rep* (colE1) Mob^+ Tra^+ Km^R	Gary Ditta
pKO3	M13ori *repA*(ts) *sacB* Cm^R	[23]
pUA826	Mob^+ R6K replicon Ap^R Str^R Spc^R	Our laboratory
pUA1089	pKO3 containing the *mob* site from pUA826	This work
pUA891	pUA826 carrying a 394-bp internal fragment of the *P. multocida fur* gene	[9]
pUA1090	pUA1089 carrying a 495-bp internal fragment of the *P. multocida galE* gene	This work

quently analyzed to corroborate that the presence of the *fur* mutation resulted in the induction of IROMPs of high molecular mass, as previously described [9,18]. These two mutants showed two different profiles (Fig. 2); the first mutant expressed the major 36-kDa OMP of *P. multocida*, OmpH [25], whereas, surprisingly, the second did not. Moreover, as previously described [35]**,** the absence of the major OMP gave rise to outer-membrane reorganization with several changes in the OMP profile (Fig. 2). Furthermore, the first mutant grew more slowly than the parental strain. In light of these different results, both *fur* mutants were retained for further protection studies.

In the complete genome sequence of *P. multocida* Pm70 [27], two copies of the *ompH* gene (separated by 154 bp), encoding the proteins OmpH1 and OmpH2, have been identified. In order to ascertain the nature of the mutation responsible for the $OmpH^-$ phenotype observed in strain PM1094, RT-PCR analysis was carried out to determine whether the *ompH* genes were transcribed. The results showed that *ompH1* and *ompH2* were indeed independently transcribed (Fig. 3); however, DNA sequencing of these genes (GenBank accession nos. EF102481 and EF102482, respectively) revealed significant differences compared to the corresponding sequences in strains PM1011 and Pm70. In *ompH1*, a nonsense mutation was found at position 76, resulting in a stop codon instead of one that encodes glutamine; this gives rise to a highly truncated protein of 24 amino acids. Similarly, the *ompH2* sequence of PM1094 strain had many nucleotide changes, including a nonsense mutation at position 670 that resulted in a truncated protein of 223 instead of 350 amino acids. These results clearly indicated that the absence of the major 36-kDa OMP in the PM1094 mutant is due to nonsense mutations in *ompH1* and *ompH2*. It is known that *fur* mutants have a high spontaneous mutagenesis rate

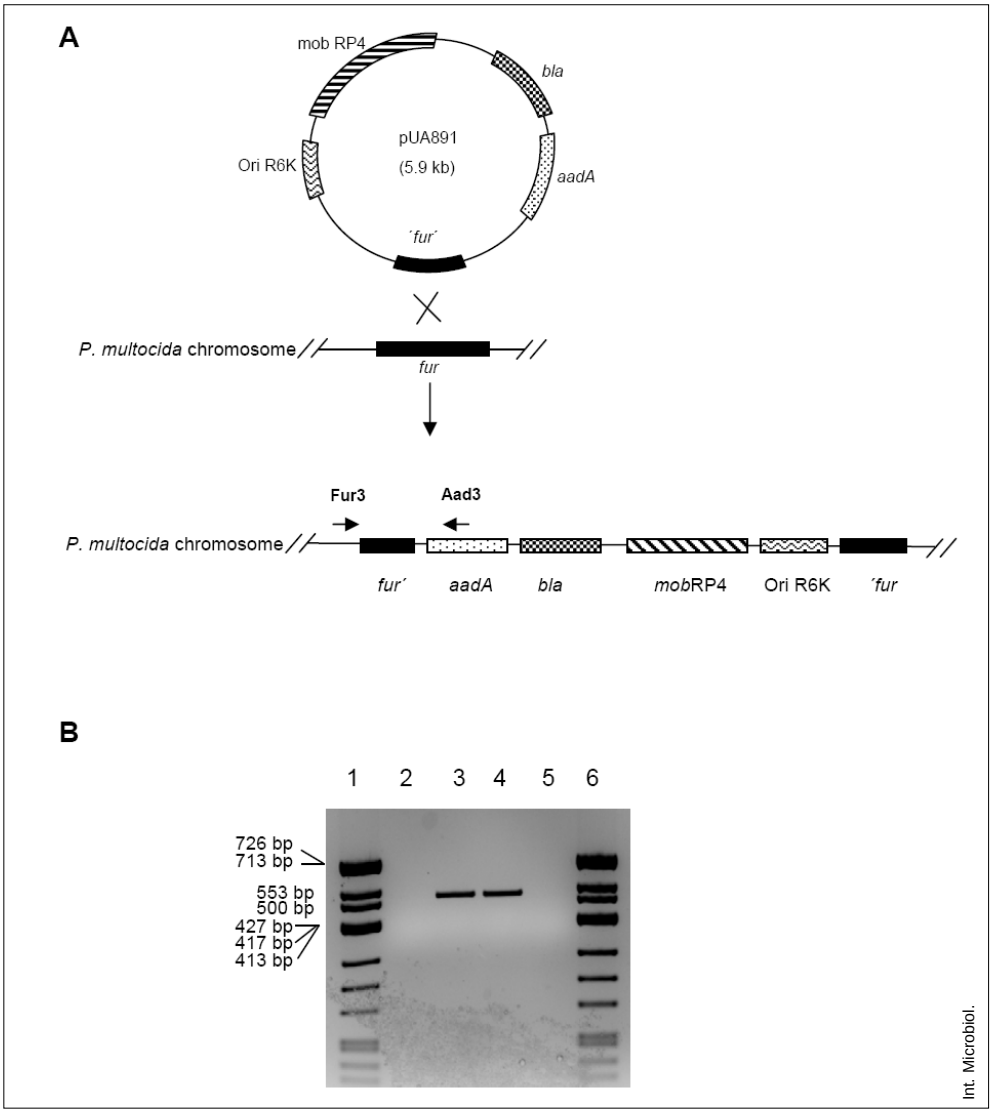

Fig. 1. PCR analysis of *Pasteurella multocida fur* mutants. (**A**) *P. multocida fur* mutant construction. Fur3 and Aad3 indicate the positions of oligonucleotide primers used to confirm the presence of the *fur* mutation. (**B**) Chromosomal DNA from wild-type strain (PM1011) (lane2), *fur* (PM1095) (lane 3), and *fur ompH* (PM1094) (lane 4) mutants was subjected to PCR analysis with the Aad3 and Fur3 oligonucleotide primers. The PCR control lacking DNA is shown in lane 5. *Hin*fI-digested Φ174 was used as a molecular size marker (lanes 1 and 6).

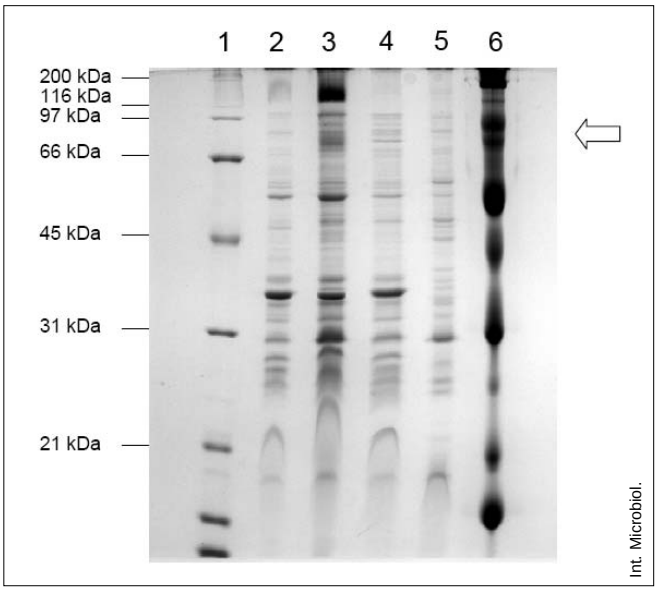

Fig. 2. SDS-PAGE (12%) profile of OMPs from *Pasteurella multocida* wild-type strain (PM1011) (lane 2), wild-type strain (PM1011) grown in the presence of DPD (lane 3), *fur* (PM1095) (lane 4), and *fur ompH* (PM1094) (lane 5). White arrow shows the high-molecular-mass proteins induced in the presence of DP or in *fur* mutants. Broad Range markers and Rainbow are shown in lanes 1 and 6, respectively.

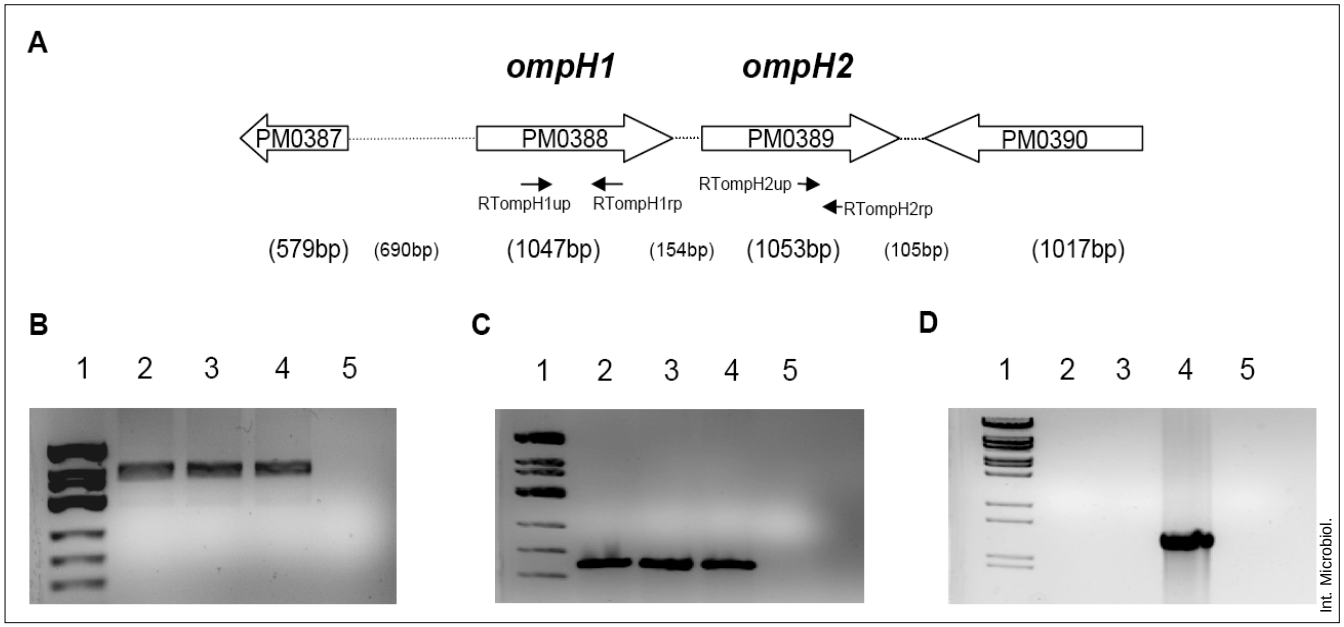

Fig. 3. Structural arrangement of *Pasteurella multocida ompH1* and *ompH2* genes. RTompH1up, RTompH1rp, RTompH2up, and RTompH2rp indicate the positions of oligonucleotide primers used for transcriptional analysis (**A**). RT-PCR analysis of transcripts of the *ompH1* (**B**), *ompH2* (**C**) genes, and the possible operon *ompH1-ompH2* (**D**) in either the wild-type strain (PM1011) (lanes 2) or the *fur ompH* mutant (PM1094) (lanes 3). Total RNA from each strain and the oligonucleotide primer pairs RTompH1up and RTompH1rp (B), RTompH2up and RTompH2rp (C) and RTompH1up and RTompH2rp (D), were used. PCRs with DNA from the wild-type strain (lanes 4) and from a negative control lacking both RNA and DNA (lanes 5) are shown. Φ174 *Hin*fI-digested (B**,** C) and *Bst*EII-digested- λ DNA (D) were used as molecular size markers (lanes 1).

because of the high oxidative stress imposed by the increased iron uptake [10,32]. This fact likely explains the high number of base-pair changes found in *ompH1* and *ompH2*.

Protection studies with *P. multocida fur* mutants. To analyze the putative protective effect of both *P. multocida fur* mutants, mice were immunized with 10 and 40 μg of OMP extracts prepared from *P. multocida* wild-type strain (PM1011) grown in the absence or presence of DPD, and from both PM1094 and PM1095 mutants. The mice were then heterologously challenged with the virulent strain PM1002 (LD_{50} = 3 CFU/animal) at a dose of 500× LD_{50}. All mice immunized with the OMP extract obtained from wild-type cells grown in the absence of DPD died 2 days after challenge (Table 2). However, in support of our initial hypothesis, equal protection (20%) was conferred in mice immunized with 40 μg of OMP extract from the wild-type strain grown under iron-depleted conditions vs. the same amount of extract from the *fur* mutant (PM1095) (Table 2). Remarkably, the OMP extract of the *fur ompH* mutant conferred total protection (Table 2). Several studies have reported that antibodies to some bacterial antigens may be detrimental to host defenses [20]. For instance, the deleterious effect of vaccination with the highly immunogenic protein PalA of *Actinobacillus pleuropneumoniae* was reported [36]. However, it was recently reported that purified OmpH protein confers homologous protection in *P. multocida* [22]; thus, the presence of OmpH does not seem to negatively affect host defenses. The high level of protection obtained with the OMP extract of the *fur ompH* mutant has at least two possible explanations: (i) an increase of the relative concentration of IROMPs in the extract due to the lack of the major OMP, and (ii) the exposure of a new antigen and/or an increase in the concentration of a particular antigen due to OMP outer-membrane reorganization, as occurred in the *fur ompH* mutant.

Since the highest level of protection was obtained with the OMP extract prepared from the double-mutant *fur ompH*, the next set of experiments was addressed at analyzing whether heat-inactivated cells of the *fur ompH* mutant promoted heterologous protection. The results showed that mice administered heat-inactivated *fur ompH* cells and then heterologously challenged with strain PM1002 at a dose of 100× LD_{50} were 60% protected (Table 3). An earlier study showed that vaccination with killed bacteria generally results in serotype-specific immunity [12], whereas heterologous protection is conferred only with attenuated strains [2]. By contrast, in this work, heterologous protection was obtained using inactivated cells. These data indicated that simple heat inactivation of *fur ompH P. multocida* cells can be used to produce a vaccine conferring heterologous protection.

Table 2. Protection conferred in mice challenged with heterologous *Pasteurella multocida* PM1002 (500× LD_{50}) following immunization with OMPs of different strains of the bacterium

Strain source of OMP	Relevant characteristics	Dose[b] (μg/animal)	Survival
PM1011	Wild-type	10	0/5
		40	0/5
PM1011	Wild-type grown in the presence of DPD[a]	10	0/5
		40	1/5
PM1095	*fur*	10	0/5
		40	1/5
PM1094	*fur ompH*	10	4/5
		40	5/5
PBS[c]			0/5

[a]The divalent-cation chelator 2,2′-dipyridyl. [b]Quantity of OMP extract inoculated per animal immunization. [c]Phosphate-buffered saline.

To determine whether the protection level could be increased by optimizing the surface exposure of antigens, a rough derivate mutant of the *fur ompH* mutant was obtained. The *galE* gene product catalyzes the epimerization of UDP-galactose to UDP-glucose and is required for the correct synthesis of the LPS core. A *galE* mutant is able to grow on glucose but unable to synthesize wild-type cell-surface LPS, thus yielding rough derivate cells. The *galE* mutant of the *fur ompH* strain (PM1094) was constructed as follows. PCR amplification with suitable oligonucleotide primers yielded a 495-bp internal fragment of *galE*. This fragment was then cloned into pUA1089 and the plasmid was introduced into strain PM1094 by triparental mating. Several putative *galE* mutants were generated by plating onto suitable selective plates. PCR analysis of the chromosomal DNA from four of the transconjugants confirmed that insertion of pUA1090 interrupted the wild-type *galE* gene. One of these clones, PM1096, was chosen for further study and analysis of its OMP profile corroborated that it had the same profile as the parental strain (data not shown). Mice were administered heat-inactivated (45ºC for 12 h) *fur ompH galE* mutant cells and then heterologously challenged with PM1002 at doses of 100 and 500× LD_{50}. As seen in Table 3, those animals immunized with the heat-inactivated strain were 60% protected at

Table 3. Protection conferred in mice challenged with heterologous *Pasteurella multocida* strain PM1002 following immunization with inactivated strains of the bacterium

Strain	Inactivation procedure[a]	Challenge dose (× LD_{50})	Survival
Wild-type	H	500	0/5
	H	100	0/5
fur ompH	H	500	0/5
	H	100	3/5
fur ompH galE	H	500	0/5
	H	100	3/5
Wild-type	S	500	0/5
fur ompH galE	S	500	3/5
PBS[b]		100	0/5

[a]Cells were heat-inactivated by incubation at 45ºC for 12 h (H), or disrupted by sonication (S), as described in Materials and methods. [b]Phosphate-buffered saline.

the lower challenge dose. Thus, although the bacterial cells expressed a shorter cell-surface LPS, this did not result in increased protection because heat-inactivated *fur ompH* and *fur ompH galE* mutants induced the same level of protection in immunized mice.

A different inactivation strategy based on cell disruption by sonication was also tested. Mice immunized with sonication-disrupted *fur ompH galE* cells were found to be 60% protected at the highest challenge dose (500× LD_{50}) (Table 3). Thus, cell disruption by sonication yielded a much better vaccine than obtained following heat-inactivation of the cells. This may be due to the heat lability of the immunogenic proteins.

In conclusion, our results suggest that *fur* mutants can be used to develop IROMP-based vaccines, particularly against pathogens presenting multiple and different iron receptors, as is the case with *P. multocida*. Furthermore, the strategy of using *fur* mutants overcomes problems associated with other methodologies, such as the poor growth of bacterial cells in the presence of divalent-cation chelators. Moreover, the absence of the OmpH protein significantly increased the cross-protection conferred by the *fur* mutant of *P. multocida.* These findings will contribute to the development of effective vaccines that protect against *P. multocida* infection.

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