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## “Phylogenomic insights into brucellaceae: The *Pseudochrobactrum algeriensis* case”

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### ABSTRACT

The genus *Pseudochrobactrum* encompasses free-living bacteria phylogenetically close to *Ochrobactrum* opportunistic pathogens and to *Brucella*, facultative intracellular parasites causing brucellosis, a worldwide-extended and grave zoonosis. Recently, *Pseudochrobactrum* strains were isolated from *Brucella* natural hosts on *Brucella* selective media, potentially causing diagnostic confusions. Strikingly, *P. algeriensis* was isolated from cattle lymph nodes, organs that are inimical to bacteria. Here, we analyse *P. algeriensis* potential virulence factors in comparison with *Ochrobactrum* and *Brucella*. Consistent with genomic analyses, Western-Blot analyses confirmed that *P. algeriensis* lacks the ability to synthesize the N-formylperosamine O-polysaccharide characteristic of the lipopolysaccharide (LPS) of smooth *Brucella* core species. However, unlike other *Pseudochrobactrum* but similar to some early diverging brucellae, *P. algeriensis* carries genes potentially synthesizing a rhamnose-based O-polysaccharide LPS. Lipid A analysis by MALDI-TOF demonstrated that *P. algeriensis* LPS bears a lipid A with a reduced pathogen-associated molecular pattern, a trait shared with *Ochrobactrum* and *Brucella* that is essential to generate a highly stable outer membrane and to delay immune activation. Also, although not able to multiply intracellularly in macrophages, the analysis of *P. algeriensis* cell lipid envelope revealed the presence of large amounts of cationic aminolipids, which may account for the extremely high resistance of *P. algeriensis* to bactericidal peptides and could favor colonization of mucosae and transient survival in *Brucella* hosts. However, two traits critical in *Brucella* pathogenicity are either significantly different (T4SS [VirB]) or absent (erythritol catabolic pathway) in *P. algeriensis*. This work shows that, while diverging in other characteristics, lipidic envelope features relevant in *Brucella* pathogenicity are conserved in *Brucellaceae*. The constant presence of these features strongly suggests that reinforcement of the envelope integrity as an adaptive advantage in soil was maintained in *Brucella* because of the similarity of some environmental challenges, such as the action of cationic peptide antibiotics and host defense peptides. This information adds knowledge about the evolution of *Brucellaceae*, and also underlines the taxonomical differences of the three genera compared.

### 1. Introduction

The *Brucellaceae* family of the  $\alpha$ -2 *Proteobacteria* currently comprises seven genera: *Brucella*, *Daeguia*, *Falsochrobactrum*, *Mycoplana*, *Ochrobactrum*, *Paenochrobactrum* and *Pseudochrobactrum* (Holmes et al., 1988; Kämpfer et al., 2014; Kämpfer et al., 2013; Kämpfer et al., 2006;

Urakami and Segers, 2015; Velasco et al., 1998; Yoon et al., 2008), bacteria that display strikingly different lifestyles, from environmental inhabitants, some of which are occasionally opportunistic pathogens (i. e., *Ochrobactrum*), to facultative intracellular pathogens of vertebrates (i. e., *Brucella*). *B. abortus*, *B. melitensis* and *B. suis*, the classical and best-characterized brucellae, cause brucellosis, one of the most common

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bacterial zoonosis affecting ruminants and other livestock (Ducrottoy et al., 2017; Perry et al., 2002). These pathogens lack classical virulence factors such as capsules, adhesins, toxins or virulence plasmids (Martirosyan et al., 2011), and their pathogenicity depends chiefly on a type IV secretion system (T4SS) (Celli, 2019), peculiar envelope free-lipids (Comerci et al., 2006; Conde-Álvarez et al., 2006; Palacios-Chaves et al., 2011) and lipopolysaccharide (LPS) (Conde-Álvarez et al., 2012; Gorvel and Moreno, 2002; Lapaque et al., 2006a; Velasco et al., 2000) and characteristic metabolic pathways (Barbier et al., 2018; Letesson et al., 2017). These classical species are phylogenetically intertwined with *B. pinnipedialis*, *B. ceti*, *B. canis*, *B. ovis*, *B. neotomae*, *B. microti* and *B. papionis* (Foster et al., 2007; Scholz et al., 2008; Whatmore et al., 2014) that together form a core brucellae group separated from the early-diverging *B. vulpis* and *B. inopinata* (Scholz et al., 2016; Scholz et al., 2010), which are closer to the free-living bacteria (occasionally opportunistic pathogens) of the  $\alpha$ -2 *Proteobacteria* (Al Dahouk et al., 2017; Soler-Lloréns et al., 2016; Alice R. Wattam et al., 2014). As suggested by the lack of classical virulence factors in brucellae, it is conceivable that their environmental relatives display traits representing an exaptation favouring adaption to intracellular pathogenicity (Batut et al., 2004; Moreno, 1992; Suárez-Esquivel et al., 2020; Whatmore and Foster, 2021).

Described long ago, *Ochrobactrum* is a large and continuously expanding genus closely related to *Brucella* (Michael P. Ryan and Pembroke, 2020). Interestingly, while *Ochrobactrum* species are primarily environmental and rhizosphere inhabitants, *O. anthropi* and *O. intermedium* have been repeatedly isolated from immunocompromised patients, and *O. pecoris* from farm animals (Berg et al., 2005; Berman et al., 1997; Gill et al., 1997; Greven and Nelson, 2001; Inoue et al., 1999; Kämpfer et al., 2011; Saeed Mahmood et al., 2000; Michael P. Ryan and Pembroke, 2020; Stiakaki et al., 2002). Another genus closely related to *Brucella* is *Pseudochrobactrum*. Several species of the latter genus have been isolated from vent covers in a seafood processing plant (Kämpfer et al., 2007), metal-working fluid (Kämpfer et al., 2009) and *P. asaccharolyticum* from a human knee (Kämpfer et al., 2006). Several species of the latter genus have been isolated from vent covers in a seafood processing plant (Kämpfer et al., 2007), metal-working fluid (Kämpfer et al., 2009) and *P. asaccharolyticum* from a human knee (Kämpfer et al., 2006). More recently, *Pseudochrobactrum* has also been isolated from various *Brucella* natural hosts (Ashford et al., 2020; Loperena-Barber et al., 2022; Pereira et al., 2023). First, Ashford et al. isolated *Pseudochrobactrum* sp. from sheep and cattle in the UK (Ashford et al., 2020). Then, Loperena-Barber et al. described the isolation of *P. algeriensis* from *Brucella* seropositive cattle on *Brucella* selective medium (Loperena-Barber et al., 2022; Khames et al., 2017) and, more recently, Pereira et al. isolated *P. saccharolyticum* from a bovine suspected of having brucellosis in Brazil causing diagnostic uncertainties (Pereira et al., 2023). *P. algeriensis* was isolated from bovine lymph nodes and, while transient presence of viable bacteria in these organs can be accidental, repeated isolation of *P. algeriensis* from the inimical environment within these organs is remarkable, particularly when considering the phylogenetically relatedness with opportunistic *Ochrobactrum* spp. (Michael P. Ryan and Pembroke, 2020) and *Brucella* (Martirosyan et al., 2011). Therefore, whereas the ability to grow on *Brucella* selective media needs attention when carrying out a bacteriological diagnosis of brucellosis, from a basic standpoint the comparison of *Brucella*, *Ochrobactrum* and *Pseudochrobactrum* can provide new information to understand the evolution of some *Brucellaceae* towards pathogenicity (Aujoulat et al., 2014; Barquero-Calvo et al., 2009; Velasco et al., 1998; Alice R. Wattam et al., 2014). To this end, we analyzed the genome, molecular features and behaviour in laboratory infection models of *P. algeriensis* in comparison with *Brucella* and *Ochrobactrum*. Here we present data supporting the hypothesis that some envelope traits shared by the extant *Brucellaceae* represent the scaffold on which *Brucella* pathogenicity was built.

## 2. Results

### 2.1. *P. algeriensis* genome

The ASM1843624v1 *P. algeriensis* C130915\_07<sup>T</sup> genomic sequence was composed by 1 chromosome (3,436,934 bp) and 4 plasmids (total 564,058 bp) with a 51.56% of GC (Fig. 1A). The annotation using RASTtk algorithm (Brettin et al., 2015) predicted 3892 protein coding sequences, 59 transfer RNA genes, and 15 ribosomal RNA genes, corresponding to 1091 hypothetical proteins plus 2801 proteins with functional assignments. The vast majority of the genes were related to metabolic processes, energy obtention and protein processing (Fig. 1B). Many other genes were putatively assigned to membrane transport, stress response or antibiotic resistances. It is significant (see Discussion) that compared to *Brucella* and *Ochrobactrum* genomes, *P. algeriensis* contains just one chromosome and a variable number of plasmids (from none to four).

### 2.2. *P. algeriensis* grew on gluconeogenic substrates but lacked the erythritol, rhamnose and ectoine metabolism operons

*In silico* analyses suggested that, like *Brucella*, *P. algeriensis* had the ability to grow on gluconeogenic media (Table A.1). Consistent with this, it grew in Gerhardt's medium (Gerhardt, 1958), a gluconeogenic medium containing lactate, glutamate and glycerol. Noteworthy, it grew faster than *B. abortus* or *O. intermedium* (Fig. 2A).

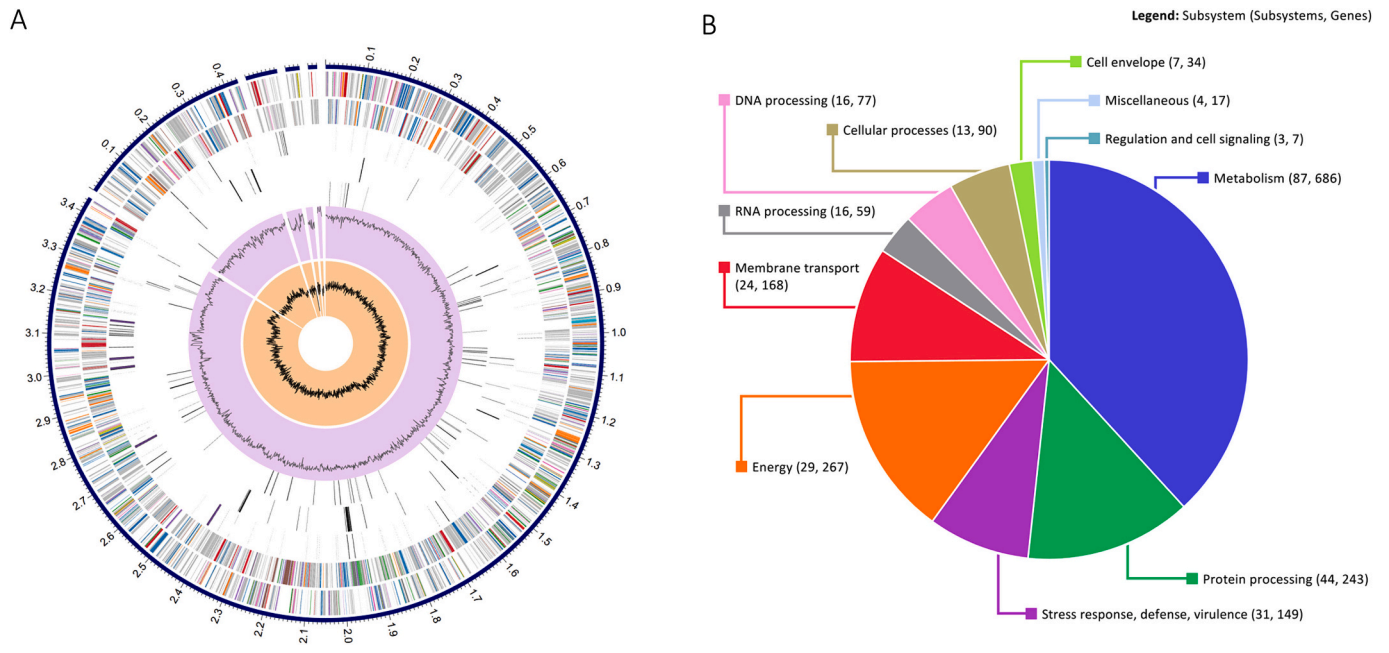
The catabolic pathway for erythritol, a preferential carbon source for *Brucella*, is driven by the *ery* operon (Barbier et al., 2017; Barbier et al., 2014; Smith et al., 1962). However, most of the homologous genes were not found in *P. algeriensis* (Table A.1). Consistent with this, and in contrast to *Brucella* and *Ochrobactrum*, *P. algeriensis* did not grow on erythritol as the sole carbon source (Fig. 2B). *Ochrobactrum* and some early diverging *Brucella* strains grow on rhamnose (a sugar with an important role in nodule formation in rhizobia (Ding et al., 2012)) and ectoine (an organic compound involved in osmoadaptation (Oren, 2008)) as the only carbon sources, and carry the respective transport and degradation operons (Soler-Lloréns et al., 2016). However, classical brucellae lack both groups of traits. *P. algeriensis* neither grew on ectoine (Fig. 2C) or L-rhamnose (Fig. 2D), nor carried the corresponding genes (Table A.1), being in both aspects like the classical *Brucella* spp. (Soler-Lloréns et al., 2016). Finally, *P. algeriensis* lacked the *nar*, *nir* and *nor* operons (Table A.1), involved in nitrate, nitrite and nitric oxide reduction, respectively, but was able to grow in microaerophilic conditions (data not shown).

### 2.3. *P. algeriensis* did not affect the survival of *Galleria mellonella*

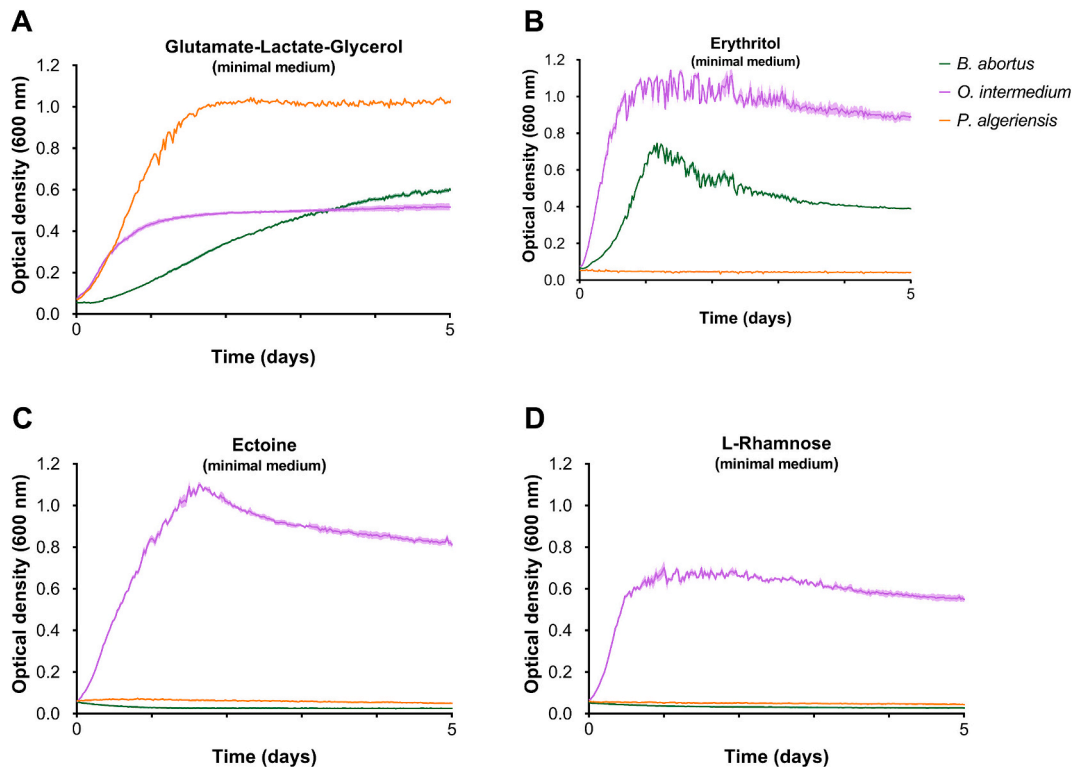
In this model, larva survival is considered a good indicator of virulence (Barnoy et al., 2017; Elizalde-Bielsa et al., 2023; Harding et al., 2013; Insua et al., 2013). Accordingly, we compared the survival of larvae infected with *K. pneumoniae* 52,145 (from  $10^3$  to  $10^6$  CFU/larva), which contains a canonical LPS readily detected by innate immunity (Insua et al., 2013), *B. abortus* ( $10^3$ – $10^6$  CFU/larva), whose LPS is not readily detected by innate immunity (Conde-Álvarez et al., 2012; Lapaque et al., 2006a; Velasco et al., 2000), and *P. algeriensis* ( $10^3$ – $10^6$  CFU/larva). A time- and dose-dependent effect on survival was observed for *B. abortus* and *K. pneumoniae* and the mortality induced by *K. pneumoniae* was noticeably higher (Fig. 3 A and 3B), as reported before (Elizalde-Bielsa et al., 2023; Insua et al., 2013). In contrast, the mortality caused by *P. algeriensis* was markedly low with a survival rate above 90% at the highest dose (Fig. 3C).

### 2.4. *P. algeriensis* did not multiply in mouse spleen or in bovine peritoneal macrophages

The ability of *P. algeriensis* to multiply or persist in the spleen of mice



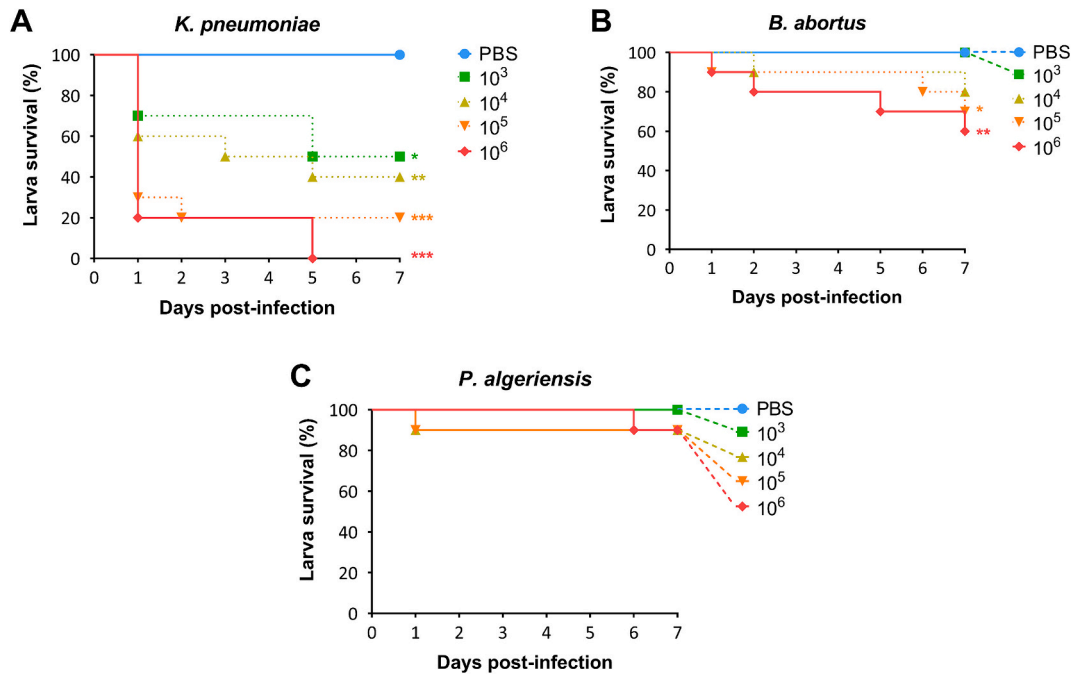
**Fig. 1.** (A) Circular graphical display of the distribution of *P. algeriensis* genome, including from outer to inner rings: the five contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content and GC skew. The colour of the CDS on the forward and reverse strands indicate the subsystem to which these genes belong. (B) Subsystem analysis of *P. algeriensis* genome; each colour represents a set of proteins that together implement a specific biological process or structural complex. In parenthesis the number of subsystems and genes belonging to each category are pointed.



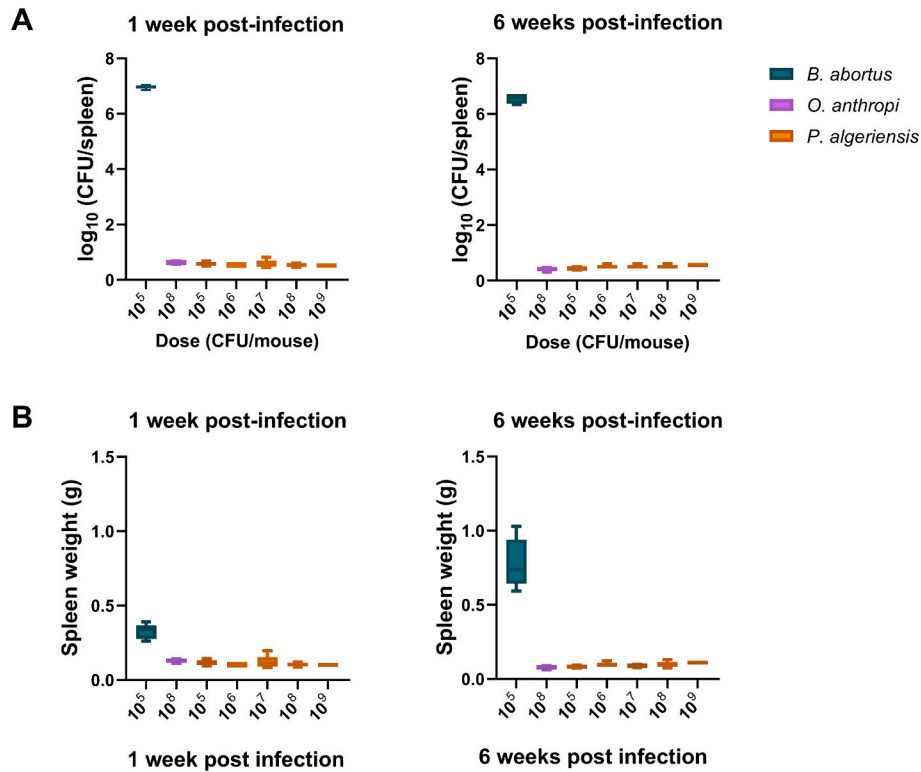
**Fig. 2.** (A) *P. algeriensis* is able to grow on gluconeogenic substrates (glutamate 1.5 g/L, lactate, 5 g/L and glycerol, 30 g/L), but not on (B) erythritol (2 g/L), (C) ectoine (20 mM) or (D) L-rhamnose (1 g/L), as the only carbon sources. Each line represents the mean  $\pm$  standard error (error bars are filled with colour) of technical triplicates. The experiment was repeated twice with similar results.

was evaluated in BALB/c mice intraperitoneally infected with doses ranging from  $10^5$  to  $10^9$  CFU/mouse. Mice were euthanized at 1 and 6 weeks after infection to determine the number of CFU/spleen (Fig. 4 A)

and spleen enlargement (Fig. 4B). Controls were mice inoculated with *B. abortus* or *O. anthropi*. As expected, (Barquero-Calvo et al., 2009), *B. abortus* at  $10^5$  CFU/mouse reached high CFU numbers 1 week after



**Fig. 3.** *P. algeriensis* does not affect the survival of *Galleria mellonella*. Larvae ( $n = 10$ ) were inoculated with doses ranging from  $10^3$  to  $10^6$  CFU/larva of (A) *K. pneumoniae* 52,145, (B) *B. abortus*, and (C) *P. algeriensis* (PBS injected larvae were used as a control). Kaplan Meyer survival curves significance was analyzed by the Log-rank Mantel-Cox test; differences between values of each sample versus the PBS control were considered significant at  $p < 0.05$  (\*),  $p < 0.005$  (\*\*) and  $p < 0.0005$  (\*\*\*)



**Fig. 4.** *P. algeriensis* is not able to multiply in mouse spleen and does not cause splenomegaly. BALB/c mice ( $n = 5$ ) were inoculated intraperitoneally with the indicated doses of *P. algeriensis*, *B. abortus* or *O. anthropi*. The plots show the mean  $\pm$  standard error of (A) CFU/spleen determined by plating on TSA and (B) the spleen weight.

infection and maintained those values throughout the experiment (Fig. 4 A) accompanied by enlargement of the spleen (Fig. 4B). *O. anthropi* at  $10^8$  CFU/mouse was not able to establish an infection and

*P. algeriensis* was not able to multiply in mouse spleens or cause splenomegaly even at the highest dose ( $10^9$  CFU/mouse).

To investigate whether *P. algeriensis* can multiply within cells, BoMac

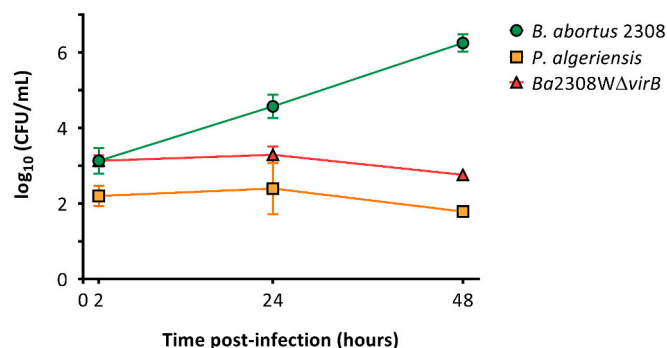


bovine peritoneal macrophages (Stabel and Stabel, 1995) were infected with virulent *B. abortus*, a highly attenuated *B. abortus*  $\Delta$ virB mutant (Lacerda et al., 2013; Sieira et al., 2000). As expected, virulent *B. abortus* multiplied extensively and the virB mutant invaded but did not multiply within the BoMac cells, highlighting the crucial role of this T4SS (Fig. 5; Fig. A.2). Remarkably, although *P. algeriensis* was not able to achieve sustained replication, the CFU did not decrease significantly at the end of the experiment, being in this regard similar to the *Brucella* VirB T4SS mutant. *P. algeriensis* genome analyses revealed a chromosomal region coding for a Trb-like T4SS and plasmidic VirB-like T4SS. The parallelism between  $\Delta$ virB and *P. algeriensis* in intracellular behaviour (Fig. 5) supports the prediction that the *P. algeriensis* systems are dedicated to DNA transfer (Fig. A.2; Table A.2), as it occurs in *Ochrobactrum* (Alice R Wattam et al., 2014).

### 2.5. *P. algeriensis* was extremely resistant to polymyxin B and its cell envelope displayed a variety of aminolipids larger than those of *Brucella* or *Ochrobactrum*

A prominent characteristic of *Brucella* is its resistance to many bactericidal cationic peptides and lipopeptides, including polymyxins (Barquero-Calvo et al., 2007; Freer et al., 1996; Manterola et al., 2007; Martínez de Tejada et al., 1995). Since *P. algeriensis* was isolated on CITA *Brucella* selective medium (De Miguel et al., 2011), which contains 7.5 mg/L of colistin (polymyxin E), it shows at least some resistance to these substances. Strikingly, the minimal inhibitory concentration (MIC) of *P. algeriensis* to polymyxin B was extremely high (>1024  $\mu$ g/mL), far higher than that of *O. anthropi* (0.75  $\mu$ g/mL) or even *B. abortus* (2  $\mu$ g/mL) (Fig. A.3). Also, the MIC values for other antimicrobials determined using the VITEK System (Table A.3) showed an extreme resistance to colistin and the cyclic lipopeptide daptomycin. Since *Brucella* resistance to cationic peptides is largely accounted for by the envelope lipids and LPS, these components of *P. algeriensis* were investigated.

The analysis of *P. algeriensis* genome predicted the existence of the main pathways involved in the synthesis of *Brucella* envelope lipids, including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), ornithine lipids (OL) and phosphatidylcholine (PC) (Aragón-Aranda et al., 2021; Comerci et al., 2006; Conde-Álvarez et al., 2006; Palacios-Chaves et al., 2011) (Table A.4). These predictions were validated by bidimensional high-performance thin layer chromatography (HPTLC). In addition to the characteristic lipids of *Brucellaceae* (i.e., PE, OL, PG, CL and PC; Fig. 6 A), *P. algeriensis* whole lipid extract displayed several unidentified lipids (ULs; dashed lines in Fig. 6 A). Ninhydrin staining revealed that most of the ULs were aminolipids (Fig. 6FB) and confirmed the identity of PE and OL, the main



**Fig. 5.** *P. algeriensis* is not able to effectively multiply in BoMac bovine macrophages. BoMac were infected with GFP-expressing bacterial strains at a MOI of 100 and extracellular bacteria were killed with 100 or 500  $\mu$ g/mL of gentamycin. For each strain, statistical comparisons between time-points were made with unpaired t-test, and comparisons between strains at each time-point were made by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test.

aminolipids of *Brucellaceae*.

### 2.6. Genomic analyses of *P. algeriensis* LPS putative genes suggested a rhamnose-based O-polysaccharide and core oligosaccharide features common to *Brucella* and *Ochrobactrum*

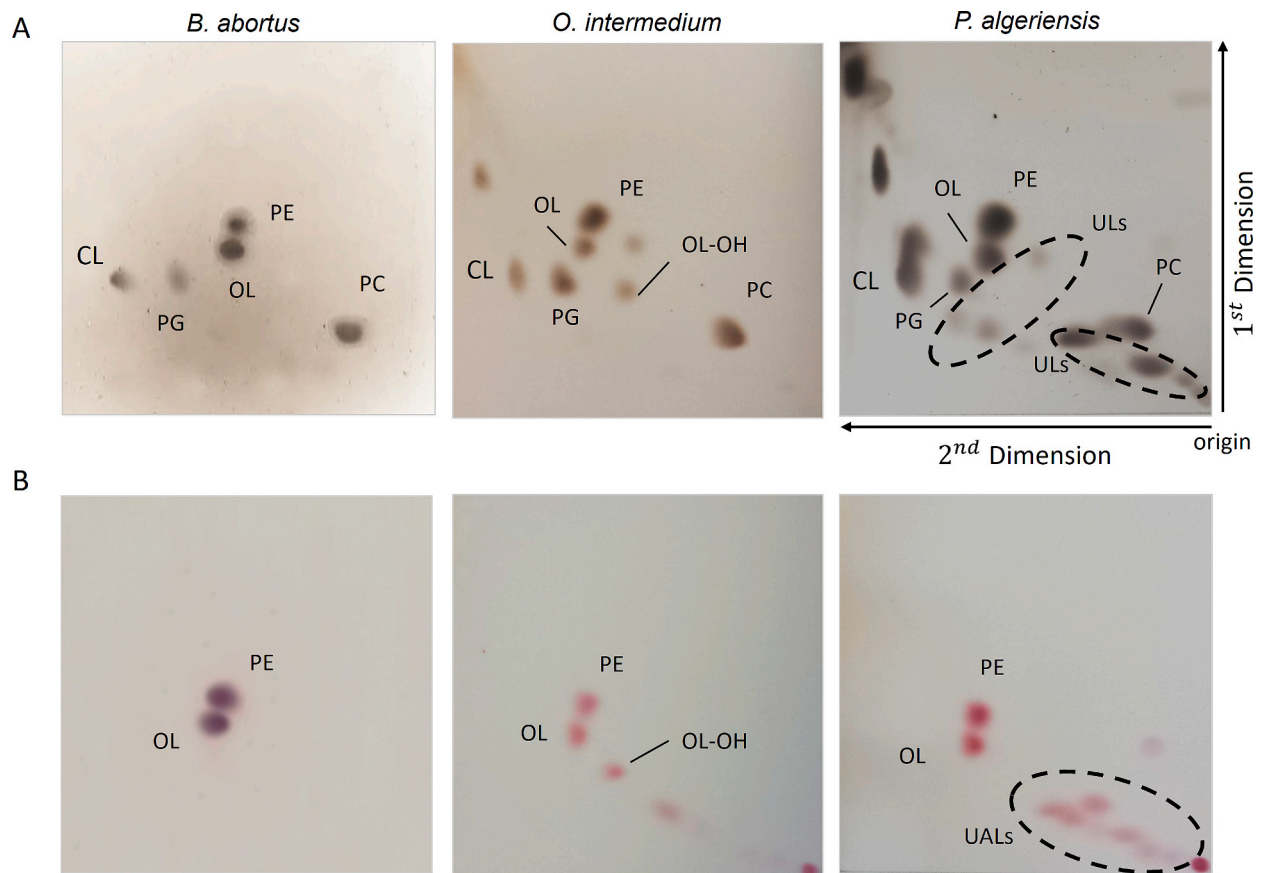
Most genes involved in the synthesis of the classical *Brucella* LPS N-formylperosamine O-chain are in the *wbk* region (Fig. 7A). In *P. algeriensis* (and other *Pseudochrobactrum*), *wbk* homologues were absent with the exception of *wbkF* and *wbkD* (Fig. 7A). Interestingly, some early-diverging *Brucella*, such as *B. inopinata* BO2, also lack most *wbk* genes and do not synthesize N-formylperosamine (Al Dahouk et al., 2017; Scholz et al., 2023; Soler-Lloréns et al., 2016; Wattam et al., 2012). Instead, they contain an operon of four genes (*rmlA-D*) shown to be involved in synthesis of a rhamnose-based O-chain in *Rhizobium* (Marie et al., 2004; Reuhs et al., 2005). Strikingly, one of the *P. algeriensis* plasmids contained homologues of the *rmlA-D* genes and, upstream of them, genes putatively encoding a rhamnosyl transferase and the *rfbB/rfbD* O-chain transporters, suggesting that *P. algeriensis* carries an S-type LPS with a rhamnose-based O-chain.

The above-data were consistent with the SDS-PAGE and Western-Blot analyses performed with *P. algeriensis*, *B. abortus*, *O. anthropi* (S-LPS) and *O. intermedium* (rough [R]-LPS). Both the migration pattern (Fig. 7B) and the reactivity of *P. algeriensis* LPS with the serum of a rabbit immunized with homologous whole cells were typical of an O-chain-containing (i.e., S-type) LPS (Fig. 7B). Also, this serum did not cross-react with *Brucella* or *O. anthropi* LPS, showing that *P. algeriensis* carries a different O-chain. We confirmed this using an antiserum to *Yersinia enterocolitica* O:9, a bacterium that shares the O-chain with *Brucella* but not proteins eliciting antibodies cross-reacting with *Brucellaceae* that could interfere in Western blot analysis of whole bacteria. As can be seen in Fig. 7B, only the *B. abortus* S LPS reacted with the anti-*Y. enterocolitica* O:9 serum. On the other hand, an anti-S *Brucella* serum did not show cross-reactivity with *P. algeriensis* or *O. intermedium* (Fig. 7B).

In *Brucella* LPS core, a lateral branch with four glucosamine units counterbalances the negatively charged Kdo increasing cationic peptide resistance and reducing recognition by innate immunity (Conde-Álvarez et al., 2012). The synthesis of this branch requires glycosyltransferase genes *wadB*, *wadC* and *wadD* (Conde-Álvarez et al., 2012; Gil-Ramírez et al., 2014; Goldman and Kohlbrenner, 1985; Iriarte et al., 2004; Monreal et al., 2003; Salvador-Bescós et al., 2018). *P. algeriensis* presents homologues of *wadC* and *wadB* but not of *wadD* (Table A.5). However, *P. algeriensis* genome did not contain homologues of *manB*Core and *manC*Core, both involved in the synthesis of mannose (Monreal et al., 2003), the sugar incorporated by *wadC*. This suggests that either *wadC* incorporates a sugar other than mannose or that *P. algeriensis* has a hitherto unknown mannose synthesis pathway. Unexpectedly, we did not find a *wadA* homologue. This absence is striking because *WadA* is responsible for linking KdoII and the glucose bridging the *Brucella* core to the O-chain, and has also been proposed to be a bifunctional enzyme acting also as an O-chain ligase (Servais et al., 2023). Moreover, we did not find *waal*, the other known O-chain ligase. This leaves the binding of the O-chain to the LPS core in *P. algeriensis* unresolved.

### 2.7. *P. algeriensis* displayed lipid A modifications consistent with a poor mononuclear cell LPS activating effect

The lipid A of some pathogens displays a modified structure that favors the evasion of the immune system, and some of them have been well-documented in *Brucella* (Conde-Álvarez et al., 2018). The lipid A backbone of *Brucella* and other *Brucellaceae* is made of diaminoglucose, while in most Gram-negative bacteria it is made of glucosamine. *GnnA* and *gnnB* genes are involved in the synthesis of this molecule in *Leptospira interrogans* (Sweet et al., 2004) and *Campylobacter* (Mourik et al., 2010). Recently, two *gnnA* and *gnnB* homologues have been found in



**Fig. 6.** *P. algeriensis* contains the characteristic *Brucella* lipids plus several unidentified aminolipids. Bidimensional HPTLC analysis of the free lipids of *B. abortus* 2308, *O. intermedium* 3301 and *P. algeriensis* (A) developed with copper (II) sulphate for total lipid detection, or (B) ninhydrin, for aminolipid detection. OL, ornithine lipids; OL-OH, hydroxylated ornithine lipids; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; UL, unidentified lipids; UL, unidentified aminolipids.

*Brucella* (Salvador-Bescós M. unpublished results; Mourik et al., 2010), and the genomic search showed two genes in *P. algeriensis* with a significant similarity to them (Table A.5), supporting the presence of a diaminoglucose backbone. Also, a feature of *Brucella* lipid A relevant in pathogenicity are the very long chain fatty acids (VLCFA) that prevent the recognition of the LPS by the Toll-like receptors (TLR) and increase the stability of the cell envelope. LpxXL and AcpXL proteins have been related to this characteristic (Basu et al., 2002; Conde-Álvarez et al., 2018; Iriarte et al., 2004). In *P. algeriensis*, there were three genes with a high percentage of similarity (Table A.5) to the three *Brucella* ORFs putatively coding for these proteins (one for LpxXL and two for AcpXL). In some bacteria, a *lptA* encoded enzyme links ethanolamine to the phosphate of the C1 of the backbone (Needham and Trent, 2013), and a phosphatase encoded by *lpxE* removes C1 and C4 phosphate. These modifications reduce the net negative charge and increase the resistance to cationic peptides (Conde-Álvarez et al., 2018; Needham and Trent, 2013; Raetz et al., 2009). *LptA* is present in at least in *B. melitensis* and there is indirect evidence suggesting that *lpxE* is active in *Brucella* (Conde-Álvarez et al., 2018). In *P. algeriensis* *lptA* was present but *lpxE* was absent (Table A.5).

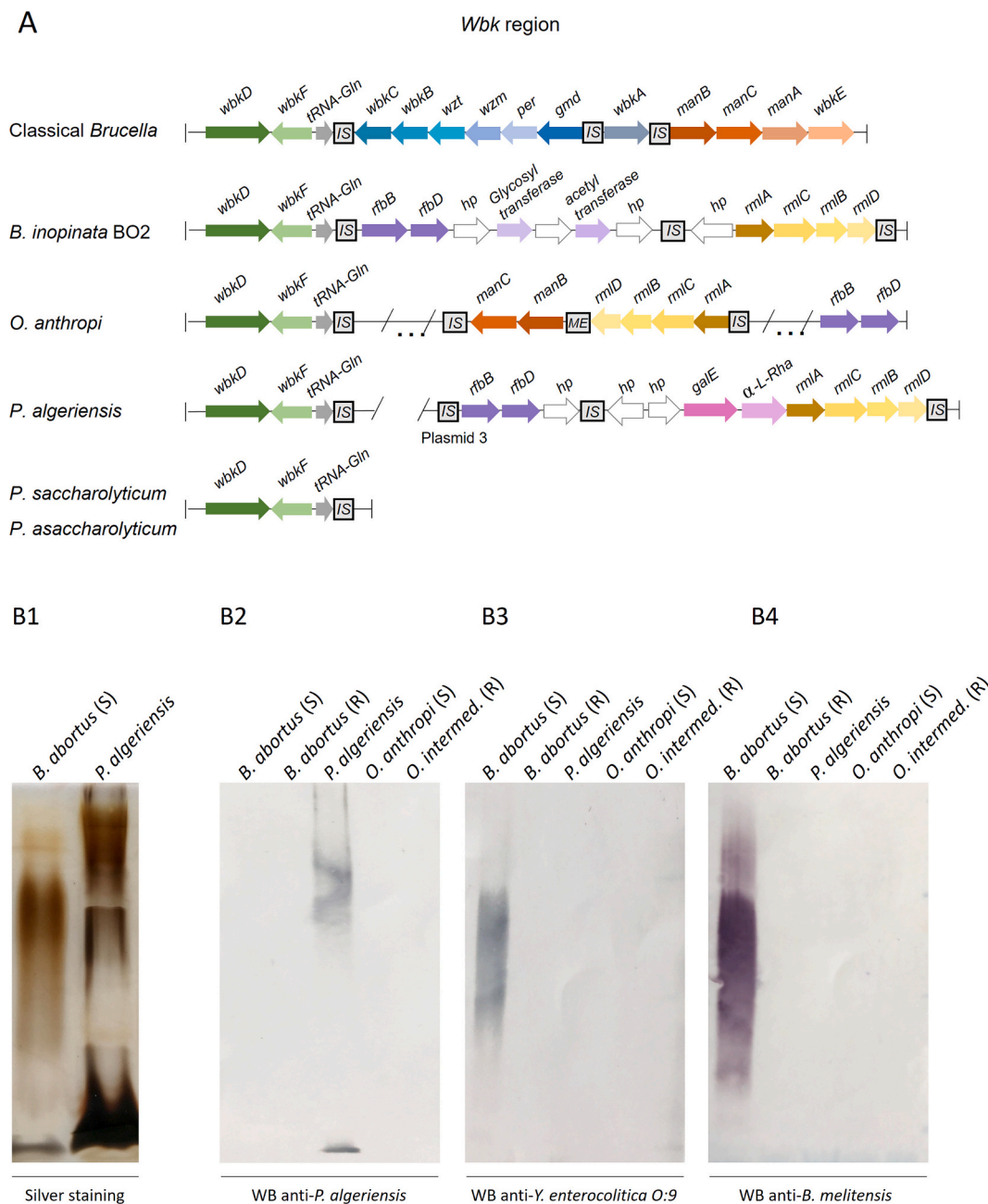
Consistent with this, the analysis of *P. algeriensis* lipid A by MALDI-TOF (Fig. 8 A) showed two predominant clusters of ions around  $m/z$  2019 (species A) and 2093 (species B). The  $m/z$  2019 was compatible with the isotopic mass of a molecule ( $C_{102}H_{196}N_4O_{25}P_2$ ) formed by an hexaacetylated and bisphosphorylated diaminoglucose disaccharide carrying hydroxylated long and very long chain acyl groups, and signals differing in a mass of 14 or 28 units (*i.e.*, 1991 and 2047  $m/z$ ) should result from the heterogeneity in acyl chain length (mass of 14 for  $CH_2$ ) typical of lipid A. Although a signal corresponding to the addition of a

phosphoethanolamine (an increase of 125  $m/z$  corresponding to  $CH_3NCH_2-CH_2-HPO_3$ ) was not observed, this might be due to the lack of expression of *lptA* under the experimental conditions used. On the other hand, the signal  $m/z$  2066 could correspond to a monophosphorylated lipid A species substituted with phosphoethanolamine suggestive of a not yet identified phosphatase that might be acting on *P. algeriensis* lipid A. Interestingly, the signal at 2093  $m/z$  (species B), previously observed in *Brucella* lipid A analyses (Conde-Álvarez et al., 2018), is consistent with a monophosphorylated lipid A ( $C_{120}H_{232}N_4O_{25}P$ ).

*Brucella* lipid A causes only a very low stimulation of cells (Lapaque et al., 2006b). Thus, we next compared human peripheral blood mononuclear cells (PBMC) activation by purified LPS from *E. coli*, *B. abortus*, *O. anthropi* and *P. algeriensis*. Upon incubation for 24 h monocyte activation markers were evaluated by flow cytometry. At 10  $\mu g/mL$ , *P. algeriensis* LPS barely or not at all induced CD80 expression, a result similar to those obtained with *B. abortus* and *O. anthropi* LPS at the same dose (Fig. 8B). In contrast, even a 100-fold less *E. coli* LPS dose induced significant CD80 expression as compared with mock-treated cells. Analogous results were obtained when other activation markers were measured (PD-L1, SLAMF7 and SLAMF8) or when analysing non-classical or intermediate monocytes (Fig. A.5). All these results strongly support the hypothesis that, like *Brucella* and *Ochrobactrum*, *Pseudochrobactrum* conserves a modified lipid A that delays immune activation.

### 3. Discussion

The *Brucellaceae* is an expanding family with members that have evolved to occupy a diversity of environmental niches and to establish



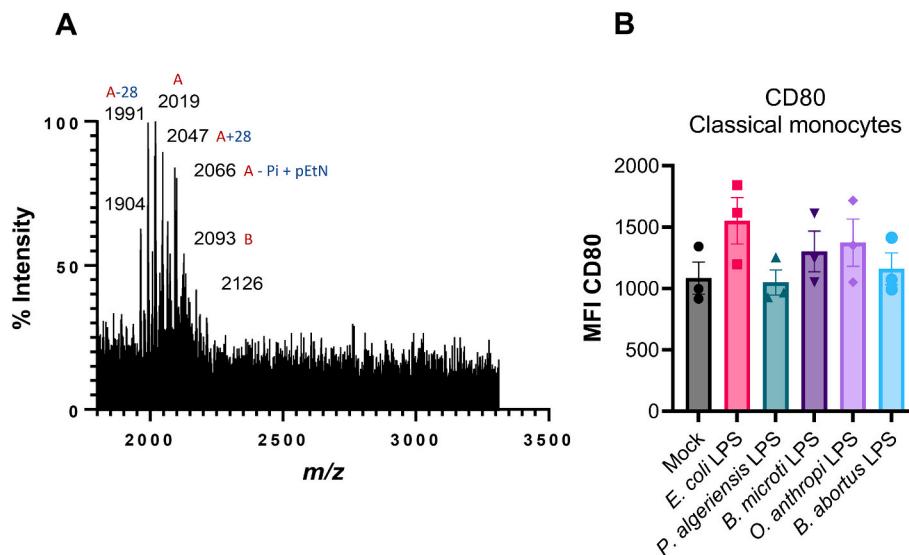
**Fig. 7.** The O-chain structure of *P. algeriensis* S-LPS differs from those of *O. anthropi*, and the classical *Brucella*. (A) *P. algeriensis*, unlike *P. saccharolyticum* and *P. asaccharolyticum* type strains, presents the genomic capacity of producing a rhamnose-based O-polysaccharide LPS. The *wbk* operon search was carried out based on the RASTtk annotation results using the PATRIC genome browser visualization tool. *B. inopinata* BO2 data were obtained from Soler-Lloréns et al. (Soler-Lloréns et al., 2016). (B) SDS-PAGE electrophoresis and silver staining of proteinase K-extracted LPS of *B. abortus* and *P. algeriensis* (B1). Western-blot analysis of LPS extracts with the serum of a rabbit immunized with whole cells of *P. algeriensis* (B2), *Yersinia enterocolitica* O:9 (B3) or *B. melitensis* (B4).

parasitic associations with vertebrates (Whatmore and Foster, 2021). *Pseudochrobactrum* bacteria are phylogenetically close to the facultative intracellular pathogen *Brucella* that have been isolated from soil, bovine lymph nodes, and a human knee aspirate (Kämpfer et al., 2009; Kämpfer et al., 2007; Kämpfer et al., 2006; Loperena-Barber et al., 2022; Pereira et al., 2023), some of which have been studied for biotechnological purposes (Long et al., 2019; Long et al., 2013; Mao et al., 2015; Mengke-Li et al., 2019; Siddique et al., 2021; Yusuf et al., 2020). However, only a *P. saccharolyticum* strain isolated from the lymph node of a cow has been genomically characterized (Pereira et al., 2023), and there are no comparative genomic and phenotypic analysis with *Brucella* that could complement those performed before with *Ochrobactrum* for a better understanding of the adaptation to pathogenicity of bacteria lacking

classical virulence factors (Aujoulat et al., 2014; Barquero-Calvo et al., 2009; Velasco et al., 1998; Alice R. Wattam et al., 2014). In this work, we took advantage of the recent isolation of *P. algeriensis* from cows (Loperena-Barber et al., 2022) to carry out these kind of studies.

One of the most significant features of *Brucella* organisms is the smaller genome size (3.1–3.4 Mbp) as compared to their closest *Ochrobactrum* relatives (4.7–8.3 Mbp), a trait in all likelihood reflects their contrasting lifestyles. The large and open genomes of free-living  $\alpha$ -*Proteobacteria* offer high genetic flexibility and exchange possibilities for a rapid adaptation to the quickly shifting and diverse niches of soil (Batut et al., 2004). On the other hand, intracellular  $\alpha$ -*Proteobacteria* usually possess closed smaller genomes (diCenzo and Finan, 2017; Eberhard, 1990; Moreno, 1998; Moreno et al., 2022). Some of these differences in





**Fig. 8.** *P. algeriensis* displays lipid A modifications consistent with a poor stimulation of PBMC. (A) Negative ion MALDI-TOF mass spectrometry spectra of purified *P. algeriensis* lipid A shows the presence of high molecular weight fatty acids, consistently with genomic findings. Data represent the mass-to-charge ( $m/z$ ) ratios; the two predominant clusters of ions around  $m/z$  2019 (species A) and 2093 (species B) are detailed. (B) Classical monocytes ( $CD14^+CD16^-$ ) were stimulated with LPS from *E. coli* (0.1  $\mu\text{g/mL}$ ), *B. abortus* (10  $\mu\text{g/mL}$ ), *O. anthropi* (10  $\mu\text{g/mL}$ ) and *P. algeriensis* (10  $\mu\text{g/mL}$ ) for 24 h and monocyte activation marker CD80 was evaluated by flow cytometry (mean fluorescence intensity, MFI). Statistical comparisons were made by parametric one-way ANOVA and Dunnett's multiple comparison post-hoc test. All values are expressed as mean  $\pm$  standard deviation (S.D.). Differences between values were considered significant at  $p < 0.05$  (\*).

genome size are associated with a variable number of plasmids in *Ochrobactrum* spp. (Teyssier et al., 2005), while in *Brucella* absence of plasmids is a constant trait (Moreno et al., 2022; Rouli et al., 2015). In the case of *Pseudochrobactrum*, the few assembled genomes available at the National Center for Biotechnology Information show intermediate-size genomes (3.47–4.64 Mbp) compared to *Brucella* and *Ochrobactrum*, and they contain just one chromosome and a variable number of plasmids (from none to four).

Most *Pseudochrobactrum* strains appear to be mainly environmental inhabitants (Kämpfer et al., 2009; Kämpfer et al., 2007; Kämpfer et al., 2006) and we cannot discard this origin for the bovine *Pseudochrobactrum* isolates. Although repeatedly isolated from the inimical environment within cattle lymph nodes (Ashford et al., 2020; Loperena-Barber et al., 2022; Pereira et al., 2023), this could be the result of transient bacteremia. Supporting the hypothesis that they are environmental bacteria, *P. algeriensis* was not able to effectively infect BALB/c mice and similar results were obtained in *G. mellonella* or bovine macrophages. Although none of these models fully replicate the natural host conditions, our results strongly suggest that *P. algeriensis* has a lifestyle like that *Ochrobactrum* (Moreno et al., 2023; Moreno et al., 2022), lacking the adaption to the intracellular life of *Brucella*. Nevertheless, neutrophils have been proposed to act as “Trojan horses” for *Brucella* entrance and dispersion (Gutiérrez-Jiménez et al., 2019), and further analyses using these cells could reveal if this ability is an ancestral trait of *Brucellaceae*, an investigation in which *P. algeriensis* could offer light.

Intracellular survival and replication of *Brucella* depend on the *virB* operon and require the translocation of protein effectors (Celli, 2019; Lacerda et al., 2013; Sieira et al., 2000). The *P. algeriensis* chromosome presents a region coding for a Trb-like T4SS, and a VirB-like T4SS in a plasmid. Since *P. algeriensis* lacks the capacity to replicate efficiently in laboratory models, these two regions are likely involved in DNA transfer as it occurs in *Ochrobactrum* where this system is proposed to be dedicated to plasmid transfer (de los Giusti et al., 2012; Wattam et al., 2014; Gohil et al., 2020; Yagel et al., 2020). In support of this hypothesis, the genomic conformation of the *virB* operon was closer to that in *Ochrobactrum* than that in *Brucella*.

Another critical aspect for intracellular pathogens is the ability to adapt the metabolism to the conditions inside host cells (Barbier et al.,

2011; Brown et al., 2008; Lamontagne et al., 2009; Viadas et al., 2010; Zúñiga-Ripa et al., 2014). Erythritol is a preferential carbon source for *Brucella* strains and is involved in *Brucella* genital tropism in animals (Barbier et al., 2017). The fact that, contrary to *Brucella* and *Ochrobactrum*, *P. algeriensis* was not able to use this pathway opens the question about the origin of the *ery* operon in *Brucellaceae*. In addition, *P. algeriensis* lacks the *nar*, *nir*, and *nor* operons, involved in nitrate, nitrite, and nitric oxide reduction, reactions that have been suggested to be important for *Brucella* persistence in the oxygen-limited environment of host tissues and/or the ability to cope with the reactive nitrogen species (Loisel-Meyer et al., 2006). These results are consistent with the lack of intracellular multiplication of *P. algeriensis*.

It has been proposed that *Brucella* have peculiar envelope structural features that represent a first barrier towards innate immunity recognition (Barquero-Calvo et al., 2007; Lapaque et al., 2006a; Velasco et al., 2000). The altered PAMP of *Brucella* lipid A highly contributes to their stealthy strategy and virulence (Barquero-Calvo et al., 2007; Conde-Alvarez et al., 2012; Lapaque et al., 2006a), and it has been hypothesized that the ancestors of *Brucella* carried envelope molecules that, without extensive remodeling, resulted in features adequate to evade innate immunity (Barquero-Calvo et al., 2009; Barquero-Calvo et al., 2007; Moriyón and Berman, 1982; Moriyón and López-Goñi, 1998). While the O-chain of *P. algeriensis* differs and may be akin to that of some early diverging brucellae, our analyses showed that, like *Brucella* and *Ochrobactrum* (Barquero-Calvo et al., 2009; Velasco et al., 1998), *P. algeriensis* conserved the modified lipid A (diaminoglucose backbone and unusual VLCFA) that hardly activates monocytes. Another molecule that seems at least partially conserved along the *Brucellaceae* is the core oligosaccharide, since *P. algeriensis* shares with *Brucella* part of the synthesis genes, and their core regions cross-react by Western Blot (data not shown).

The evolution of the cell envelope towards pathogenicity could have involved lipids. *P. algeriensis* presented a wider variety of lipids than *Brucella*, most of them aminolipids, which could explain at least partially the extreme resistance of *P. algeriensis* to the bactericidal peptide surrogate polymyxin B. It has been postulated that *Brucella* takes advantage of PC to gain eukaryotic-like features and to display structural and molecular mimicry at the membrane level (Comerci et al., 2006; Conde-

Álvarez et al., 2006), and it has been demonstrated that PC-rich membranes are more resistant to bactericidal peptides (Willumeit et al., 2005). *P. algeriensis* have genome capability to produce PC through both the Pcs and the PmtA pathway. In some bacteria, the anionic glycerophospholipids PG and CL are decorated with aminoacyl residues to lower the negative charge of their cellular envelope and increase resistance to some antibacterial agents (Jones and Smith, 1979; Roy and Ibbá, 2009). Based on migration pattern and genomic content, some of the ULs found in *P. algeriensis* could correspond to decorated PG. Thus far this kind of compounds have not been identified in the *Brucella* species investigated, indicating that more research is necessary on the cell envelope lipids of *Brucellaceae* to clarify their role in the different environments in which these bacteria thrive. Since these results might contribute to the current understanding of *Brucellaceae* resistance to cationic peptides, work is in progress to elucidate the identity of aminolipids of the *P. algeriensis* lipid extract by mass spectrometry using untargeted and targeted lipidomics.

In summary, this research shows that *P. algeriensis* bear both similarities and differences with the key metabolic pathways and VirB system present in *Brucella*. *P. algeriensis* also bears cell envelope traits (lipid A and free lipids) that represent a first scaffold in the evolution towards intracellular parasitism, thus accounting for at least a transient colonization of vertebrate lymph nodes, and perhaps potential opportunistic pathogenicity. In *Pseudochrobactrum* and *Ochrobactrum* species it seems that these traits are primarily adaptations to some of the diverse environmental challenges of soil. Thus, the homologous traits of *Brucella* represent an outstanding example of exaptive evolution in the microbial world, since originally evolved for extracellular survival in soil were later adapted to hamper innate immunity of vertebrates.

## 4. Material and methods

### 4.1. Comparative genomics

We retrieved 23 genomic assemblies from *Brucellaceae* including: 10 *Brucella*, 5 *Ochrobactrum*, 1 *Paenochrobactrum*, 2 *Falschrobactrum* and 5 *Pseudochrobactrum* from public repositories (Supplemental material Table A.6). We annotated all complete and incomplete assemblies with the software RASTtk (Brettin et al., 2015), to identify the coding sequences. After the genome annotation we visualized operon searches with the tool Genome Browser available in The PATRIC Bioinformatics Resource Center (<https://www.bv-brc.org>) (Davis et al., 2019). Once we annotated the coding sequences, we identified the homologous genes between genera and the unique genes of each group, using Proteome Comparison and Protein Family Sorter (available in Davis et al., 2019, 09/2021). To compare specific biological processes between taxa we used Comprehensive Genome Analysis (available in Davis et al., 2019, 09/2021). Additionally, we validated the presence/absence of relevant proteins using discontinuous megablast and tblastn of BLAST v2.13.0+ (Altschul et al., 1990) with the following thresholds: alignment identity >65%, and E-values <1·10<sup>5</sup>.

### 4.2. Bacterial strains and growth conditions

The bacterial strains used in this work are listed in Table A.7. All bacteria were grown either on tryptic soy agar plates (European Bacteriological Agar, Condalab), in tryptic soy broth (TSB, Scharlau) or BAB2 (Blood Agar Base N° 2, OXOID) at 37 °C. Where indicated, growth media were supplemented with kanamycin (50 µg/mL) or polymyxin (2 µg/mL). All strains were stored in skim milk at -80 °C. Work with *Brucella* was performed at the Biosafety Level 3 (BSL-3) laboratory facilities of the University of Navarra and “Centro de Investigación Médica Aplicada” (CIMA). For the construction of *P. algeriensis* strains expressing GFP (Green Fluorescent Protein), the *E. coli* S17λpir strain bearing the pBBR1MCS-2 GFP plasmid (Celli et al., 2005), resistant to kanamycin, was introduced by conjugation. The selection of clones containing the

plasmid was done on TSA polymyxin (2 µg/mL)-kanamycin (50 µg/mL). BD GasPak™ EZ container systems were used for generating micro-aerophilic conditions.

### 4.3. Growth curves

Growth rate studies were performed as previously described (Zúñiga-Ripa et al., 2018). Briefly, cells were inoculated into 10 mL of TSB in a 50-mL flask and incubated at 37 °C with orbital agitation for 18 h. The number of bacteria was determined by optical density (O.D.) at 600 nm in a spectrophotometer. Bacteria were harvested by centrifugation at 15,700 xg for 5 min and resuspended in 10 mL of the different studied media at an O.D. 600 nm of 0.1. Starting with this O.D., cells were cultivated in a Bioscreen C (Lab Systems) with continuous shaking in a working volume of 200 µL/well. Temperature was controlled at 37 °C and O.D. at 420–580 nm. Absorbance values of cell suspensions were automatically read at regular intervals of 0.5 h. All experiments were performed in triplicate. Tubes containing only the culture medium were used as sterility control. The media used for the different growth curves were Gerhardt's defined medium (glutamate [1.5 g/L], lactate [5 g/L], glycerol [30 g/L]) (Gerhardt, 1958) and the vitamin-salt mixture described by Plommet (Plommet, 1991), modified as described by Barbier et al. (Zúñiga-Ripa et al., 2014), and supplemented with filter-sterilized erythritol (2 g/L), glucose (2 g/L) ectoine (20 mM) or L-rhamnose (1 g/L). BAB2 was used as rich medium.

### 4.4. Virulence in mice

Eight-week-old female BALB/c mice (ENVIGO, Harlan) were lodged in cages with water and food *ad libitum* for 6 weeks. The animal handling and procedures were in accordance with the current European (directive 86/609/EEC) and Spanish (RD 53/2013) legislations, supervised by the corresponding Ethical and Animal Welfare Committee of the Institution and authorized by “Gobierno de Navarra” (Protocol number R077–20). Groups of 5 mice each were inoculated with *Brucella abortus* 2308 (10<sup>5</sup> colony forming units, CFU), *Ochrobactrum anthropi* 3331 (10<sup>8</sup> CFU) and *Pseudochrobactrum algeriensis* (10<sup>5</sup> - 10<sup>9</sup> CFU). Inocula were prepared in sterile PBS and each mouse was administered intraperitoneally with the corresponding dose in 0.1 mL. To assess the exact dose retrospectively, dilutions of each inoculum were plated by triplicate on TSA plates. Spleen CFUs in infected mice were counted at 1 and 6 weeks after inoculation. The spleens were weighed and homogenized in 9 volumes of PBS and serial ten-fold dilutions were plated by triplicate on TSA plates. The data were normalized by logarithmic transformation and the mean log CFU/spleen values and the standard deviations were calculated.

### 4.5. Virulence in *Galleria mellonella* model

*G. mellonella* larvae TruLarv™ were obtained from BioSystems Technology (Exeter, United Kingdom) and kept at 15 °C in dark and starvation prior use. For the experiments, larvae with homogeneous parameters of length (2–3 cm) and weight (180–250 mg) were selected. Bacterial inocula were prepared from bacteria grown on TSA or BAB2 plates in sterile PBS (0.14 M NaCl, 0.0027 M KCl and 0.010 M Phosphate buffer pH 7.4) (Medicago). Bacterial concentrations were then adjusted by a visible light spectrophotometer (Genesis 20, Thermo Scientific) to an optical density at an O.D. 600 nm of 0.25, equivalent to approximately 10<sup>9</sup> CFU/mL. Finally, proper serial 10-fold dilutions were obtained and the exact doses were retrospectively assessed by plating countable dilutions. Larvae inoculation was performed as follows. The Hamilton syringe (25 µL Microliter Syringe Model 802 N, Cemented Needle, 22 s gauge; Hamilton) was disinfected by taking and ejecting 70% ethanol several times and cleaned with sterile PBS. Larvae were injected with 10 µL of inoculum, at the level of the posterior second proleg. Once injected, each 10 larvae experimental group was placed in a

Petri dish containing a circular filter paper and incubated at 37 °C in the dark. Larva survival was monitored every day by the observation of no movement of the larvae in response to being rolled over.

#### 4.6. Cell infections and fluorescence processing

The BoMac cell line, obtained from bovine peritoneal macrophages immortalized with a plasmid containing the SV40 genomic DNA (Stabel and Stabel, 1995), was used for the *in vitro* infection experiments. Cells were cultured in IMDM (Iscove's Modified Dulbecco's Medium) GlutaMAX™ Supplement (Gibco, 31,980–022) + 10% FBS + MEM NEAA 1× (Minimum Essential Medium, MEM, Non-Essential Amino Acids Solution [100×]; Gibco, 11,120–037) + MEM Vitamins 1× (MEM Vitamin Solution [100×]; Gibco, 11,120–037) + Pen Strep 1× + 2-Mercaptoethanol 50 μM (2-Mercaptoethanol [50 mM]; Gibco, 31,350–010). The cell line was stored at –146 °C in IMDM +10% FBS + 10% Dimethyl sulfoxide (DMSO) and negative-tested for *Mycoplasma* prior to each experimental assay employing the LooKOut® *Mycoplasma* PCR Detection Kit (Sigma, MP0035). Cells were maintained at 37 °C with 5% CO<sub>2</sub> atmosphere for one week prior to infection. They were seeded in 24 well-plates containing coverslips at  $2 \times 10^4$  cells/well and infected 24 h later with a multiplicity of infection (MOI) of 100. Centrifugation was carried out at 400 g for 10 min at 4 °C, and then cells were incubated for 30 min at 37 °C with 5% CO<sub>2</sub>. After removing extracellular bacteria by washing with fresh medium, cells were incubated for 1 h with medium supplemented with 500 μg/mL gentamicin for *P. algeriensis* and 100 μg/mL for *B. abortus* strains, for removing remaining extracellular bacteria. Then, gentamicin concentrations were decreased to 100 μg/mL and 25 μg/mL, respectively, for maintenance.

For CFU-counts, cells were washed three times with DPBS and incubated with 0.1% Triton X100 in DPBS for 5 min at room temperature. After the detergent treatment, the lysates were collected, 10-fold diluted in PBS and plated on BAB2 or TSA, and incubated at 37 °C for approximately 4–5 days to determine the number of intracellular bacteria. All experiments were performed in biological triplicate. In addition, after 2, 24 or 48 h cells were washed three times with Dulbecco's Phosphate Buffered Saline (DPBS) and fixed for 30 min with paraformaldehyde (Merck) 3.7% in 0.2 M N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) at pH 7.4 at room temperature and washed again three times with DPBS. Then, cells were permeabilized with 0.1% Triton X-100 (Sigma) in DPBS for 5 min. After washing with DPBS the coverslips were incubated with tetramethylrhodamine (TRITC)-phalloidin (1 μg/mL in PBS) for 30 min at room temperature and washed three times with DPBS. Finally, coverslips were mounted on microscope slides using a DAPI-based mounting solution (Abbott Molecular). Fluorescence imaging was carried out by a LED epifluorescence Axiolab 5 microscope (Carl Zeiss, 430,037–9021-000) and the filters for 4',6-diamidino-2-phenylindole (DAPI), GFP and TRITC. Images were processed with the program Zen 3.5 (blue edition) (Carl Zeiss Microscopy GmbH, 2011).

#### 4.7. Sensitivity to polymyxin B and other antibiotics

The minimal inhibitory concentration (MIC) of polymyxin B was determined in cation-adjusted Mueller-Hinton agar (BioMérieux) using Sahm's method (Sahm and Washington, 1991). Exponentially growing bacteria were adjusted to an O.D. equivalent to 1 of the McFarland scale, and exposed to a gradient of polymyxin B concentrations on an E-test strip (BioMérieux). The MIC was determined after 3 days of incubation at 37 °C. The other antimicrobial susceptibility tests were performed using the VITEK 2 compact system (BioMérieux, USA) according to the manufacturer's instructions (BioMérieux, USA).

#### 4.8. Polar lipid extraction and analysis

Free lipids were extracted as described by Bligh and Dyer (Bligh and

Dyer, 1959) and analyzed on silica gel 60 high-performance thin layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany). The samples were applied to the plate and chromatography was performed bidimensionally with chloroform-methanol-water (14:6:1) first and chloroform-methanol-acetic acid (13:5:2) for the second dimension. Plates were developed with ninhydrin in acetone (2 g/L) and warm air for aminolipid detection, or with copper (II) sulfate at 120 °C for the identification of total lipids.

#### 4.9. LPS extraction

LPS was extracted by the proteinase-K sodium dodecyl sulphate (SDS) protocol (Dubray and Limet, 1987; Garin-Bastuji et al., 1990) with some modifications for WB-. Bacteria grown overnight in 10 mL of TSB were killed with 0.5% phenol during 3 days in agitation at 37 °C. After that, samples were weighed and pipetted into small polycarbonate cap tubes and then suspended by ultrasounds in 2% SDS-60 mM Tris-HCl buffer (pH 6.8) at a concentration of 0.5 g (wet weight) of bacteria per 10 mL of buffer. Samples were then heated at 100 °C for 10 min and lysates were cooled to 55 °C. This treatment was followed by digestion with 60 μL of proteinase-K at 2.5 mg/mL in HCl-Tris per mL of sample (Merck KGaA) for 3 h at 55 °C and overnight incubation at 20 °C. Afterwards, they were centrifuged at 20,000 xg for 30 min at room temperature, and the LPS was precipitated from the supernatant by addition of 3 volumes of methanol containing 1% sodium acetate-saturated methanol at –20 °C. After 12 h, the precipitate was harvested by centrifugation at 5000 xg for 15 min at 4 °C and resuspended by sonication in 10 mL of distilled water. After a second methanol precipitation and centrifugation, the pellets were resuspended by sonication in 2–3 mL of 60 mM HCl-Tris (pH 6.8) and left at 37 °C. Then samples were treated with 20 μg/mL of RNase and DNase stock solutions at 0.5 mg/mL in HCl-Tris (MP Biomedicals and Sigma-Aldrich, respectively) at 37 °C for 30 min. Subsequently, the LPS was treated again with 5 μL/mL of proteinase K at 2.5 mg/mL in HCl-Tris, at 55 °C for 3 h and then, at room temperature overnight. After a third methanol precipitation in the same conditions described above, the pellet containing LPS was recovered in 1 mL of distilled water and frozen at –20 °C.

In addition, purified LPS for PBMC stimulation was obtained by the method of with modifications. (Leong et al., 1970; Velasco et al., 2000). When applying this protocol to classical *Brucella* spp., the S-LPS is obtained in the phenol phase, due to the solubility properties of the N-formyl-perosamine O-chain (Caroff et al., 1984; Perry and Bundle, 1990). Remarkably, the LPS of *P. algeriensis* mainly partitions to the aqueous phase when performing phenol-water extraction (Fig. A.3). Noticeably, the LPS of the ancestral BO2 strain partitions almost equally into the water and the phenol (Wattam et al., 2012) and the rhamnose containing LPS of *Rhizobium* NGR234 partitions in the aqueous phase (Broughton et al., 2006). In the case of *P. algeriensis* it was evidenced by the KDO content of the aqueous and phenol phases (*P. algeriensis* aqueous phase 1.7%; *P. algeriensis* phenol phase 0.004%). Kdo was determined colorimetrically by the thiobarbituric acid method using pure Kdo and deoxyribose as the standards, with the modifications described previously (Diaz-Aparicio et al., 1993). Moreover, SDS-PAGE and WB (Fig. A.4) analyses with the anti-*P. algeriensis* serum confirmed that the S-LPS is enriched in the aqueous phase. This result also supports the different nature of *P. algeriensis* and classical *Brucella* spp. LPS O-chain.

#### 4.10. Lipid A extraction and mass spectrometry

Lipid A was extracted using the ammonium hydroxide/isobutyric acid method (El Hamidi et al., 2005). Negative ion MALDI-TOF mass spectrometry analysis (Bruker Daltonics) of the samples was undertaken using an equal volume of dihydroxybenzoic acid matrix (Sigma-Aldrich) dissolved in (1:2) acetonitrile-0.1% trifluoroacetic acid.



#### 4.11. Peripheral blood mononuclear cell stimulation

Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by centrifugation over Ficoll-Hypaque. Cells were suspended at  $10^6$  / mL in flat-bottom 48-well plates with RPMI 1640 (Roswell Park Memorial Institute 1640 Medium; Gibco, 21,875–034) supplemented with L-Glutamine (2 mM, Sigma) and 10% FBS. PBMC were then stimulated with *E. coli* LPS (0.1 µg/mL), *P. algeriensis* LPS (10 µg/mL), *O. anthropi* LPS (10 µg/mL) or *B. abortus* LPS (10 µg/mL) for 24 h and monocyte activation markers were evaluated by flow cytometry. Briefly, cells were blocked in Fc-block solution for 15 min and then stained for surface expression with fluorophore-marked antibodies against CD80, PD-L1, SLAMF7 and SLAMF8 on classical monocytes (CD14<sup>+</sup>CD16<sup>-</sup>), non-classical monocytes (CD14<sup>+</sup>CD16<sup>+</sup>) and intermediate monocytes (CD14<sup>low</sup>CD16<sup>+</sup>). Viability was corroborated by staining with Live/Dead (ThermoFisher). Negative control samples were incubated with irrelevant isotype-matched monoclonal antibodies. Samples were analyzed on a BD LSRFortessa™ X-20 Cell Analyzer cytometer (BD, San Jose, CA, USA).

#### 4.12. Antibodies for immunological tests and anti-*P. algeriensis* serum obtention

The following polyclonal sera were used in this work: anti-*B. melitensis* (from rabbits infected with *B. melitensis* 16 M whole cells  $10^9$  CFU, bled at day 45, and diluted 1:500; (Aragón et al., 1996), anti-*Y. enterocolitica* O:9 (from rabbits immunized with *Y. enterocolitica* O:9, diluted 1:500; (Díaz et al., 1984), and anti-*P. algeriensis*. To obtain the serum reacting with *P. algeriensis*, rabbits were inoculated intravenously with 1 mL of 1 mg/mL of phenol-inactivated whole cells. Then, the animals were bled 4 weeks later. The rabbits (2.5 Kg New-Zealand female rabbits; San Bernardo, Spain) were kept in cages with water and food *ad libitum* in the animal facilities of CIFA (“Centro de Investigación en Farmacobiología Aplicada”; University of Navarra). Rabbits were handled, bled and sacrificed according to the current European (directive 86/609/EEC) and Spanish (RD 53/2013) legislations, supervised by the corresponding Ethical and Animal Welfare Committee of the Institution and authorized by “Gobierno de Navarra” (Protocol number R113–19).

#### 4.13. SDS-PAGE and Western blots

Samples were analyzed in Tris-HCl-glycine 12 or 15% polyacrylamide gels. 15 µL of each sample were run at 30 mA constant current for 140 min. Finally, LPS molecules were revealed by the periodate-alkaline silver method and whole cells with Coomassie Blue staining (Tsai and Frasch, 1982). For Western blot, gels were electrotransferred onto nitrocellulose sheets (0.2 µm pore size) in a transfer buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 20% (vol/vol) methanol. Transfer was performed at a constant voltage of 8 V and 200 mA for 30 min in a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). After transference, membranes were blocked overnight with 3% skim milk in PBS containing 0.05% Tween 20, and next washed with the same buffer. They were then incubated overnight at 4 °C with immune sera in the same solution. Membranes were washed again as described above and incubated for 4 h with peroxidase-conjugated G-protein. Bound immunoglobulins were detected using 4-chloro-1-naphthol.

#### 4.14. Statistical analysis

Statistical analyses were done with the GraphPad Prism (version 8) software. Kaplan Meyer survival curves significance was analyzed by the Log-rank Mantel-Cox test. All values are expressed as mean ± standard deviation (S.D.). Statistical comparisons were made by non-parametric Kruskal-Wallis test or by parametric unpaired Student's t-test or one-way ANOVA and Dunnett's multiple comparison post-hoc test. All

values are expressed as mean ± S.D. Differences between values were considered significant at  $p < 0.05$  (\*),  $p < 0.005$  (\*\*) and  $p < 0.0005$  (\*\*\*)

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#### Author contributions

ML-B, IM and RC-Á conceived the study and wrote the manuscript. ML-B, AE-B, MS-B, PR-R, JMP, CR-M, RL, AZ-R, MI, JA B, MC, J-PG participated in the experimental work. All authors participated in the discussion of the results and the revision of the manuscript. All authors read and approved the final version of the manuscript.

#### CRedit authorship contribution statement

**Maite Loperena-Barber:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aitor Elizalde-Bielsa:** Writing – review & editing, Methodology, Investigation. **Miriam Salvador-Bescós:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Paula Ruiz-Rodríguez:** Writing – review & editing, Methodology, Investigation, Data curation. **Joaquín Miguel Pellegrini:** Writing – review & editing, Methodology, Investigation. **Chantal Renau-Mínguez:** Writing – review & editing, Methodology, Investigation. **Rebecca Lancaster:** Writing – review & editing, Methodology, Investigation. **Amaia Zúñiga-Ripa:** Writing – review & editing, Methodology, Investigation. **Maite Iriarte:** Writing – review & editing, Investigation. **Jose A. Bengochea:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition. **Mireia Coscollá:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition. **Jean-Pierre Gorvel:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition. **Ignacio Moriyón:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Raquel Conde-Álvarez:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

#### Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

#### Data availability

Data will be made available on request.

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## References

- Al Dahouk, S., Köhler, S., Occhialini, A., Jiménez de Bagüés, M.P., Hammerl, J.A., Eisenberg, T., Vergnaud, G., Cloeckaert, A., Zygmunt, M.S., Whatmore, A.M., Melzer, F., Drees, K.P., Foster, J.T., Wattam, A.R., Scholz, H.C., 2017. *Brucella* spp. of amphibians comprise genomically diverse motile strains competent for replication in macrophages and survival in mammalian hosts. *Sci. Rep.* 7, 44420. <https://doi.org/10.1038/srep44420>.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
- Aragón, V., Díaz, R., Moreno, E., Moriyón, I., 1996. Characterization of *Brucella abortus* and *Brucella melitensis* native haptenes as outer membrane O-type polysaccharides independent from the smooth lipopolysaccharide. *J. Bacteriol.* 178, 1070–1079. <https://doi.org/10.1128/jb.178.4.1070-1079.1996>.
- Aragón-Andara, B., Palacios-Chaves, L., Salvador-Bescós, M., de Miguel, M.J., Muñoz, P. M., Vences-Guzmán, M.A., Zúñiga-Ripa, A., Lázaro-Antón, L., Sohlenkamp, C., Moriyón, I., Iriarte, M., Conde-Álvarez, R., 2021. The phospholipid N-methyltransferase and phosphatidylcholine synthase pathways and the ChoXWV choline uptake system involved in phosphatidylcholine synthesis are widely conserved in most, but not all *Brucella* species. *Front. Microbiol.* 12 <https://doi.org/10.3389/fmicb.2021.614243>.
- Ashford, R.T., Muchowski, J., Koylass, M., Scholz, H.C., Whatmore, A.M., 2020. Application of whole genome sequencing and pan-family multi-locus sequence analysis to characterize relationships within the family *Brucellaceae*. *Front. Microbiol.* 11 <https://doi.org/10.3389/fmicb.2020.01329>.
- Aujoulat, F., Romano-Bertrand, S., Masnou, A., Marchandin, H., Jumas-Bilak, E., 2014. Niches, population structure and genome reduction in *Ochrobactrum intermedium*: clues to technology-driven emergence of pathogens. *PLoS One* 9, e83376. <https://doi.org/10.1371/journal.pone.0083376>.
- Barbier, T., Nicolas, C., Letesson, J.J., 2011. *Brucella* adaptation and survival at the crossroad of metabolism and virulence. *FEBS Lett.* 585, 2929–2934. <https://doi.org/10.1016/j.febslet.2011.08.011>.
- Barbier, T., Collard, F., Zúñiga-Ripa, A., Moriyón, I., Godard, T., Becker, J., Wittmann, C., Van Schaftingen, E., Letesson, J.-J., 2014. Erythritol feeds the pentose phosphate pathway via three new isomerases leading to D-erythrose-4-phosphate in *Brucella*. *Proc. Natl. Acad. Sci.* 111, 17815–17820. <https://doi.org/10.1073/pnas.1414622111>.
- Barbier, T., Machelart, A., Zúñiga-Ripa, A., Plovier, H., Hougard, C., Lobet, E., Willems, K., Muraille, E., De Bolle, X., Van Schaftingen, E., Moriyón, I., Letesson, J.-J., 2017. Erythritol availability in bovine, murine and human models highlights a potential role for the host aldose reductase during *Brucella* infection. *Front. Microbiol.* 8 <https://doi.org/10.3389/fmicb.2017.01088>.
- Barbier, T., Zúñiga-Ripa, A., Moussa, S., Plovier, H., Sternon, J.F., Lázaro-Antón, L., Conde-Álvarez, R., De Bolle, X., Iriarte, M., Moriyón, I., Letesson, J.J., 2018. *Brucella* central carbon metabolism: an update. *Crit. Rev. Microbiol.* 44, 182–211. <https://doi.org/10.1080/1040841X.2017.1332002>.
- Barnoy, S., Gancz, H., Zhu, Y., Honnold, C.L., Zurawski, D.V., Venkatesan, M.M., 2017. The *Galleria mellonella* larvae as an in vivo model for evaluation of *Shigella* virulence. *Gut Microbes* 8, 335–350. <https://doi.org/10.1080/19490976.2017.1293225>.
- Barquero-Calvo, E., Chaves-Olarte, E., Weiss, D.S., Guzmán-Verri, C., Chacón-Díaz, C., Rucavado, A., Moriyón, I., Moreno, E., 2007. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. *PLoS One* 2, e631. <https://doi.org/10.1371/journal.pone.0000631>.
- Barquero-Calvo, E., Conde-Álvarez, R., Chacón-Díaz, C., Quesada-Lobo, L., Martirosyan, A., Guzmán-Verri, C., Iriarte, M., Mancek-Keber, M., Jerala, R., Gorvel, J.P., Moriyón, I., Moreno, E., Chaves-Olarte, E., 2009. The differential interaction of *Brucella* and *Ochrobactrum* with innate immunity reveals traits related to the evolution of stealthy pathogens. *PLoS One* 4, e5893. <https://doi.org/10.1371/journal.pone.0005893>.
- Basu, S.S., Karbar, M.J., Raetz, C.R.H., 2002. Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in *Rhizobium leguminosarum*. *J. Biol. Chem.* 277, 28959–28971. <https://doi.org/10.1074/jbc.M204525200>.
- Batut, J., Andersson, S.G.E., O'Callaghan, D., 2004. The evolution of chronic infection strategies in the  $\alpha$ -*Proteobacteria*. *Nat. Rev. Microbiol.* 2, 933–945. <https://doi.org/10.1038/nrmicro1044>.
- Berg, G., Eberl, L., Hartmann, A., 2005. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ. Microbiol.* 7, 1673–1685. <https://doi.org/10.1111/j.1462-2920.2005.00891.x>.
- Berman, A.J., Del Priore, L.V., Fischer, C.K., 1997. Endogenous *Ochrobactrum anthropi* endophthalmitis. *Am. J. Ophthalmol.* 123, 560–562. [https://doi.org/10.1016/S0002-9394\(14\)70190-4](https://doi.org/10.1016/S0002-9394(14)70190-4).
- Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917. <https://doi.org/10.1139/o59-099>.
- Brettin, T., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Olsen, G.J., Olson, R., Overbeek, R., Parrello, B., Pusch, G.D., Shukla, M., Thomason, J.A., Stevens, R., Vonstein, V., Wattam, A.R., Xia, F., 2015. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep.* 5, 8365. <https://doi.org/10.1038/srep08365>.
- Broughton, W.J., Hanin, M., Relić, B., Kopcińska, J., Golinowski, W., Šimšek, S., Ojanen-Reuhs, T., Reuhs, B., Marie, C., Kobayashi, H., Bordogna, B., Le Quéré, A., Jabbouri, S., Fellay, R., Perret, X., Deakin, W.J., 2006. Flavonoid-inducible modifications to rhamnan O antigens are necessary for *Rhizobium* sp. strain NGR234-legume symbioses. *J. Bacteriol.* 188, 3654–3663. <https://doi.org/10.1128/JB.188.10.3654-3663.2006>.
- Brown, S.A., Palmer, K.L., Whiteley, M., 2008. Revisiting the host as a growth medium. *Nat. Rev. Microbiol.* 6, 657–666. <https://doi.org/10.1038/nrmicro1955>.
- Caroff, M., Bundle, D.R., Perry, M.B., 1984. Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O:9. *Eur. J. Biochem.* 139, 195–200. <https://doi.org/10.1111/j.1432-1033.1984.tb07994.x>.
- Celli, J., 2019. The intracellular life cycle of *Brucella* spp. *Microbiol Spectr* 7. <https://doi.org/10.1128/microbiolspec.BAI-0006-2019>.
- Celli, J., Salcedo, S.P., Gorvel, J.-P., 2005. *Brucella* coopts the small GTPase Sar1 for intracellular replication. *Proc. Natl. Acad. Sci.* 102, 1673–1678. <https://doi.org/10.1073/pnas.0406873102>.
- Comerci, D.J., Altabe, S., de Mendoza, D., Ugalde, R.A., 2006. *Brucella abortus* synthesizes phosphatidylcholine from choline provided by the host. *J. Bacteriol.* 188, 1929–1934. <https://doi.org/10.1128/JB.188.5.1929-1934.2006>.
- Conde-Álvarez, R., Grilló, M.J., Salcedo, S.P., de Miguel, M.J., Fugier, E., Gorvel, J.P., Moriyón, I., Iriarte, M., 2006. Synthesis of phosphatidylcholine, a typical eukaryotic phospholipid, is necessary for full virulence of the intracellular bacterial parasite *Brucella abortus*. *Cell. Microbiol.* 8, 1322–1335. <https://doi.org/10.1111/j.1462-5822.2006.00712.x>.
- Conde-Álvarez, R., Arce-Gorvel, V., Iriarte, M., Mancek-Keber, M., Barquero-Calvo, E., Palacios-Chaves, L., Chacón-Díaz, C., Chaves-Olarte, E., Martirosyan, A., von Bargen, K., Grilló, M.-J., Jerala, R., Brandenburg, K., Llobet, E., Bengochea, J.A., Moreno, E., Moriyón, I., Gorvel, J.-P., 2012. The lipopolysaccharide core of *Brucella abortus* acts as a shield against innate immunity recognition. *PLoS Pathog.* 8, e1002675 <https://doi.org/10.1371/journal.ppat.1002675>.
- Conde-Álvarez, R., Palacios-Chaves, L., Gil-Ramírez, Y., Salvador-Bescós, M., Bárcena-Varela, M., Aragón-Andara, B., Martínez-Gómez, E., Zúñiga-Ripa, A., de Miguel, M. J., Bartholomew, T.L., Hanniffy, S., Grilló, M.J., Vences-Guzmán, M.A., Bengochea, J.A., Arce-Gorvel, V., Gorvel, J.P., Moriyón, I., Iriarte, M., 2018. Identification of *lptA*, *lpxE*, and *lpxO*, three genes involved in the remodeling of *Brucella* cell envelope. *Front. Microbiol.* 8, 1–14. <https://doi.org/10.3389/fmicb.2017.02657>.
- Davis, J.J., Wattam, A.R., Aziz, R.K., Brettin, T., Butler, R., Butler, R.M., Chlenski, P., Conrad, N., Dickerman, A., Dietrich, E.M., Gabbard, J.L., Gerdes, S., Guard, A., Kenyon, R.W., Machi, D., Mao, C., Murphy-Olson, D., Nguyen, M., Nordberg, E.K., Olsen, G.J., Olson, R.D., Overbeek, J.C., Overbeek, R., Parrello, B., Pusch, G.D., Shukla, M., Thomas, C., VanOeffelen, M., Vonstein, V., Warren, A.S., Xia, F., Xie, D., Yoo, H., Stevens, R., 2019. The PATRIC bioinformatics resource center: expanding data and analysis capabilities. *Nucleic Acids Res.* <https://doi.org/10.1093/nar/gkz943>.
- de los Giusti, M.A., Pistorio, M., Lozano, M.J., Torres Tejerizo, G.A., Salas, M.E., Martini, M.C., López, J.L., Draghi, W.O., Del Papa, M.F., Pérez-Mendoza, D., Sanjuán, J., Lagares, A., 2012. Genetic and functional characterization of a yet-unclassified rhizobial Dtr (DNA-transfer-and-replication) region from a ubiquitous plasmid conjugal system present in *Sinorhizobium meliloti*, in *Sinorhizobium medicae*, and in other nonrhizobial gram-negative bacteria. *Plasmid* 67, 199–210. <https://doi.org/10.1016/j.plasmid.2011.12.010>.
- De Miguel, M.J., Marín, C.M., Muñoz, P.M., Dieste, L., Grilló, M.J., Blasco, J.M., 2011. Development of a selective culture medium for primary isolation of the main *Brucella* species. *J. Clin. Microbiol.* 49, 1458–1463. <https://doi.org/10.1128/JCM.02301-10>.
- Díaz, R., Toyos, J., Salvo, M.D., Fernandez-Lago, L., Alonso, B., Moriyón, I., Dorronsoro, I., 1984. Studies on the polysaccharide B and native hapten of *Brucella* and *Yersinia enterocolitica* serotype 9. *Dev. Biol. Stand.* 56, 213–220.
- Díaz-Aparicio, E., Aragón, V., Marín, C., Alonso, B., Font, M., Moreno, E., Perez-Ortiz, S., Blasco, J.M., Díaz, R., Moriyón, I., 1993. Comparative analysis of *Brucella* serotype a and M and *Yersinia enterocolitica* O:9 polysaccharides for serological diagnosis of brucellosis in cattle, sheep, and goats. *J. Clin. Microbiol.* 31, 3136–3141. <https://doi.org/10.1128/JCM.31.12.3136-3141.1993>.
- diCenzo, G.C., Finan, T.M., 2017. The divided bacterial genome: structure, function, and evolution. *Microbiol. Mol. Biol. Rev.* 81 <https://doi.org/10.1128/MMBR.00019-17>.
- Ding, H., Yip, C.B., Geddes, B.A., Oresnik, I.J., Hynes, M.F., 2012. Glycerol utilization by *Rhizobium leguminosarum* requires an ABC transporter and affects competition for nodulation. *Microbiology (N Y)* 158, 1369–1378. <https://doi.org/10.1099/mic.0.057281-0>.
- Dubray, G., Limet, J., 1987. Evidence of heterogeneity of lipopolysaccharides among *Brucella* biovars in relation to a and M specificities. *Ann. Inst. Pasteur Microbiol.* 1985 (138), 27–37. [https://doi.org/10.1016/0769-2609\(87\)90051-2](https://doi.org/10.1016/0769-2609(87)90051-2).
- Ducrotot, M., Bertu, W.J., Matope, G., Cadmus, S., Conde-Álvarez, R., Gusi, A.M., Welburn, S., Ocholi, R., Blasco, J.M., Moriyón, I., 2017. Brucellosis in sub-Saharan Africa: current challenges for management, diagnosis and control. *Acta Trop.* 165, 179–193. <https://doi.org/10.1016/j.actatropica.2015.10.023>.
- Eberhard, W.G., 1990. Evolution in bacterial plasmids and levels of selection. *Q. Rev. Biol.* 65, 3–22. <https://doi.org/10.1086/416582>.
- El Hamidi, A., Tirsoaga, A., Novikov, A., Hussein, A., Caroff, M., 2005. Microextraction of bacterial lipid: a easy and rapid method for mass spectrometric characterization. *J. Lipid Res.* 46, 1773–1778. <https://doi.org/10.1194/jlr.D500014-JLR200>.
- Elizalde-Bielsa, A., Aragón-Andara, B., Loperena-Barber, M., Salvador-Bescós, M., Moriyón, I., Zúñiga-Ripa, A., Conde-Álvarez, R., 2023. Development and evaluation of the *Galleria mellonella* (greater wax moth) infection model to study *Brucella* host-pathogen interaction. *Microb. Pathog.* 174, 105930 <https://doi.org/10.1016/j.micpath.2022.105930>.
- Foster, G., Osterman, B.S., Godfroid, J., Jacques, I., Cloeckaert, A., 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int. J. Syst. Evol. Microbiol.* 57, 2688–2693. <https://doi.org/10.1099/ijs.0.65269-0>.
- Freer, E., Moreno, E., Moriyón, I., Pizarro-Cerdá, J., Weintraub, A., Gorvel, J.P., 1996. *Brucella-Salmonella* lipopolysaccharide chimeras are less permeable to hydrophobic

- probes and more sensitive to cationic peptides and EDTA than are their native *Brucella* sp. counterparts. *J. Bacteriol.* 178, 5867–5876. <https://doi.org/10.1128/jb.178.20.5867-5876.1996>.
- Garin-Bastuji, B., Bowden, R.A., Dubray, G., Limet, J.N., 1990. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting analysis of smooth-lipopolysaccharide heterogeneity among *Brucella* biovars related to a and M specificities. *J. Clin. Microbiol.* 28, 2169–2174. <https://doi.org/10.1128/jcm.28.10.2169-2174.1990>.
- Gerhardt, P., 1958. The nutrition of *Brucellae*. *Bacteriol. Rev.* 22, 81–98. <https://doi.org/10.1128/br.22.2.81-98.1958>.
- Gill, M.V., Ly, H., Mueenuddin, M., Schoch, P.E., Cunha, B.A., 1997. Intravenous line infection due to *Ochrobactrum anthropi* (CDC Group Vd) in a normal host. *Heart Lung* 26, 335–336. [https://doi.org/10.1016/S0147-9563\(97\)90092-3](https://doi.org/10.1016/S0147-9563(97)90092-3).
- Gil-Ramírez, Y., Conde-Álvarez, R., Palacios-Chaves, L., Zúñiga-Ripa, A., Grilló, M.-J., Arce-Gorvel, V., Hanniffy, S., Moriyón, I., Iriarte, M., 2014. The identification of *wadB*, a new glycosyltransferase gene, confirms the branched structure and the role in virulence of the lipopolysaccharide core of *Brucella abortus*. *Microb. Pathog.* 73, 53–59. <https://doi.org/10.1016/j.micpath.2014.06.002>.
- Gohil, K., Rajput, V., Dhame, M., 2020. Pan-genomics of *Ochrobactrum* species from clinical and environmental origins reveals distinct populations and possible links. *Genomics* 112, 3003–3012. <https://doi.org/10.1016/j.ygeno.2020.04.030>.
- Goldman, R.C., Kohlbrenner, W.E., 1985. Molecular cloning of the structural gene coding for CTP: CMP-3-deoxy-manno-octulosonate cytidyltransferase from *Escherichia coli* K-12. *J. Bacteriol.* 163, 256–261. <https://doi.org/10.1128/jb.163.1.256-261.1985>.
- Gorvel, J.P., Moreno, E., 2002. *Brucella* intracellular life: from invasion to intracellular replication. *Vet. Microbiol.* 90, 281–297. [https://doi.org/10.1016/S0378-1135\(02\)00214-6](https://doi.org/10.1016/S0378-1135(02)00214-6).
- Greven, C.M., Nelson, K.C., 2001. Chronic postoperative endophthalmitis secondary to *Ochrobactrum anthropi*. *Retina* 21, 279–280. <https://doi.org/10.1097/00006982-200106000-00021>.
- Gutiérrez-Jiménez, C., Mora-Carrión, R., Altamirano-Silva, P., Chacón-Díaz, C., Chaves-Olarte, E., Moreno, E., Barquero-Calvo, E., 2019. Neutrophils as trojan horse vehicles for *Brucella abortus* macrophage infection. *Front. Immunol.* 10 <https://doi.org/10.3389/fimmu.2019.01012>.
- Harding, C.R., Schroeder, G.N., Collins, J.W., Frankel, G., 2013. Use of *Galleria mellonella* as a model organism to study *Legionella pneumophila* infection. *J. Vis. Exp.* <https://doi.org/10.3791/50964>.
- Holmes, B., Poppof, M., Kiredjian, M., Kersters, K., 1988. *Ochrobactrum anthropi* gen. Nov., sp. nov. from human clinical specimens and previously known as group Vd. *Int. J. Syst. Bacteriol.* 38, 406–416. <https://doi.org/10.1099/00207713-38-4-406>.
- Inoue, K., Numaga, J., Nagata, Y., Sakurai, M., Aso, N., Fujino, Y., 1999. *Ochrobactrum anthropi* endophthalmitis after vitreous surgery. *Br. J. Ophthalmol.* 83, 501b. <https://doi.org/10.1136/bjo.83.4.501b>.
- Insua, J.L., Llobet, E., Moranta, D., Pérez-Gutiérrez, C., Tomás, A., Garmendia, J., Bengochea, J.A., 2013. Modeling *Klebsiella pneumoniae* pathogenesis by infection of the wax moth *Galleria mellonella*. *Infect. Immun.* 81, 3552–3565. <https://doi.org/10.1128/IAI.00391-13>.
- Iriarte, M., González, D., Delrue, R.-M., Monreal, D., Conde, R., López-Goñi, I., 2004. *Brucella* lipopolysaccharide: structure, biosynthesis and genetics. In: *Brucella. Molecular and Cellular Biology*, pp. 159–192.
- Jones, D.E., Smith, J.D., 1979. Phospholipids of the differentiating bacterium *Caulobacter crescentus*. *Can. J. Biochem.* 57, 424–428.
- Kämpfer, P., Rosselló-Mora, R., Scholz, H.C., Welinder-Olsson, C., Falsen, E., Busse, H.-J., 2006. Description of *Pseudochrobactrum* gen. Nov., with two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int. J. Syst. Evol. Microbiol.* 56, 1823–1829. <https://doi.org/10.1099/ijs.0.64256-0>.
- Kämpfer, P., Scholz, H., Huber, B., Thummes, K., Busse, H.-J., Maas, E.W., Falsen, E., 2007. Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int. J. Syst. Evol. Microbiol.* 57, 755–760. <https://doi.org/10.1099/ijs.0.64714-0>.
- Kämpfer, P., Huber, B., Lodders, N., Warfolomeow, I., Busse, H.-J., Scholz, H.C., 2009. *Pseudochrobactrum lubricantis* sp. nov., isolated from a metal-working fluid. *Int. J. Syst. Evol. Microbiol.* 59, 2464–2467. <https://doi.org/10.1099/ijs.0.008540-0>.
- Kämpfer, P., Huber, B., Busse, H.J., Scholz, H.C., Tomaso, H., Hotzel, H., Melzer, F., 2011. *Ochrobactrum pecoris* sp. nov., isolated from farm animals. *Int. J. Syst. Evol. Microbiol.* 61, 2278–2283. <https://doi.org/10.1099/IJS.0.027631-0/CITE/REFWORKS>.
- Kämpfer, P., Glaeser, S., Busse, H.J., Eisenberg, T., Scholz, H., 2013. *Falsochrobactrum ovis* gen. Nov., sp. nov., isolated from a sheep. *Int. J. Syst. Evol. Microbiol.* 63, 3841–3847. <https://doi.org/10.1099/ijs.0.049627-0>.
- Kämpfer, P., Poppel, M.T., Wilharm, G., Glaeser, S.P., Busse, H.-J., 2014. *Paenochrobactrum pullorum* sp. nov. isolated from a chicken. *Int. J. Syst. Evol. Microbiol.* 64, 1724–1728. <https://doi.org/10.1099/ijs.0.061101-0>.
- Khames, M., Mick, V., de Miguel, M.J., Girault, G., Conde-Álvarez, R., Khelef, D., Oumouna, M., Moriyón, I., Muñoz, P.M., Zúñiga-Ripa, A., 2017. The characterization of *Brucella* strains isolated from cattle in Algeria reveals the existence of a *B. abortus* lineage distinct from European and sub-Saharan Africa strains. *Vet. Microbiol.* 211, 124–128. <https://doi.org/10.1016/j.vetmic.2017.10.008>.
- Lacerda, T.L.S., Salcedo, S.P., Gorvel, J.-P., 2013. *Brucella* T4SS: the VIP pass inside host cells. *Curr. Opin. Microbiol.* 16, 45–51. <https://doi.org/10.1016/j.mib.2012.11.005>.
- Lamontagne, J., Forest, A., Marazzo, E., Denis, F., Butler, H., Michaud, J.F., Boucher, L., Pedro, I., Villeneuve, A., Sitnikov, D., Trudel, K., Nassif, N., Boudjelti, D., Tomaki, F., Chaves-Olarte, E., Guzmán-Verri, C., Brunet, S., Côté-Martin, A., Hunter, J., Moreno, E., Paramithiotis, E., 2009. Intracellular adaptation of *Brucella abortus*. *J. Proteome Res.* 8, 1594–1609. <https://doi.org/10.1021/pr800978p>.
- Lapaque, N., Forquet, F., de Chastellier, C., Mishal, Z., Jolly, G., Moreno, E., Moriyón, I., Heuser, J.E., He, H.-T., Gorvel, J.-P., 2006a. Characterization of *Brucella abortus* lipopolysaccharide macrodomains as mega rafts. *Cell. Microbiol.* 8, 197–206. <https://doi.org/10.1111/j.1462-5822.2005.00609.x>.
- Lapaque, N., Takeuchi, O., Corrales, F., Akira, S., Moriyón, I., Howard, J.C., Gorvel, J.-P., 2006b. Differential inductions of TNF-alpha and IGTP, IIGP by structurally diverse classic and non-classic lipopolysaccharides. *Cell. Microbiol.* 8, 401–413. <https://doi.org/10.1111/j.1462-5822.2005.00629.x>.
- Leong, D., Diaz, R., Milner, K., Rudbach, J., Wilson, J.B., 1970. Some structural and biological properties of *Brucella* endotoxin. *Infect. Immun.* 1, 174–182. <https://doi.org/10.1128/iai.1.2.174-182.1970>.
- Letesson, J.J., Barbier, T., Zúñiga-Ripa, A., Godfroid, J., De Bolle, X., Moriyón, I., 2017. *Brucella* genital tropism: What's on the menu. *Front. Microbiol.* 8, 506. <https://doi.org/10.3389/fmicb.2017.00506>.
- Loisel-Meyer, S., Jiménez de Bagüés, M.P., Bassères, E., Dornand, J., Köhler, S., Liautard, J.-P., Jubier-Maurin, V., 2006. Requirement of *norD* for *Brucella suis* virulence in a murine model of in vitro and in vivo infection. *Infect. Immun.* 74, 1973–1976. <https://doi.org/10.1128/IAI.74.3.1973-1976.2006>.
- Long, D., Tang, X., Cai, K., Chen, G., Chen, L., Duan, D., Zhu, J., Chen, Y., 2013. Cr(VI) reduction by a potent novel alkaliphilic halotolerant strain *Pseudochrobactrum saccharolyticum* LY10. *J. Hazard. Mater.* 256–257, 24–32. <https://doi.org/10.1016/j.jhazmat.2013.04.020>.
- Long, D., Hashmi, M.Z., Su, X., Pongpiachan, S., 2019. Cr(VI) reduction by an extracellular polymeric substance (EPS) produced from a strain of *Pseudochrobactrum saccharolyticum*. *Biotech* 9, 111. <https://doi.org/10.1007/s13205-019-1641-8>.
- Loperena-Barber, M., Khames, M., Leclercq, S.O., Zygmunt, M.S., Babot, E.D., Zúñiga-Ripa, A., Gutiérrez, A., Oumouna, M., Moriyón, I., Clockaert, A., Álvarez, R.C., 2022. *Pseudochrobactrum algeriensis* sp. nov., isolated from lymph nodes of Algerian cattle. *Int. J. Syst. Evol. Microbiol.* 72, 1–7. <https://doi.org/10.1099/ijsem.0.005223>.
- Manterola, L., Guzmán-Verri, C., Chaves-Olarte, E., Barquero-Calvo, E., de Miguel, M.-J., Moriyón, I., Grilló, M.-J., López-Goñi, I., Moreno, E., 2007. BvrR/BvrS-controlled outer membrane proteins Omp3a and Omp3b are not essential for *Brucella abortus* virulence. *Infect. Immun.* 75, 4867–4874. <https://doi.org/10.1128/IAI.00439-07>.
- Mao, Z., Yu, C., Xin, L., 2015. Enhancement of phenol biodegradation by *Pseudochrobactrum* sp. through ultraviolet-induced mutation. *Int. J. Mol. Sci.* 16, 7320–7333. <https://doi.org/10.3390/ijms16047320>.
- Marie, C., Deakin, W.J., Ojanen-Reuhs, T., Diallo, E., Reuhs, B., Broughton, W.J., Perret, X., 2004. TslI, a key regulator of *Rhizobium* species NGR234 is required for type III-dependent protein secretion and synthesis of rhamnose-rich polysaccharides. *Mol. Plant-Microbe Interact.* 17, 958–966. <https://doi.org/10.1094/MPMI.2004.17.9.958>.
- Martínez de Tejada, G., Pizarro-Cerdá, J., Moreno, E., Moriyón, I., 1995. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. *Infect. Immun.* 63, 3054–3061. <https://doi.org/10.1128/iai.63.8.3054-3061.1995>.
- Martirosyan, A., Moreno, E., Gorvel, J.-P., 2011. An evolutionary strategy for a stealthy intracellular *Brucella* pathogen. *Immunol. Rev.* 240, 211–234. <https://doi.org/10.1111/j.1600-065X.2010.00982.x>.
- Mengke-Li, Yuting-Zhuo, Yuting-Hu, Shuzhen-Li, Liang-Hu, Hui-Zhong, Zhiguo-He, 2019. Exploration on the bioreduction mechanism of Cr(VI) by a gram-positive bacterium: *Pseudochrobactrum saccharolyticum* W1. *Ecotoxicol. Environ. Saf.* 184, 109636. <https://doi.org/10.1016/j.ecoenv.2019.109636>.
- Monreal, D., Grilló, M.J., González, D., Marín, C.M., De Miguel, M.J., López-Goñi, I., Blasco, J.M., Clockaert, A., Moriyón, I., 2003. Characterization of *Brucella abortus* O-polysaccharide and core lipopolysaccharide mutants and demonstration that a complete core is required for rough vaccines to be efficient against *Brucella abortus* and *Brucella ovis* in the mouse model. *Infect. Immun.* 71, 3261–3271. <https://doi.org/10.1128/IAI.71.6.3261-3271.2003>.
- Moreno, E., 1992. Evolution of *Brucella*. In: Plommet, M. (Ed.), *Prevention of Brucellosis in the Mediterranean Countries*. Pudoc Scientific Publishers, Wageningen, pp. 198–218.
- Moreno, E., 1998. Genome evolution within the alpha *Proteobacteria*: why do some bacteria not possess plasmids and others exhibit more than one different chromosome? *FEMS Microbiol. Rev.* 22, 255–275. <https://doi.org/10.1111/j.1574-6976.1998.tb00370.x>.
- Moreno, E., Blasco, J.M., Letesson, J.J., Gorvel, J.P., Moriyón, I., 2022. Pathogenicity and its implications in taxonomy: the *Brucella* and *Ochrobactrum* case. *Pathogens* 11. <https://doi.org/10.3390/pathogens11030377>.
- Moreno, E., Middlebrook, E.A., Altamirano-Silva, P., Al Dahouk, S., Araj, G.F., Arce-Gorvel, V., Arenas-Gamboa, Á., Ariza, J., Barquero-Calvo, E., Battelli, G., Bertu, W.J., Blasco, J.M., Bosilkovski, M., Cadmus, S., Caswell, C.C., Celli, J., Chacón-Díaz, C., Chaves-Olarte, E., Comerchi, D.J., Conde-Álvarez, R., Cook, E., Cravero, S., Dadar, M., De Boelle, X., De Massis, F., Díaz, R., Escobar, G.I., Fernández-Lago, L., Ficht, T.A., Foster, J.T., Garin-Bastuji, B., Godfroid, J., Gorvel, J.-P., Güler, L., Erdenli-Gürbülk, S., Gusi, A.M., Guzmán-Verri, C., Hai, J., Hernández-Mora, G., Iriarte, M., Jacob, N.R., Keriel, A., Khames, M., Köhler, S., Letesson, J.-J., Loperena-Barber, M., López-Goñi, I., McGiven, J., Melzer, F., Mora-Carrión, R., Moran-Gilad, J., Muñoz, P.M., Neubauer, H., O'Callaghan, D., Ochoi, R., Onate, A., Pandey, P., Pappas, G., Pembroke, J.T., Roop, M., Ruiz-Villalobos, N., Ryan, M.P., Salcedo, S.P., Salvador-Bescós, M., Sangari, F.J., de Lima Santos, R., Seimenis, A., Splitter, G., Suárez-Esquivel, M., Tabbaa, D., Trangoni, M.D., Tsolis, R.M., Vizcaino, N., Wareth, C., Welburn, S.C., Whatmore, A., Zúñiga-Ripa, A., Moriyón, I., 2023. If you're not confused, you're not paying attention: *Ochrobactrum* is not *Brucella*. *J. Clin. Microbiol.* <https://doi.org/10.1128/jcm.00438-23>.
- Moriyón, I., Berman, D.T., 1982. Effects of nonionic, ionic, and dipolar ionic detergents and EDTA on the *Brucella* cell envelope. *J. Bacteriol.* 152, 822–828.



- Moriyón, I., López-Goiñi, I., 1998. Structure and properties of the outer membranes of *Brucella abortus* and *Brucella melitensis*. *Int. Microbiol.* 1, 19–26.
- Mourik, A., Steeghs, L., van Laar, J.J., Meiring, H.D., Hamstra, H.-J., van Putten, J.P.M., Wosten, M.M.S.M., 2010. Altered linkage of hydroxyacyl chains in lipid a of *Campylobacter jejuni* reduces TLR4 activation and antimicrobial resistance. *J. Biol. Chem.* 285, 15828–15836. <https://doi.org/10.1074/jbc.M110.102061>.
- Needham, B.D., Trent, M.S., 2013. Fortifying the barrier: the impact of lipid a remodeling on bacterial pathogenesis. *Nat. Rev. Microbiol.* 11, 467–481. <https://doi.org/10.1038/nrmicro3047>.
- Oren, A., 2008. Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Syst* 4, 2. <https://doi.org/10.1186/1746-1448-4-2>.
- Palacios-Chaves, L., Conde-Álvarez, R., Gil-Ramírez, Y., Zúñiga-Ripa, A., Barquero-Calvo, E., Chacón-Díaz, C., Chaves-Olarte, E., Arce-Gorvel, V., Gorvel, J.-P., Moreno, E., de Miguel, M.-J., Grilló, M.-J., Moriyón, I., Iriarte, M., 2011. *Brucella abortus* ornithine lipids are dispensable outer membrane components devoid of a marked pathogen-associated molecular pattern. *PLoS One* 6, e16030. <https://doi.org/10.1371/journal.pone.0016030>.
- Pereira, C.R., de Jesus Sousa, T., Lima da Silva, A., Gonçalves dos Santos, R., Minharmo, S., Costa Custódio, D.A., Pickard, D.J., O'Callaghan, D., Foster, J.T., de Castro Soares, S., Juca Ramos, R.T., Góes-Neto, A., Matiuzzi da Costa, M., Lage, A.P., Azevedo, V., Seles Dorneles, E.M., 2023. First report and whole-genome sequencing of *Pseudochrobactrum saccharolyticum* in Latin America. *Microbes Infect.* 25, 105018. <https://doi.org/10.1016/j.micinf.2022.105018>.
- Perry, M.B., Bundle, D.R., 1990. Lipopolysaccharide antigens and carbohydrates of *Brucella*. In: Adams, L.G. (Ed.), *Advances in Brucellosis Research*. Texas A & M University Press, Texas, pp. 76–88.
- Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R., Thornton, P.K., 2002. *Animal Diseases and their Impact on the Poor*, in: Investing in Animal Health Research to Alleviate Poverty. ILRI, Nairobi.
- Plommet, M., 1991. Minimal requirements for growth of *Brucella suis* and other *Brucella* species. *Zentralblatt für Bakteriologie* 275, 436–450. [https://doi.org/10.1016/S0934-8840\(11\)80165-9](https://doi.org/10.1016/S0934-8840(11)80165-9).
- Raetz, C.R.H., Guan, Z., Ingram, B.O., Six, D.A., Song, F., Wang, X., Zhao, J., 2009. Discovery of new biosynthetic pathways: the lipid a story. *J. Lipid Res.* 50 (Suppl), S103–S108. <https://doi.org/10.1194/jlr.R800060-JLR200>.
- Reuhs, B.L., Relić, B., Forsberg, L.S., Marie, C., Ojanen-Reuhs, T., Stephens, S.B., Wong, C.-H., Jabbouri, S., Broughton, W.J., 2005. Structural characterization of a flavonoid-inducible *Pseudomonas aeruginosa* a-band-like O antigen of *Rhizobium* sp. strain NGR234, required for the formation of nitrogen-fixing nodules. *J. Bacteriol.* 187, 6479–6487. <https://doi.org/10.1128/JB.187.18.6479-6487.2005>.
- Rouli, L., Merhej, V., Fournier, P.-E., Raoult, D., 2015. The bacterial pangenome as a new tool for analysing pathogenic bacteria. *New Microb. New Infect* 7, 72–85. <https://doi.org/10.1016/j.nmni.2015.06.005>.
- Roy, H., Ibba, M., 2009. Broad range amino acid specificity of RNA-dependent lipid remodeling by multiple peptide resistance factors. *J. Biol. Chem.* 284, 29677–29683. <https://doi.org/10.1074/jbc.M109.046367>.
- Ryan, Michael P., Pembroke, J.T., 2020. The genus *Ochrobactrum* as major opportunistic pathogens. *Microorganisms* 8, 1797. <https://doi.org/10.3390/microorganisms8111797>.
- Saeed Mahmood, M., Sarwari, A.R., Khan, M.A., Sophie, Z., Khan, E., Sami, S., 2000. Infective endocarditis and septic embolization with *Ochrobactrum anthropi*: case report and review of literature. *J. Inf. Secur.* 40, 287–290. <https://doi.org/10.1053/jinf.2000.0644>.
- Sahm, D.F., Washington, J.A., 1991. *Antibacterial susceptibility tests: dilution methods*. In: ASM press (Ed.), *Manual of Clinical Microbiology, fifth edition ed*, pp. 1105–1116.
- Salvador-Bescós, M., Gil-Ramírez, Y., Zúñiga-Ripa, A., Martínez-Gómez, E., de Miguel, M. J., Muñoz, P.M., Cloeckaert, A., Zygmunt, M.S., Moriyón, I., Iriarte, M., Conde-Álvarez, R., 2018. WadD, a new *Brucella* lipopolysaccharide core glycosyltransferase identified by genomic search and phenotypic characterization. *Front. Microbiol.* 9. <https://doi.org/10.3389/fmicb.2018.022293>.
- Scholz, H.C., Hubalek, Z., Nesvadbova, J., Tomaso, H., Vergnaud, G., Le Flèche, P., Whatmore, A.M., Al Dahouk, S., Krüger, M., Lodri, C., Pfeffer, M., 2008. Isolation of *Brucella microti* from soil. *Emerg. Infect. Dis.* 14, 1316–1317. <https://doi.org/10.3201/eid1408.080286>.
- Scholz, H.C., Nöckler, K., Lner, C.G., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kämpfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M.S., Whatmore, A.M., Pfeffer, M., Huber, B., Busse, H.J., De, B.K., 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int. J. Syst. Evol. Microbiol.* 60, 801–808. <https://doi.org/10.1099/ijs.0.011148-0>.
- Scholz, H.C., Revilla-Fernández, S., Al Dahouk, S., Hammerl, J.A., Zygmunt, M.S., Cloeckaert, A., Koylass, M., Whatmore, A.M., Blom, J., Vergnaud, G., Witte, A., Aistleitner, K., Hofer, E., 2016. *Brucella vulpis* sp. Nov., isolated from mandibular lymph nodes of red foxes (*Vulpes vulpes*). *Int. J. Syst. Evol. Microbiol.* 66, 2090–2098. <https://doi.org/10.1099/ijsem.0.000998>.
- Scholz, H.C., Heckers, K.O., Appelt, S., Geier-Dömling, D., Schlegel, P., Wattam, A.R., 2023. Isolation of *Brucella inopinata* from a White's tree frog (*Litoria caerulea*): pose exotic frogs a potential risk to human health? *Front. Microbiol.* 14. <https://doi.org/10.3389/fmicb.2023.1173252>.
- Servais, C., Vassen, V., Verhaeghe, A., Küster, N., Carlier, E., Phégnon, L., Mayard, A., Auberger, N., Vincent, S., De Bolle, X., 2023. Lipopolysaccharide biosynthesis and traffic in the envelope of the pathogen *Brucella abortus*. *Nat. Commun.* 14, 911. <https://doi.org/10.1038/s41467-023-36442-y>.
- Siddique, K., Shahid, M., Shahzad, T., Mahmood, F., Nadeem, H., Saif Ur Rehman, M., Hussain, S., Sadak, O., Gunasekaran, S., Kamal, T., Ahmad, I., 2021. Comparative efficacy of biogenic zinc oxide nanoparticles synthesized by *Pseudochrobactrum* sp. C5 and chemically synthesized zinc oxide nanoparticles for catalytic degradation of dyes and wastewater treatment. *Environ. Sci. Pollut. Res. Int.* 28, 28307–28318. <https://doi.org/10.1007/s11356-021-12575-9>.
- Sieira, R., Comerci, D.J., Sánchez, D.O., Ugalde, R.A., 2000. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. *J. Bacteriol.* 182, 4849–4855. <https://doi.org/10.1128/JB.182.17.4849-4855.2000>.
- Smith, H., Williams, A.E., Pearce, J.H., Keppie, J., Harris-Smith, P.W., Fitz-George, R.B., Witt, K., 1962. Fetal erythritol: a cause of the localization of *Brucella abortus* in bovine contagious abortion. *Nature* 193, 47–49. <https://doi.org/10.1038/193047a0>.
- Soler-Lloréns, P.F., Quance, C.R., Lawhon, S.D., Stuber, T.P., Edwards, J.F., Ficht, T.A., Robbe-Austerman, S., O'Callaghan, D., Keriel, A., 2016. A *Brucella* spp. isolate from a Pac-man frog (*Ceratophrys ornata*) reveals characteristics departing from classical *Brucellae*. *Front. Cell. Infect. Microbiol.* 6, 116. <https://doi.org/10.3389/fcimb.2016.00116>.
- Stabel, J.R., Stabel, T.J., 1995. Immortalization and characterization of bovine peritoneal macrophages transfected with SV40 plasmid DNA. *Vet. Immunol. Immunopathol.* 45, 211–220. [https://doi.org/10.1016/0165-2427\(94\)05348-V](https://doi.org/10.1016/0165-2427(94)05348-V).
- Stiakaki, E., Galanakis, E., Samonis, G., Christidou, A., Maraka, S., Tselentis, Y., Kalmanti, M., 2002. *Ochrobactrum anthropi* bacteremia in pediatric oncology patients. *Pediatr. Infect. Dis. J.* 21, 72–74. <https://doi.org/10.1097/00006454-2002101000-00018>.
- Suárez-Esquivel, M., Chaves-Olarte, E., Moreno, E., Guzmán-Verri, C., 2020. *Brucella* genomics: macro and micro evolution. *Int. J. Mol. Sci.* 21, 7749. <https://doi.org/10.3390/ijms21207749>.
- Sweet, C.R., Ribeiro, A.A., Raetz, C.R.H., 2004. Oxidation and transamination of the 3'-position of UDP-N-acetylglucosamine by *Acidithiobacillus ferrooxidans*. *J. Biol. Chem.* 279, 25400–25410. <https://doi.org/10.1074/jbc.M400596200>.
- Teyssier, C., Marchandin, H., Masnou, A., Jeannot, J.L., de Buochberg, M.S., Jumas-Bilak, E., 2005. Pulsed-field gel electrophoresis to study the diversity of whole-genome organization in the genus *Ochrobactrum*. *Electrophoresis* 26, 2898–2907. <https://doi.org/10.1002/elps.200410323>.
- Tsai, C.-M., Frasch, C.E., 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119, 115–119. [https://doi.org/10.1016/0003-2697\(82\)90673-X](https://doi.org/10.1016/0003-2697(82)90673-X).
- Urakami, T., Segers, P., 2015. *Mycoplana*. In: *Bergey's manual of systematics of Archaea and Bacteria*. Wiley, pp. 1–7. <https://doi.org/10.1002/9781118960608.gbm00808>.
- Velasco, J., Romero, C., Lopez-Goiñi, I., Leiva, J., Diaz, R., Moriyón, I., 1998. Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int. J. Syst. Bacteriol.* 48, 759–768. <https://doi.org/10.1099/00207713-48-3-759>.
- Velasco, J., Bengochea, J.A., Brandenburg, K., Lindner, B., Seydel, U., González, D., Zähringer, U., Moreno, E., Moriyón, I., 2000. *Brucella abortus* and its closest phylogenetic relative, *Ochrobactrum* spp., differ in outer membrane permeability and cationic peptide resistance. *Infect. Immun.* 68, 3210–3218. <https://doi.org/10.1128/IAI.68.6.3210-3218.2000>.
- Viadas, C., Rodríguez, M.C., Sangari, F.J., Gorvel, J.P., García-Lobo, J.M., López-Goiñi, I., 2010. Transcriptome analysis of the *Brucella abortus* BvrR/BvrS two-component regulatory system. *PLoS One* 5, 1–8. <https://doi.org/10.1371/journal.pone.0010216>.
- Wattam, A.R., Inzana, T.J., Williams, K.P., Mane, S.P., Shukla, M., Almeida, N.F., Dickerman, A.W., Mason, S., Moriyón, I., O'Callaghan, D., Whatmore, A.M., Sobral, B.W., Tiller, R.V., Hoffmaster, A.R., Frace, M.A., De Castro, C., Molinaro, A., Boyle, S.M., De, B.K., Setubal, J.C., 2012. Comparative genomics of early-diverging *Brucella* strains reveals a novel lipopolysaccharide biosynthesis pathway. *mBio* 3. <https://doi.org/10.1128/mBio.00246-12>.
- Wattam, A.R., Foster, J.T., Mane, S.P., Beckstrom-Sternberg, S.M., Beckstrom-Sternberg, J.M., Dickerman, A.W., Keim, P., Pearson, T., Shukla, M., Ward, D.V., Williams, K.P., Sobral, B.W., Tsolis, R.M., Whatmore, A.M., O'Callaghan, D., 2014. Comparative phylogenomics and evolution of the *Brucellae* reveal a path to virulence. *J. Bacteriol.* 196, 920–930. <https://doi.org/10.1128/JB.01091-13>.
- Whatmore, A.M., Foster, J.T., 2021. Emerging diversity and ongoing expansion of the genus *Brucella*. *Infect. Genet. Evol.* 92, 104865. <https://doi.org/10.1016/j.meegid.2021.104865>.
- Whatmore, A.M., Davison, N., Cloeckaert, A., Al Dahouk, S., Zygmunt, M.S., Brew, S.D., Perrett, L.L., Koylass, M.S., Vergnaud, G., Quance, C., Scholz, H.C., Dick, E.J., Hubbard, G., Schlabritz-Loutsevitch, N.E., 2014. *Brucella papionis* sp. nov., isolated from baboons (*Papio* spp.). *Int. J. Syst. Evol. Microbiol.* 64, 4120–4128. <https://doi.org/10.1099/ijs.0.065482-0>.
- Willumeit, R., Kumpugdee, M., Funari, S.S., Lohner, K., Navas, B.P., Brandenburg, K., Linser, S., Andrä, J., 2005. Structural rearrangement of model membranes by the peptide antibiotic NK-2. *Biochim. Biophys. Acta Biomembr.* 1669, 125–134. <https://doi.org/10.1016/j.bbmem.2005.01.011>.
- Yagel, Y., Sestito, S., Motro, Y., Shnaiderman-Torban, A., Khalfin, B., Sagi, O., Navon-Venezia, S., Steinman, A., Moran-Gilad, J., 2020. Genomic characterization of antimicrobial resistance, virulence, and phylogeny of the genus *Ochrobactrum*. *Antibiotics* 9. <https://doi.org/10.3390/antibiotics9040177>.
- Yoon, J.-H., Kang, S.-J., Park, S., Oh, T.-K., 2008. *Daeguia caeni* gen. nov., sp. nov., isolated from sludge of a textile dye works. *Int. J. Syst. Evol. Microbiol.* 58, 168–172. <https://doi.org/10.1099/ijs.0.65483-0>.
- Yusuf, I., Garba, L., Shehu, M.A., Oyiza, A.M., Kabir, M.R., Haruna, M., 2020. Selective biodegradation of recalcitrant black chicken feathers by a newly isolated thermotolerant bacterium *Pseudochrobactrum* sp. IY-BUK1 for enhanced production

- of keratinase and protein-rich hydrolysates. *Int. Microbiol.* 23, 189–200. <https://doi.org/10.1007/s10123-019-00090-4>.
- Zúñiga-Ripa, A., Barbier, T., Conde-Álvarez, R., Martínez-Gómez, E., Palacios-Chaves, L., Gil-Ramírez, Y., Grilló, M.J., Letesson, J.-J., Iriarte, M., Moriyón, I., 2014. *Brucella abortus* depends on pyruvate phosphate dikinase and malic enzyme but not on Fbp and GlpX fructose-1,6-bisphosphatases for full virulence in laboratory models. *J. Bacteriol.* 196, 3045–3057. <https://doi.org/10.1128/JB.01663-14>.
- Zúñiga-Ripa, A., Barbier, T., Lázaro-Antón, L., de Miguel, M.J., Conde-Álvarez, R., Muñoz, P.M., Letesson, J.J., Iriarte, M., Moriyón, I., 2018. The fast-growing *Brucella suis* biovar 5 depends on phosphoenolpyruvate carboxykinase and pyruvate phosphate dikinase but not on Fbp and GlpX fructose-1,6-bisphosphatases or isocitrate lyase for full virulence in laboratory models. *Front. Microbiol.* 9 <https://doi.org/10.3389/fmicb.2018.00641>.