Brucella, nitrogen and virulence

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Abstract

The brucellae are α-Proteobacteria causing brucellosis, an important zoonosis. Although multiplying in endoplasmic reticulum-derived vacuoles, they cause no cell death, suggesting subtle but efficient use of host resources. Brucellae are amino-acid prototrophs able to grow with ammonium or use glutamate as the sole carbon–nitrogen source in vitro. They contain more than twice amino acid/peptide/polyamine uptake genes than the amino-acid auxotroph Legionella pneumophila, which multiples in a similar vacuole, suggesting a different nutritional strategy. During these two last decades, many mutants of key actors in nitrogen metabolism (transporters, enzymes, regulators, etc) have been described to be essential for full virulence of brucellae. Here, we review the genomic and experimental data on Brucella nitrogen metabolism and its connection with virulence. An analysis of various aspects of this metabolism (transport, assimilation, biosynthesis, catabolism, respiration and regulation) has highlighted differences and similarities in nitrogen metabolism with other α-Proteobacteria. Together, these data suggest that, during their intracellular life cycle, the brucellae use various nitrogen sources for biosynthesis, catabolism and respiration following a strategy that requires prototrophy and a tight regulation of nitrogen use.

Introduction

We are what we eat. As with other organisms, bacteria must find in their environment sources for energy generation and cellular component biosynthesis. Obviously, these requirements exist during a symbiotic or pathogenic interaction with eukaryotic hosts (Barbier et al., 2011; Brown et al., 2008; Kafsack & Llinas, 2010), and as most of the bacteria able to establish these relationships are chemo-heterotrophs, they rely on materials provided by the host. Indeed, many of these bacteria are grown in the laboratory on media that not only provide all the essential nutrients but also often contain a surplus of other compounds that alleviate the biosynthetic needs and speed growth. Thus, inferences on their dietary habits and available menus in the real world are not straightforward.

Brucellae are typically facultative intracellular pathogens responsible for brucellosis, a serious and worldwide zoonosis. They are aerobic, Gram-negative bacteria belonging to the order Rhizobiales of the α-Proteobacteria class and are thus closely related to bacteria that also establish intimate relationships with plant or animal cells (e.g. Agrobacterium, Rhizobium or Bartonella; Moreno & Moriyón, 2006). The Brucella genus is divided into several species, most of which show a preferential mammalian host. As these bacteria have a tropism for the male and female reproductive tract, brucellosis often causes abortion and infertility. Most Brucella species can infect humans, causing a chronic and debilitating disease known as Malta fever. From this perspective, the most important species are B. melitensis, B. abortus and B. suis (some but not all biovars), which will henceforth be referred to as the classical smooth Brucella spp.

The virulence of this pathogen relies on its ability to avoid or modulate the host immune response and to survive and replicate in an endoplasmic-reticulum derived vacuole; several key molecular players of this strategy have been identified (Martirosyan et al., 2011). In addition, the emerging view is that Brucella virulence is also closely linked to the regulation of its metabolism in response to the changing nutritional environments encountered during both the intra-vacuolar trafficking and in the various organs or host cells infected (Barbier et al., 2011; Xavier et al., 2013). However, Brucella metabolism is not a preferential focus of present-day research, and when research in this area does occur, it is from the perspective of central carbon metabolism (Essenberg et al., 2002; Poncet et al., 2009; Zúñiga-Ripa et al., 2014) and/or of the preferential carbon sources (i.e. erythritol). Nevertheless, nitrogen, along with carbon, oxygen and hydrogen, are some of the main bio-elements. Nitrogen represents approximately 10% of the dry weight of a bacteria and is required for the biosynthesis of amino acids, puric and
pyrimidic bases, amino sugars, amino lipids and some coenzymes (Gottschalk, 1986). Moreover, many bacteria use nitrate (NO$_3^-$) and nitrite (NO$_2^-$) in anaerobic respiration. Consequently, bacteria have developed a variety of strategies to utilize the nitrogen sources available in the environments where they multiply. Indeed, a picture encompassing the range and availability of nitrogen compounds and the regulation of their metabolism in correlation with central carbon metabolism is essential to understand the complex relationships that the brucellae establish with their hosts. Based on genomic data (http://www.broadinstitute.org/annotation/genome/brucella_group/MultiHome.html) on attenuated mutants described in the last decade (Delrue et al., 2001, 2004; Foulongne et al., 2000; Hong et al., 2000; Kim et al., 2012; Lestrade et al., 2000, 2003) and on data from proteomics/transcriptomics analyses (Eskra et al., 2001; Köhler et al., 1999; Lamontagne et al., 2009; Rossetti et al., 2011), we recapitulate here what is currently known about nitrogen metabolism and Brucella virulence.¹

Nitrogen sources and transport

Generalities

Bacteria can use inorganic or organic sources of nitrogen. It is likely that all bacteria can use ammonium (NH$_4^+$), which is a favored nitrogen source because it is directly available for biosynthesis, while the assimilatory reduction of nitrate (NO$_3^-$), nitrite (NO$_2^-$) or N$_2$ to ammonium implies a high-energy cost. Whereas NH$_3$ crosses most biological membranes by simple diffusion, NH$_4^+$ uptake requires specific active transporters² (Kleiner, 1985; Tate et al., 1998). Nitrate also needs to be actively transported either by primary transport [involving proteins of the ATP-binding cassette (ABC) superfamily] or secondary transport systems (nitrate/proton symport and nitrate/nitrite antiport; Moir & Wood, 2001).

Nitrogen originating from organic compounds consists of amino or imino groups, or heterocyclic nitrogen, all of which are metabolized without changing the oxidation state of the nitrogen atom. For biosynthetic purposes, they are either deaminated to ammonium or the substrate of transaminases that transfer amino groups to carbon backbones carrying keto groups, such as to α-ketoglutarate to form glutamate. Usually, these pathways are activated below an adequate NH$_4^+$ concentration. Amino acids, polypeptides, and short peptides are usually imported through ABC or secondary transport systems (Saier, 2000), whereas urea uptake depends on either an ABC transporter or a mono-component permease (Sebbane et al., 2002).

Nitrogen sources used by Brucella in vitro

Brucella are devoid of the extracellular hydrolytic enzymes that would allow the use of nitrogen-containing macromolecules (e.g., proteins, polymers of amino sugars, nucleic acids, etc.; Moreno & Moriyón, 2006). Accordingly, these bacteria depend exclusively on low molecular weight nitrogen sources.

In early investigations carried out in chemically defined media, the classical smooth Brucella spp. were found to be able to use ammonium readily as a source of inorganic nitrogen while nitrate or nitrite did not support growth (Gerhardt, 1958; Gerhardt & Wilson, 1947). This is coherent with the absence of cytoplasmic assimilatory nitrate reductase homologues (Nas family) in the available Brucella genomes (http://img.jgi.doe.gov/cgi-bin/w/main.cgi); indeed, the use of nitrate as the sole nitrogen source has never been reported for these bacteria. The brucellae are thus different from most Rhizobium spp. because although they are phylogenetically close, most Rhizobium spp. can use nitrate as the sole nitrogen source (Patriarca et al., 2002). Although all species of Brucella except for B. ovis are positive in the nitrate reduction test (Moreno & Moriyón, 2006; Pickett & Nelson, 1954; Zobell & Meyer, 1932a), this is the result of a dissimilatory anaerobic respiration process (i.e. denitrification) carried out by a membrane-located enzymatic activity (Nar family; Haine et al., 2006; refer the “Brucella and denitrification” section).

Whereas little attention has been given to other organic nitrogen sources, many studies have indicated that the growth of brucellae in vitro is optimal when a balanced amino acid proportion is provided (Gerhardt, 1958). Moreover, in early studies carried out with B. abortus S19³, it was found that, while other amino acids support little or no growth (Gerhardt & Wilson, 1948; Gerhardt et al., 1950), asparagine, histidine or glutamate can be used as the only nitrogen source with glycerol and lactate as the carbon sources. Only natural L isomers of these amino acids are used by these bacteria (Gerhardt et al., 1950; Yaw & Kakavas, 1952). A correlation between glutamate, asparagine and histidine deamination by Brucella resting cells and the ability to use them as the nitrogen source for growth was noted in those early studies, and the possibility that the ammonium released could be used for biosynthesis was also suggested (Gerhardt et al., 1950). For asparagine, the experiments were extended to several B. abortus, B. melitensis and B. suis strains, and it was observed that the CO$_2$-dependent B. abortus strains strictly required this gas for growth with this amino acid, while some strains were not able to use it as the sole nitrogen source (Gerhardt & Wilson, 1948). Further studies with S19 and two additional B. abortus strains proved that only glutamate could be used as its sole source of carbon, nitrogen and energy (Gerhardt et al., 1950). As supported by later metabolic studies in another attenuated B. abortus strain (Marr et al., 1953), this double role of glutamate can be explained by its conversion to α-ketoglutarate, which is then metabolized through the tricarboxylic acid (TCA) cycle and gluconeogenesis to provide energy, reducing power and biosynthetic precursors. This capacity to use glutamate as the sole carbon,

³Unless indicated otherwise, this review uses primarily the sequences of B. melitensis 16M (reference strain for biovar 1 of this species), B. abortus 2308 (USDA virulent challenge strain) and B. suis 1330 (reference strain for biovar 1 of this species) because these have been the strains used in the large majority of virulence studies.

²Active transport requires energy. In primary active transport, the energy is derived directly from ATP hydrolysis. In secondary active transport, the energy is derived from energy stored in the form of an electrochemical gradient (typically Na$^+$ or H$^+$) between the two sides of a membrane.

³B. abortus S19, also known as US19 or strain 19, is a vaccine strain carrying multiple mutations. It was extensively used in early metabolic studies because its attenuation made laboratory work safer.
energy and nitrogen source is common in free-living *Rhizobium* spp. as opposed to *Enterobacteriaceae* members (Patriarca et al., 2002). No systematic studies have been carried out to test whether this holds true in all brucellae, but the ability to oxidatively deaminate this amino acid by resting cells is extended in the genus, with intensities that depend on both the species (Jacques et al., 2007) and on the envelope permeability of the particular strain (Gerhardt et al., 1953; Wilson & Dasinger, 1960; refer “Brucella amino acid catabolism” section). In summary, the results of the nutritional studies are compatible with a picture in which the host provides nitrogen as ammonium or amino acids, the latter being then either a source of ammonium by deamination and/or transamination or used directly for protein synthesis, or both. Glutamate could play a central role by virtue of its role in transamination reactions and its connection with the TCA and urea cycles.

**Brucella nitrogen transport**

Before being assimilated, \( \text{NH}_4^+ \), amino acids and peptides have to be actively transported into the cytoplasm.

**What the genomes tell us**

**General comparison.** Table 1 presents a quantitative summary of those *B. melitensis* 16M ORFs whose annotations suggest a role in nitrogen transport, and Supplemental Figure S1 presents a schematic overview. Approximately 9% of the encoding ability of *Brucella* is devoted to ABC transporters, most of which are involved in nutrient import. They comprise approximately 100 systems (326 genes), of which c.a. 25% (81 of 326 genes) are devoted to the import of nitrogenous compounds (Jenner et al., 2009). Table 1 also compares the transport systems annotated in the genomes of *B. melitensis*, *Ochrobactrum anthropi* (a *Brucella*-related opportunistic pathogen), *Sinorhizobium meliloti* (a *Brucella*-related plant endosymbiont) and three unrelated facultative intracellular pathogens (*Salmonella enterica* serovar *Typhimurium*, *Legionella pneumophila* and *Mycobacterium tuberculosis*). For *B. melitensis*, *O. anthropi* and *S. meliloti*, the data show that the total number of putative primary or secondary transport systems in the former is commensurate to the genomic reduction noted before for the intracellular animal pathogens of the \( \alpha \)-*Proteobacteria* (Moreno, 1998; Sällström & Andersson, 2005). The data also show that the number and proportion of putative ATP-dependent nitrogen transport systems is substantially higher in *B. melitensis* than in *L. pneumophila*, which has a similar genome size, or than in *M. tuberculosis* or *S. enterica Typhimurium*, both of which have approximately 25–30% larger genomes. Interestingly, the annotations suggest an almost reverse picture for the secondary transporters (Table 1). These differences may be a consequence of an ancestrally higher and lower number, respectively, of primary and secondary transport systems in the \( \alpha \)-*Proteobacteria*, as suggested by the comparison with *O. anthropi* and *S. meliloti*. In addition, *Brucella*, *L. pneumophila* and *M. tuberculosis* may have different nitrogen sources in their replicating vacuoles (see below).

**Table 1. Quantitative distribution of total and nitrogen putative uptake systems annotated in the genomes of three \( \alpha \)-*Proteobacteria* and selected facultative intracellular bacteria.**

|                        | \( \alpha \)-*Proteobacteria* | \( \gamma \)-*Proteobacteria* | Actinobacteria |
|------------------------|--------------------------------|--------------------------------|---------------|
|                        | *B. melitensis*                 | *O. anthropi*                  | *S. meliloti*  |
| Genome Size in Mb      | 3294.93                        | 5205.78                        | 6691.69       |
| No. primary transport genes | 2 (0.4%)                  | 2 (0.2%)                      | 4 (0.4%)    |
| Total (No./Mb)         | 476 (0.14)                     | 855 (0.16)                     | 1024 (0.15)   |
| ATP-dependent          | 326 (68.5%)                    | 598 (69.9%)                    | 705 (68.8%)   |
| ATP-dependent nitrogen uptake | 81 (17.0%)                | 178 (20.8%)                    | 188 (18.4%)   |
| Ion channels           | 13 (2.7%)                      | 16 (1.9%)                      | 26 (2.5%)    |
| Ionic nitrogen channels | 2 (0.4%)                      | 2 (0.2%)                       | 1 (0.1%)    |
| PTS                    | 4 (0.8%)                       | 11 (1.3%)                      | 4 (0.4%)   |
| No. secondary transport genes | Total 122 (25.6%)         | 213 (24.9%)                    | 271 (26.5%)   |
| Nitrogen\(^b\)         | 9 (1.8%)                       | 11 (1.2%)                      | 24 (2.3%)    |
| Unclassified           | 8 (1.7%)                       | 15 (1.8%)                      | 13 (1.3%)   |
|                        |                                |                                |               |
|                        | *S. enterica Typhimurium*      | *L. pneumophila*               | *M. tuberculosis* |
| Genome Size in Mb      | 4951.37                        | 3359                           | 4421.2       |
| No. primary transport genes | 3 (0.5%)                  | 3 (1.1%)                      | NA           |
| Total (No./Mb)         | 627 (0.13)                     | 275 (0.08)                     | 268 (0.06)   |
| ATP-dependent          | 252 (40.2%)                    | 133 (48.4%)                    | 155 (57.8%)   |
| ATP-dependent nitrogen uptake | 55 (9.0%)                  | 16 (5.8%)                      | 10 (3.7%)    |
| Ion channels           | 25 (4%)                        | 11 (4%)                        | 6 (2.2%)    |
| Ionic nitrogen channels | 1 (0.1%)                      | NA\(^a\)                       | 1 (0.4%)    |
| PTS                    | 61 (9.7%)                      | 2 (0.7%)                       | NA\(^a\) |
| No. secondary transport genes | Total 275 (43.9%)         | 119 (43.3%)                    | 98 (36.6%)   |
| Nitrogen\(^b\)         | 59 (9.4%)                      | 20 (7.2%)                      | 18 (6.7%)    |
| Unclassified           | 10 (1.6%)                      | 7 (2.5%)                       | 9 (3.4%)    |

From: http://www.membranetransport.org/index_v2_rc1.html.

\(^a\)NA, not available.

\(^b\)Only those dedicated to import are scored.
In most *Rhizobiales, amtB* is close to *ftsK*, which encodes the translocase involved in resolving the chromosomal dimers during septation (Allemand & Maier, 2009). Indeed, the presence of this highly conserved ammonium transporter is in agreement with the in vitro nutritional studies.

**Nitrate and urea transporter.** An ABC transporter gene predicted to import nitrate from the periplasm is most likely connected to the first enzymatic step of the denitrification process (Jenner et al., 2009; refer “Nitrogen and respiration” section). This transporter gene is present even in *B. ovis*, although this species is negative in the standard nitrate reduction tests.

Similarly, all *Brucella* species appear to have a single-component urea transport system encoded in the last gene of the second urease operon (*ure2*). This system is homologous to the eukaryotic urea transporter that allows urea to have access to the cytoplasmic activity of urease (Sangari et al., 2010; refer the “*Brucella* urea cycle and urease activity” section). This gene is inactivated in *B. ovis*, the only species of this genus that is negative in the standard urease test (Tsolis et al., 2009).

**Amino acid, di- or oligo-peptide and polyamine transporters.** In contrast to the scarcity of transport systems for inorganic nitrogen and urea, *Brucella* genomes encode a large number of primary (ABC) transport systems for branched-chain amino acids (isoleucine, leucine and valine), di- and oligo-peptides, and also genes for polyamine (spermidine/putrescine) transport. *B. ovis*, however, is predicted to lack functional polyamine transporters (Jenner et al., 2009). Other amino acids could also be imported by the less numerous secondary transport systems (Table 1). *Brucella* has three members of the amino acid/polyamine/organocation (APC) family (Jack et al., 2000), among which an antiporter glutamate/γ-aminobutyrate has been recently described in *B. microti* (Occhialini et al., 2012). This antiporter, however, is not present in *B. suis, B. canis* or *B. ovis*. Another transporter belongs to the alanine or glycine:cation symporter (AGCS) family catalyzing the reaction: alanine or glycine (out) + Na⁺ or H⁺ (out) → alanine or glycine (in) + Na⁺ or H⁺ (in).

It has been speculated that the abundance of peptides or amino acid transporters relates to the growth of *Brucella* in a replicative vacuole derived from the endoplasmic reticulum, where peptides and amino acids might be abundant (Paulsen et al., 2002; refer the “Mutants and/or data from transcriptomic or proteomic experiments” and “Biosynthesis of amino acids by *Brucella*” sections). Although this hypothesis is seemingly consistent with the fact that the total number of transporters for amino acids and derivatives is only slightly lower than that in *S. enterica* (90 versus 114), another intravacuolar pathogen, *Brucella* transport abilities seem to be shifted towards peptide uptake (35 versus 17; Table 2). This could be due to differences in lifestyle because while both bacteria are amino acid prototrophs, *S. enterica* but not *Brucella* has a life cycle outside the host. However, *L. pneumophila* has fewer transporters, particularly for peptides (Table 2), and also lives in an endoplasmic reticulum-derived niche. This absence of a common pattern may not only reflect the phylogenetic background of each bacterium but also the differences in the intracellular compartments created under the influence of the pathogen (refer the “Biosynthesis of amino acids by *Brucella*” section).

**Mutants and/or data from transcriptomic or proteomic experiments**

Compared to their importance in the genome of brucellae, relatively few mutants in peptide and amino acid transporters have been described as attenuated (Table 3). This could be the result of a redundancy of function or an indication that they are not strictly needed during the infectious cycle in the standard models of infection. In fact, the substrates of many of these transporters might not be available (refer the “Biosynthesis of amino acids by *Brucella*” section). In addition, random transposon mutagenesis approaches may have been biased with regards to the insertion sites of the transposon agent (Wu et al., 2006). According to these investigations, a mutation of three systems involved in dipeptide or oligopeptide import (Table 3) causes attenuation, a result compatible with the hypothesis that the cognate compounds are available and needed at some point during the infectious process. Nevertheless, the mutagenic system used does not always preclude polarity effects, and the phenotypes of some of these mutants are not obviously related to a metabolic defect. For example, *B. abortus* mutants in BAB2_1040 and BAB2_1056 (Table 3) are reported to have a higher internalization in HeLa cells than the wild type even though they putatively encode elements of a dipeptide uptake system (Kim et al., 2012).

Transcriptomic analyses of *Brucella* genes that are differentially expressed during intracellular growth also give clues about the nutrients encountered (Eskra et al., 2001; Köhler et al., 1999; Rossetti et al., 2009; Table 3). During infection, the nitrate import gene is induced, which suggests a microaerobic intracellular environment and the use of nitrate respiration consistent with other evidence (refer previous

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Table 2. Comparison of the relative proportions of transporters for amino acids and derivatives annotated in the genomes of three alpha-2 Proteobacteria and three facultative intracellular bacteria.

| Amino acid, di- or oligo-peptide and polyamine transporters | α-Proteobacteria | γ-Proteobacteria | Actinobacteria |
|-----------------------------------------------------------|------------------|------------------|---------------|
| *B. melitensis*                                           | 29 (36%)         | 74 (74%)         | 102           |
| *O. anthropi*                                             | 78 (45%)         | 22 (65%)         | 34            |
| *S. meliloti*                                             | 68 (34%)         | 9 (26%)          | 3             |
| *S. enterica* Typhimurium                                 | 11 (11%)         | 11 (14%)         | 22            |
| *L. pneumophila*                                          | 17 (17%)         | 17 (17%)         | 34            |
| *M. tuberculosis*                                         | 27 (16%)         | 9 (26%)          | 2             |
| *M. tuberculosis*                                         | 46 (23%)         | 3 (9%)           |               |
| *M. tuberculosis*                                         | 19 (11%)         | 2 (14%)          |               |
| *B. abortus*                                              | 81               | 81               |               |
| *B. suis*                                                 | 173              | 173              |               |
| *B. canis*                                                | 201              | 201              |               |

Data from http://www.membranetransport.org/ (Ren & Paulsen, 2007).
Table 3. *Brucella* nitrogen transport genes reported to be related to virulence or modulated in transcriptomic or proteomic studies in vivo.

| Gene name or putative function | *B. melitensis* | *B. abortus* | *B. suis* | Model (study)a | References |
|-------------------------------|----------------|--------------|------------|----------------|------------|
| **Attenuated mutants**        |                |              |            |                |            |
| Dipeptide import              | BMEI0433       | PGb          | BRA0859    | Macrophage, HeLa, mice | Lestrate et al. (2003) |
| Oligopeptide import           | BMEI0220       | BAB2_1020    | BRA1081    | Macrophage, HeLa | Kim et al. (2012) |
| Dipeptide import              | BMEI0285       | BAB2_0952    | BRA1009    | Macrophage, HeLa, mice | Kim et al. (2012); Wu et al. (2006) |
| Methionine transport          | BMEI0336       | BAB2_0273    | BRA0962    | Macrophage, HeLa, mice | Delrue (2002) |
| Spermidine/putrescine import  | BMEI0923       | BAB2_0879    | BRA0326    | Mice           | Delrue (2002) |
| **Modulated**                 |                |              |            |                |            |
| Nitrate import                | BMEI0798       | BAB2_0769    | BRA0467    | HeLa-Transcriptomic/Up (12 hpi) HeLa-DFI/Up | Rossetti et al. (2011); Eskra et al. (2001) |
| Polar amino acid import       | BMEI1104       | BAB1_0181    | BR0955     | HeLa-DFI/Up    | Köhler et al. (1999) |
| Branched amino acid transport | BMEI0258       | BAB1_1799    | BR1791     | HeLa-DFI/Up    | Köhler et al. (1999) |
| *gadC* E/GABA antiporter      | BMEI0909       | BAB2_0864    | PGb        | HeLa-Transcriptomic/Up (4 hpi) | Rossetti et al. (2011) |
| Polyamine import              | BMEI0922       | BAB2_0878    | BRA0327    | HeLa-Transcriptomic/Up (4 hpi) | Rossetti et al. (2011) |
| Dipeptide import              | BMEI0207       | BAB2_1051    | BRA1093    | HeLa-Transcriptomic/Up (4 hpi) | Rossetti et al. (2011) |
| Dipeptide import              | BMEI0284       | BAB2_0974    | BRA1012    | HeLa-Transcriptomic/Down (4 hpi) | Rossetti et al. (2011) |
| Dipeptide import              | BMEI0433       | PGb          | BRA0859    | HeLa-Transcriptomic/Down (4 hpi) | Rossetti et al. (2011) |
| Arginine binding protein      | BMEI1627       | BAB1_0325    | BR0295     | HeLa-Transcriptomic/Up (12 hpi) | Rossetti et al. (2011) |
| Branched amino acid transport | BMEI0070       | BAB2_0023    | BRA0024    | HeLa-Transcriptomic/Up (12 hpi) | Rossetti et al. (2011) |
| Branched amino acid transport | BMEI0631       | PGb          | BRA0650    | HeLa-Transcriptomic/Up (12 hpi) | Rossetti et al. (2011) |
| Polyamine import              | BMEI0196       | BAB2_1062    | PGb        | HeLa-Transcriptomic/Up (12 hpi) | Rossetti et al. (2011) |
| Ser/ala/gly import            | BMEI0038       | BAB2_0055    | BRA0056    | HeLa-Transcriptomic/Down (12 hpi) | Rossetti et al. (2011) |
| Branched amino acid transport | BMEI0263       | BAB1_1792c   | BR1785     | Macrophage-Proteomic/Down (3, 20, 48 hpi) | Lamontagne et al. (2009) |
| Branched amino acid transport | BMEI0344       | BAB2_0282    | BRA0953    | Macrophage-Proteomic/Down (3, 20, 48 hpi) | Lamontagne et al. (2009) |
| Oligopeptide import           | BMEI0734       | BAB2_0699c   | BRA0538    | Macrophage-Proteomic/Down (3, 20, 48 hpi) | Lamontagne et al. (2009) |

ORF in boldface indicate the species for which the information was obtained. The orthologous genes were collected from http://biocyc.org/.

aUp and Down, positively or negatively modulated, respectively; (hpi), hours post-infection of the observation; DFI: differential fluorescence induction.
bPG: pseudogene.
cOnly the first of several contiguous genes concerned is included in the table.
section and “Brucella and denitrification” section). Concerning other transporters detected in these experiments, a few are already down or upregulated 4h after infection, notably the glutamate/γ-aminobutyrate antipporter, but most are upregulated at later times (Table 3) once Brucella is in the replicative niche. This suggests that amino acids are used for protein synthesis and/or as ammonium and/or carbon sources during active growth. The same overall picture results from proteomic analyses (Lamontagne et al., 2009; Table 3). It is striking that there is little overlapping among the different studies: of the systems (or ORFs) listed in Table 3, only a dipeptide ABC transporter (BMEII0285 and thus BMII0284) has been identified in more than one study. Indeed, being mostly membrane proteins, these transporters may be more difficult to identify in proteomic studies. Interestingly, the substrate binding component (BAB1_1794) of the branched amino acid transporter identified in these studies (BAB1_1799 [inner-membrane translocator]; Table 3) has been reported to be regulated by AbcR small RNAs in B. abortus (Caswell et al., 2012).

Ammonium assimilation

Generalities

Once transported into the bacteria, ammonium can be incorporated into glutamate by two pathways: directly by a reductive amination of α-ketoglutarate catalyzed by the NADPH-dependent glutamate dehydrogenase (assimilatory glutamate dehydrogenase or GdhA [EC 1.4.1.4], different from the dissipatory NAD-dependent GdhB [EC 1.4.1.2]; Figure 1, reactions 1 and 4, respectively); or indirectly through a cycle of two reactions involving the synthesis of glutamine from ammonium and glutamate by the ATP-dependent glutamine synthetase (GS; Figure 1, reaction 2), followed by the transfer of the amide group onto glutamate and glutamine synthetase isoform (GSI, encoded by glnT) (Espin et al., 1990; Shatters et al., 1993). Both GS and GSI appear to have a high affinity for glutamate and ammonium (Espin et al., 1990; Shatters et al., 1993) and GlnT displays a much lower affinity (Shatters et al., 1993) and was thus postulated to carry another but still unknown activity. Both GSII and GlnT form homo-octamers (Manco et al., 1992; Merrick & Edwards, 1995).

Brucella nitrogen assimilation

Based on genome annotations and on curated pathways from SEED (http://www.theseed.org/wiki/Home_of_the_SEED; Overbeek, 2005 and IMG databases (https://img.jgi.doe.gov/cgi-bin/w/main.cgi), Brucella should be able to assimilate glutamate and NH\textsubscript{4}\textsuperscript{+} via both GS-GOGAT and GdhA systems (Table 4), a prediction fully consistent with nutritional studies in vitro. Figure 2 compares the conservation of the major players in the nitrogen assimilation of several α-Proteobacteria and E. coli.

GS-GOGAT system

There are three ORFs annotated as glutamine synthetase in all the available Brucella genomes (Table 4). ORF BMEI0979 (and its orthologues) is in chromosome I downstream of glnB, which encodes the nitrogen regulatory protein PII (refer the “Nitrogen regulation in Brucella” section), a genomic arrangement conserved in most Rhizobiales. The predicted amino acid sequence of this ORF is 66% identical to that of E. coli GlnA, making BMEI0979 a good candidate for the GSI gene. Moreover, a transpositional mutant of glnA is attenuated for intramacrophagic multiplication in B. suis (Köhler et al., 2002b). Interestingly, glnA is also essential for virulence in other intracellular pathogens such as S. typhimurium and M. tuberculosis, suggesting that the glutamine concentration in the phagosomal compartment is quite low (Klose & Mekalanos, 1997; Tullius et al., 2003). In spite of their phylogenetic proximity with plant-associated bacteria, no orthologs of GlnI (Dombrecht et al., 2002) or GlnT have been identified in Brucella. The two other ORFs annotated as encoding glutamine synthetases (BMEII0523 and BMEII0554) have only approximately 30% identity with E. coli GlnA. A miniTn5 B. abortus mutant in one of these genes has been reported as attenuated in HeLa cells (Kim et al., 2012; Table 4). Whether these genes encode “bona fide” glutamine synthetase isoforms and under which conditions they may be active remains to be determined.

Figure 1. Nitrogen assimilation in bacteria. GdhA: NADPH-dependent glutamate dehydrogenase; GdhB: NAD-dependent glutamate dehydrogenase; GS: glutamine synthetase; GOGAT: glutamate-α-aminotransferase; AKG: α-ketoglutarate; Gln: glutamine; Glu: glutamate. Reaction 1 and 2 occur at high and low NH\textsubscript{4} concentration, respectively.
The glutamate synthase enzyme (GOGAT) is an NADPH-dependent heteromultimeric enzyme complex (Cottevieille et al., 2008) formed by the products of \textit{gltB} (large or \( \alpha \) subunit) and \textit{gltD} (small or \( \beta \) subunit). Mutants in these genes are attenuated both in cellular and mouse models of infection (Hong et al., 2000; Wu et al., 2006; Table 4). These results suggest that not only glutamine/glutamate is not provided in sufficient amounts by the host but also the assimilatory NADP+-dependent GDH (GdhA) is not efficient enough in assimilating NH\(_4^+\) to maintain glutamine and glutamate pools during the intravacuolar life of \textit{Brucella}. The nutritional studies (refer the ‘‘Nitrogen sources used by \textit{Brucella in vitro}’’ section) showed that \textit{Brucella} cannot grow with glutamine as its nitrogen source (Gerhardt et al., 1950), but the reason is not known.

\textit{Gdh} system

Contrary to \textit{E. coli}, all members of the \textit{Brucella} genus seem to have both the NADP\(^+\)- (gdhA) and the NAD\(^+\)-dependent glutamate dehydrogenase (gdhB) genes (Table 4 and Figure 2). The presence of catabolic GdhB is consistent with nutritional studies that showed growth on glutamate as the sole source of nitrogen, carbon and energy (refer the ‘‘Nitrogen sources used by \textit{Brucella in vitro}’’ section). It must be noted that, under these conditions, ammonia from glutamate is required for the synthesis of glutamine (and derived compounds; refer the ‘‘Generalities’’ section) catalyzed by GS. The coexistence of GdhA and GS does not seem constant in all \textit{Rhizobiales} as some lack gdhA and only carry gdhB. In these bacteria, the net assimilation of NH\(_4^+\) is entirely dependent on the GS–GOGAT system (Patriarca et al., 2002; Tate et al., 2004), which could explain why \textit{R. leguminosarum} grows better when glutamine is used as sole N source instead of NH\(_4^+\) (Rossi et al., 1989). As noted above, this is not the case of \textit{Brucella}.

No \textit{Brucella} gdhA or gdhB attenuated mutants have been identified in random searches, and the proteomic analysis in murine macrophages shows that \textit{B. suis} GdhA is considerably reduced 48 h after infection while \textit{B. abortus} GdhB decreases during the infectious cycle (Al Dahouk et al., 2008; Lamontagne et al., 2009). Although these data do not allow drawing a firm conclusion on the importance of these glutamate dehydrogenases \textit{in vivo}, the evidence discussed in the previous section indicates that GdhA cannot compensate for defects in the GS–GOGAT system.

\textbf{Nitrogen in biosynthesis}

\textbf{Generalities}

According to the precursor from which they derive their carbon skeleton, amino acids are classified into the following families: aromatic (phenylalanine, tyrosine and tryptophan); serine (serine, glycine, cysteine and methionine); aspartate (aspartate, asparagine, threonine and the sulfur-containing amino acids cysteine and methionine); pyruvate (alanine and the branched amino acids valine, leucine and isoleucine); and glutamate (glutamate, glutamine, arginine, proline and lysine). Finally, histidine is connected to the nucleotide biosynthetic pathway. Glutamate and glutamine are the nitrogen donors for the synthesis of amino acids, amino sugars, purines, pyrimidines, NAD\(^+\) and \( p \)-aminobenzoate (refer the ‘‘Generalities’’ section). In addition, \textit{Brucella} envelopes contain

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Degree of conservation of major players in nitrogen assimilation and regulation in several \( \alpha \)-Proteobacteria and \textit{E. coli}. The percentage of homology obtained by blast analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparing \textit{Brucella melitensis} 16M protein sequences with \textit{Brucella abortus}, \textit{Sinorhizobium meliloti}, \textit{Caulobacter crescentus} and \textit{Escherichia coli} counterparts. Contrary to \textit{E. coli}, \( \alpha \)-Proteobacteria encode three glutamine synthetase-like enzymes (GlnA, GlnA2 and GlnA3), a second NtrY/NtrX two-component system and an NAD-dependant glutamate dehydrogenase (GdhB).}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
System and genes (putative function) & Reaction No. & \textit{B. melitensis} & \textit{B. abortus} & \textit{B. suis} & References \\
\hline
\textbf{GS-GOGAT} & & & & & \\
\textit{glnA} (glutamine synthetase; GSI) & 2 & BMEI0979 & BAB1\_1023 & BR1004 & Köhler et al. (2002b) \\
\( ^{gb} \) glutamine synthetase (GS?) & 2& BMEI0523 & BAB2\_0471 & BRA0768 & Kim et al. (2012), \\
\( ^{gb} \) glutamine synthetase (GS?) & & BMEI0554 & BAB2\_0507 & BRA0732 & Kim et al. (2012), \\
gltB (GOGAT \( \alpha \) [large] subunit) & 3 & BMEI0040 & BAB2\_0053 & BRA0054 & Wu et al. (2006) \\
gltD (GOGAT \( \beta \) [small] subunit) & 3 & BMEI0039 & BAB2\_0054 & BRA0055 & Wu et al. (2006); \\
& & & & & Hong et al. (2000) \\
\hline
\textbf{Gdh} & & & & & \\
gdhA (NADP\(^+\)-dependent glutamate dehydrogenase) & 1 & BMEI1723 & BAB1\_0228 & BR0227 & Al Dahouk et al. (2008) \\
gdhB (NAD\(^+\)-dependent glutamate dehydrogenase) & 4 & BMEI2031 & BAB1\_1827 & BR1819 & Lamontagne et al. (2009) \\
\hline
\end{tabular}
\caption{The GS-GOGAT and Gdh systems of brucellae.}
\end{table}

All genes in the Table are present and similarly organized in all \textit{Brucella} genomes available at the Biocyc website (http://biocyc.org/). ORF in boldface indicate the species in which attenuation or modulation during infection was reported.

\(^a\)See Figure 1.

\(^b\)Genbank annotation but function unknown. Refer the ‘‘GS-GOGAT system’’ section.
phosphatidylethanolamine and phosphatidylcholine, two phospholipids whose amino groups are derived from serine or choline and amino acid-containing lipids.

**Biosynthesis of amino acids by Brucella**

When provided with ammonium, brucellae can synthesize all L-amino acids (refer the “Nitrogen sources used by Brucella in vitro” section), and although only the roles of proA and leuB (proline and leucine genes, respectively) have been confirmed (Essenberg & Sharma, 1993), this prototrophy is consistent with genomic analyses (http://patricbrc.org/portal/portal/patric/PathwayFinder?cType=taxon&cld=234&dm=) (Wattam et al., 2013). This is worth commenting on because in the process of becoming adapted to (and thus more dependent on) their host, pathogenic bacteria lose the genomic information that becomes superfluous for nutrient acquisition (Casadevall, 2008). *L. pneumophila*, for example, is auxotrophic for cysteine, arginine, isoleucine, leucine, methionine, valine and threonine (Cazalet et al., 2004). As *Brucella* can be described as a facultative extracellular intracellular parasite and, like *L. pneumophila*, multiplies in an endoplasmic reticulum-derived vacuole, its amino acid prototrophy could seem a paradox (refer the “What the genomes tell us” section). However, *L. pneumophila* is able to manipulate the proteasome machinery to increase amino acid availability in the replicative vacuole (Price et al., 2011); as there is increasing evidence that infected host cells are able to limit amino acid availability, keeping the biosynthetic pathways can also be an alternative strategy (Zhang & Rubin, 2013). In fact, for several intracellular pathogens, amino acid auxotrophic mutants are often attenuated. This appears to also be true for *Brucella*.

No transcriptomic study has provided information on amino acid biosynthetic pathways during intracellular infection. However, 17 different mutants in amino acid biosynthetic pathways have been described as attenuated for *Brucella* (Supplemental Table S1). All families are concerned, but the pyruvate family (alanine and the branched amino acids valine, leucine and isoleucine) represents five mutants in different biosynthetic steps of the branched amino acid pathway, strongly suggesting that these amino acids are limiting in the infection models used. Although these results should parallel those concerning the transport systems (refer the “What the genomes tell us” section), the diversity of the models and *Brucella* strains used makes it difficult to draw definite conclusions. While the attenuation of the mutants in the amino acid biosynthetic pathways suggests that the intracellular replication site of *Brucella* is poor in amino acids (Köhler et al., 2002a), those focusing on transport genes (Table 3) suggest that at some point amino acids and/or peptides are available, perhaps before reaching the replicative compartment (Delrue et al., 2004). In addition, these imported amino acids could be used catabolically, and proteomic data suggest that *B. abortus* preferentially derives TCA cycle precursors from amino acids at early and mid-point times of infection (Lamontagne et al., 2009).

**Biosynthesis of envelope amino compounds in Brucella**

Several amino compounds have been shown to be important in the *Brucella* envelope properties that are relevant in pathogenicity, and because their biosynthesis is tightly connected to nitrogen metabolism, these pathways are briefly reviewed here.

**Ethanolamine, choline and aminoacyl containing free lipids**

*Brucella* envelopes contain phosphatidylethanolamine and phosphatidylcholine, both of which are required for full virulence (Bukata et al., 2008; Comerci et al., 2006; Conde-Alvarez et al., 2006). Phosphatidylethanolamine synthesis requires serine (Figure 3A), and accordingly, an important membrane defect should contribute to the attenuation observed for *serB* mutants (Supplemental Table S1). In bacteria, phosphatidylcholine results from the methylation of phosphatidylethanolamine (*pmtA* pathway; Figure 3A) or from the condensation of diacylglycerol and exogenous choline (*pcs* pathway; Figure 3A). There are apparently contradictory results with respect to the attenuation or lack thereof in *B. abortus* mutants regarding the *pcs* gene (Comerci et al., 2006; Conde-Alvarez et al., 2006) or the gene(s) encoding the binding protein(s) of choline ABC transporter(s) (Herrmann et al., 2013; LLeyre, 2010). Moreover, the consensus sequence of the PmtA methylase is conserved in *B. melitensis* and *B. suis* but not in *B. abortus*, suggesting that the picture of choline use and phosphatidylcholine synthesis changes depending on the *Brucella* species. Indeed, if choline is not available, *serB* mutants should also be affected in phosphatidylcholine synthesis.

In some bacteria, phosphatidylglycerol and cardiolipin are decorated with cationic aminoacyl residues. This simple modification decreases the binding of cationic antimicrobial peptides and is also involved in resistance to osmotic or acidic stresses (Roy & Ibba, 2009). These modifications require an aminoacyl-phosphatidylglycerol synthase plus the corresponding aminoacyl-tRNA. Genomic analyses reveal the presence of two putative lysil-phosphatidylglycerol synthases in *Brucella* (Figure 3A), and although they have not been studied, aminoacyl-phosphatidylglycerol synthases play an important role in several extracellular pathogens including *Mycobacterium* (Klein et al., 2009; Maloney et al., 2009). In *Rhizobium*, lysil-phosphatidylglycerol confers resistance to cationic peptides under acidic conditions (Sohlenkamp et al., 2007). Thus, as in the case of the *serB* mutation, the attenuation associated with the *lysA* mutation (Supplemental Table S1) may be due in part to a significant envelope defect (Köhler et al., 2002a).

Acyl–oxyacyl lipids, in which the lipid part is amide-linked to an amino acid, are widely distributed among bacteria, and ornithine-containing lipids are the most common version (Figure 3B). This diamino acid participates in the urea cycle (refer the “Brucella urea cycle and urease activity” section) and is not a protein constituent, so these amino lipids represent the only significant contribution of ornithine to the structural building blocks. *Brucella* spp. present this phosphorus-free membrane eubacterial lipid in considerable amounts, and it has been speculated that it contributes to the stability of the outer membrane by interacting with anionic groups of the lipopolysaccharide (Freer et al., 1996). Dysfunction of the OlsB acylase (Figure 3B) does not upset the outer membrane stability or generate attenuation (Palacios-Chaves et al., 2011).
Nevertheless, these mutants seem to compensate the ornithine lipid deficiency by producing other amino lipids (Conde-Alvarez, R., unpublished results).

Lipopolysaccharide amino sugars and ethanolamine

With the exception of \textit{B. inopinata} (Wattam et al., 2012), all smooth \textit{Brucella} spp. have lipopolysaccharides with O-antigens made of N-formyl perosamine (N-formyl-4-amino, 4,6-dideoxy-D-mannose) polysaccharides plus two mannoses and one quinovosamine (2-amino, 2,6-dideoxy-D-glucose) linking this polysaccharide to the core (Kubler-Kielb & Vinogradov, 2012). Perosamine is derived from mannose through GDP-4-dehydro-6-deoxy-\alpha-D-mannose, and glutamate is the putative donor of the amino group used by perosamine synthetase (Per) (Figure 3C). By virtue of both their structural arrangements and the positive charges associated with the amino group, the core oligosaccharide of \textit{Brucella} lipopolysaccharides shows an exceedingly low affinity for cationic bactericidal peptides and complement (Conde-Alvarez et al., 2012; Freer et al., 1996; Martínez de Tejada et al., 1995). Four glucosamine (2-amino, 2-deoxy-D-glucose) units are present in a core branch (Kubler-Kielb & Vinogradov, 2012). They are synthesized from fructose, and the aminotransferase (GlmS; BMEI0685) uses glutamine. Quinovosamine is derived from glucosamine through UDP-linked 2-acetamido-2,6-dideoxy-L-lyxo-4-hexulose and 2-acetamido-2,6-dideoxy-D-mannose (Kneidinger et al., 2003). Interestingly, in contrast to biologically potent lipids A that usually have a glucosamine disaccharide backbone, \textit{Brucella} lipid A displays a low biological activity and contains a backbone made of diaminoglucose (2,6-amino-2,6-dideoxy-D-glucose). In \textit{Acidithiobacillus ferroxidans} (Sweet, 2004), diaminoglucose is synthesized from glucosamine through the activity of a dehydrogenase (GnnA) and a transaminase (GnnB) that uses glutamine as a donor, and \textit{Brucella} carries homologues (BMEI0420 and BMEI0421) of both enzymes (Figure 3C).

Several bacteria are endowed with transferases that add phosphoethanolamine to the lipid A/core oligosaccharide, thereby reducing the binding of cationic bactericidal peptides (Needham & Trent, 2013). Genomic analyses have revealed the presence of a gene (BMEI0118 in \textit{B. melitensis} 16 M) encoding the homologue of LptA, one of these phosphoethanolamine transferases. This gene is highly conserved in all \textit{Brucella} spp. except for \textit{B. abortus}, which contains a frameshift causing the premature stop of translation. Consistent with these genomic data, ethanolamine has not been detected in the lipopolysaccharides of \textit{B. abortus} (Moreno et al., 1990), but at least in \textit{B. melitensis}, mass spectra revealed ethanolamine-substituted lipid A forms that disappeared upon \textit{lptA} mutation (Gil-Ramírez, 2011).

Polyamines

Biogenic polyamines play a variety of roles in bacteria, and their uptake and metabolism is regulated to maintain intracellular polyamine levels. These organic polycations can interact with and modulate the properties and/or activities of molecules that carry phosphate and free carboxylic groups, such as lipopolysaccharides, lipids, porins and nucleic acids. They can also act as free radical scavengers and take part in the response to osmotic and acid stresses in several bacteria (Shah & Swiatlo, 2008). Putrescine (1,4-diaminobutane) is common and results directly from the decarboxylation of ornithine or is produced by the decarboxylation of arginine to yield agmatine, followed by the hydrolysis of the latter to putrescine and urea (Shah & Swiatlo, 2008). Whereas \textit{Brucella} genomes have an annotation for ornithine decarboxylase (BMEI1133 in \textit{B. melitensis} 16 M), the agmatine

![Figure 3. Biosynthesis of Brucella cell envelope amino lipids and amino sugars. Panel A. Amino glycerophospholipid synthesis. Pcs: phosphatidylcholine synthase; Pss: phosphatidylserine synthase; Psd: phosphatidylserine decarboxylase; PmtA: phospholipid N-methyltransferase; PgsA: phosphatidylglycerol phosphate synthase; Pgg: phosphatidylglycerol phosphate phosphatase; LPGs: Lysýl-phosphatidylglycerol synthase. Panel B. Amino acid-containing acyl-oxacyl lipid synthesis. OlsB: ornithine lipid synthase B; OlsA: ornithine lipid synthase A; OlsC: ornithine lipid synthase C. Panel C. Amino sugars involved in peptidoglycan and lipopolysaccharide synthesis. Per: perosamine synthase; GlcN-6-P: D-Glucosamine 6-phosphate; UDP-GlnAc: UDP-N-acetylgalactosamine; UDP-GlnAc3N: UDP-N-acetyldiaminoglucose; Gt: glycosyltransferase; GlmS: glucosamine-P acetyltransferase; GnnA: UDP-GlnAc dehydrogenase; GnnB: UDP-GlnAc3N transaminase. DOI: 10.3109/1040841X.2014.962480 Brucella, nitrogen and virulence 515]
pathway seems to be missing. Like many other \(\alpha\)-Proteobacteria (Shaw et al., 2010), there are homologues (BMEI0015 and BMEI0016) of the homospermidine \([\text{N-(4-aminobutyl)butane-1,4-diamine}]\) synthases that convert putrescine plus spermidine into propionate-1,3-diamine plus homospermidine. However, homologues of the genes involved in the synthesis of spermidine or cadaverine, two common polyamines, are seemingly absent. These genomic characteristics suggest that the brucellae would need to import spermidine to generate homospermidine, and in fact, they have annotations for two spermidine/putrescine ABC transport systems. Interestingly, a \(B.\) \textit{melitensis} mutant in the periplasmic binding component of one of these transporters (BMMI0923) is attenuated in mice (Table 3), and two components of the \(B.\) \textit{abortus} orthologue system (BAB2_0879 and BAB2_0878) have been identified as regulated by AbcR small RNAs (Caswell et al., 2012). Because homospermidine is required for the normal growth of \(R.\) \textit{leguminosarum} and is also involved in the response to osmotic and acid stresses in \(S.\) \textit{Sinorhizobium fredii} (Fujihara & Yoneyama, 1993; Shaw et al., 2010), it is tempting to speculate that this triamine is also relevant in \textit{Brucella} biology.

**Amino acid catabolism**

**Generalities**

All 20 amino acids are degraded to seven intermediates (pyruvate, acetyl-CoA, \(\alpha\)-acetoacetyl-CoA, \(\alpha\)-ketoglutarate, succinyl-CoA, fumarate and oxaloacetate) that feed the TCA cycle, and the first reaction is always the removal of the amino group. Amino acids are often oxidatively deaminated into the corresponding ketoacid by a non-specific flavoprotein oxidase coupled directly to the electron transport chain. Some specific NAD(P)\(^+\) dehydrogenases (for example, alanine or \(\alpha\)-ketoglutarate dehydrogenases) can also take part, and serine, threonine, aspartate and histidine can also be deaminated by specific enzymes. Thus, growth with amino acids as the only source of both carbon and energy not only requires gluconeogenesis but also implies the release of a considerable quantity of ammonium, which would create a nitrogen imbalance if it were not for the activity of the urea cycle. In this cycle, ornithine plays a "catalytic" role, and two ammonium molecules (one from carbamoyl-phosphate and the other from aspartate) are converted into urea (Figure 4).

**Brucella amino acid catabolism**

Since the pioneering work of Meyer (Meyer & Cameron, 1961; Zobell & Meyer, 1932b), reference laboratories have used respirometric studies of amino acid oxidative catabolism for \textit{Brucella} biotyping (Alton et al., 1975; Jacques et al., 2007). Although glutamate, asparagine and histidine can be used as the sole nitrogen source (refer the "Nitrogen sources used by \textit{Brucella in vitro}" section), the former causes a rate of oxygen uptake that is considerably greater, most likely because it is the only amino acid that can also be used as a carbon and energy source, and it feeds directly into the TCA cycle (refer the "Nitrogen sources used by \textit{Brucella in vitro}" section). Indeed, the oxidation of glutamate was also shown to be coupled to the urea cycle (Cameron et al., 1952; refer the "Brucella urea cycle and urease activity" section). Initially, it was observed that attenuated strains oxidize glutamate at rates higher than those observed for virulent strains, and a possible link between glutamate catabolism and virulence was investigated. However, further work demonstrated that, rather than being a result of differences in enzymatic activity, such high rates were due to an increased permeability of the attenuated strains attributable to envelope defect references (Dasinger & Wilson, 1962; Wilson & Dasinger, 1960), the latter most likely accounting for the attenuation.

**Brucella urea cycle and urease activity**

In early studies, \(B.\) \textit{abortus} and \(B.\) \textit{melitensis} resting cells were shown to metabolize glutamate with a transient accumulation of arginine, ornithine and citrulline, and arginase activity was demonstrated in \(B.\) \textit{abortus}, \(B.\) \textit{melitensis} and \(B.\) \textit{suis} (Cameron & Meyer, 1954; Cameron et al., 1952). Arginase is hallmark of the urea cycle (Figure 4); therefore, these results indicate that at least the three classical smooth \textit{Brucella} species should have this cycle. Indeed, genomic analyses (http://patricbrc.org/portal/portal/patric/Taxon?cType=taxon&cId=234) have extended these results by showing that all the sequenced \textit{Brucella} genomes contain all the urea cycle genes.

Except for \(B.\) \textit{ovis}, all \textit{Brucella} \textit{spp.} show urease activity in the standard bacteriology urease test (Corbel & Hendry, 1985). This enzyme is located in the cytoplasmic fraction (Brun & Descous, 1984). All \textit{Brucella} (including \(B.\) \textit{ovis}) bear two urease operons (\textit{ure1} and \textit{ure2}) on chromosome I, but the urease activity derives mostly from \textit{ure1} (Bandara et al., 2007). Although, it was known that the \textit{ure2} operon is transcribed in vivo, its function has been unraveled only recently: it appears to code for an acid-activated urea and nickel transporter crucial for maximal urease activity, the latter enzyme being encoded in the \textit{ure1} cluster (Sangari et al., 2007). Consistent with this, the urea negative phenotype of \(B.\) \textit{ovis} relates to a 30 bp deletion in one of the \textit{ure1} genes (Tsolis et al., 2009).

In all likelihood, urease is needed to remove intracellular urea when amino acids are the main source of carbon and energy. In this cycle, ornithine plays a "catalytic" role, and two ammonium molecules (one from carbamoyl-phosphate and the other from aspartate) are converted into urea (Figure 4).

**Figure 4.** The urea cycle in \textit{Brucella}. (\textit{B. melitensis} 16M ORFs are in brackets). (1) arginase (BMEI0396); (2) ornithine carbamoyltransferase (BMEI1620); (3) argininosuccinate synthase (BMEI1870); (4) arginino-succinate lyase (BMEI0086); (5) carbamoyl-P synthase (large subunit, BMEI0522; small subunit, BMEI0526).
energy (Cameron et al., 1952), and in fact, *B. suis* urease-negative mutants grow slowly in amino acid-rich media (Bandara et al., 2007). This could be the case 24 h after macrophage infection, when the bacteria are already in the endoplasmic reticulum-derived vacuoles because several enzymes associated with protein and amino acid catabolism are increased at this time (Lamontagne et al., 2009). However, at least *B. abortus* and *B. suis* urease-negative mutants do not display attenuation in murine macrophages (Bandara et al., 2007; Sangari et al., 2007), suggesting that the production of urea in this model is not as intense as to become deleterious. Similarly, no attenuation is observed when these urease mutants are injected intraperitoneally in mice (Bandara et al., 2007; Sangari et al., 2007). Indeed, this could not only reflect a relatively low production of urea in the replicating niche but also some limitations of these virulence models. In addition to the catabolic role of urease, it is conceivable that the ammonium released in the hydrolysis of urea could serve to generate resistance to acidity, and accordingly, that urease is a virulence factor in the classical *Brucella* spp. Concerning intracellular invasion and multiplication, the above-mentioned urease mutants are not attenuated in macrophages, where they are at least transitorily exposed to an acidic pH. However, it is also possible that the urease helps *Brucella* to survive the passage through the stomach; this has been investigated in *B. abortus* (Sangari et al., 2007), *B. suis* (Bandara et al., 2007) and *B. melitensis* (Paixão et al., 2009) in mice. Whereas *ure1* mutants behave such as the wild-type when injected intraperitoneally, their numbers in the intestine or in the spleen are considerably lower than in oral infections. Nevertheless, a gavage using large numbers of bacteria (10^10–10^5) in mice does not necessarily reflect an intestinal infection (Grilló et al., 2012), and moreover, a number of factors show that this route is unlikely to be significant in ruminants and humans (Gorvel et al., 2009).

**Nitrogen and respiration**

**Generalities**

In respiring prokaryotes, the electron flow through a chain of carriers from primary donors to a terminal acceptor is coupled to the electrochemical proton gradient and represents the main energy yielding process. When the terminal acceptor is not oxygen, the process is called anaerobic respiration and requires a terminal reductase that varies according to the acceptor. Denitrification is a common case of anaerobic respiration in which nitrate, the initial acceptor, is finally reduced to N₂. The complete process involves a nitrate reductase, a nitrite reductase, a nitric oxide reductase and a nitrous oxide reductase organized so that the product of one reductase becomes the acceptor in the subsequent step (i.e. nitrite produced by nitrate reductase is the acceptor of nitrite reductase, and so forth). The first reaction occurs on the cytoplasmic side, and in Gram-negative bacteria, nitrite is then transported to the periplasm where the rest of the process occurs. Not all bacteria carry those four reductases, and a common variant is the nitrate/nitrite respiration, in which only the nitrate reductase takes part. When anaerobic respiration is facultative, oxygen is the preferred terminal acceptor due to its higher E₀, and the expression of the alternative reductases is tightly controlled.

**Brucella and denitrification**

Except where indicated, all genes mentioned in this section are present and organized similarly in the genomes of *Brucella* that are available at the Biocyc website (http://biocyc.org/; Caspi et al., 2013), and the *B. melitensis* 16M genes will be used as a reference.

Nitrate–nitrite reduction is observed in all *Brucella* classical species except for *B. ovis* (Pickett & Nelson, 1954; Zobell & Meyer, 1932a). During the characterization of the electron transport system of *B. abortus* 19 (British strain; see below), Rest and Robertson found a membrane-linked nitrate reductase activity (Rest, 1975), and they reasoned that, just as in other aerobic bacteria, its presence was due to a failure to supply enough oxygen to meet its consumption by actively growing brucellae even in vigorously stirred broths. Subsequently, Sperry & Robertson (1975) noticed that nitrate was almost as effective as oxygen in stimulating the breakdown of erythritol by the *S19* British strain4, and because nitrite had no effect, they suggested that *B. abortus* flavoproteins, ubiquinone and cytochromes are linked to a terminal nitrate reductase. This insight was partially confirmed when the first *Brucella* genome became available (DelVecchio et al., 2002).

The *B. melitensis* genome contains the narKGHJI (BMEI10948-0953) cluster coding for the four subunits of the respiratory nitrate reductase plus the nitrite extrusion protein (NarK). By working as a nitrate/nitrite antiporter, the latter would prevent intracellular accumulation of toxic levels of nitrite and allow for further detoxification by the periplasmic nitrite reductase (see below). The narK gene appears as a pseudogene in *B. ovis*, a species that does not reduce nitrate to nitrite, but also in *B. abortus* 2308 (Chain et al., 2005; Tsolis et al., 2009). In addition to the nar cluster, *B. melitensis* also appears to have the gene clusters encoding the nitrite (nrKV; BMEII0988-0987), nitric oxide (norEFCBQD; BMEII1001-0996) and nitrous oxide (nosRZDFYSLX; BMEII0975-0967) reductases necessary for full denitrification (Haine et al., 2006). The genes *norB* (encoding a subunit of nitric oxide reductase) and *nosX* (unknown function but located in the nitrous oxide reductase cluster) are presumed to be non-functional in *B. ovis* (Tsolis et al., 2009).

In both *B. suis* and *B. abortus*, *narG* (the catalytic subunit of the nitrate reductase) mutants are unable to form nitrite from nitrate and are attenuated in macrophages (Kim et al., 2012; Köhler et al., 2002a). The brucellae most likely encounter a micro-aerobic environment in the vacuole and respire nitrate for intracellular survival and growth. In keeping with this hypothesis, it has been demonstrated recently that the redox sensing two-component systems PrrBA (also called RegBA) and NutYX coordinately regulate the expression of denitrification genes and of the high-affinity cytochrome oxidase, the latter being an indicator of reduced oxygen tension (Abdou et al., 2013; Carrica et al., 2013). Moreover, a gene encoding a subunit of the nitrous oxide

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4Contrary to *B. abortus* S19 (US19 or strain 19), this British 19 variant is able to catabolize erythritol.
promotes the survival of inhibition of the nitric oxide synthase of the host cell that is crucial in host cell defense. In fact, the chemical in the that, in addition to a role in respiration, the proteins encoded cells and mice (Lestrate et al., 2003). It is indeed possible paradigm is the E. coli the global nitrogen regulatory (ntr) system, and its to sense and respond to the availability of nitrogen is called the cellular response (Leigh & Dodsworth, 2007). The capacity deficit or excess has to be translated into a general paradigm Regulation of nitrogen metabolism Generalities Nitrogen deficiency or excess has to be translated into a cellular response (Leigh & Dodsworth, 2007). The capacity to sense and respond to the availability of nitrogen is called the global nitrogen regulatory (ntr) system, and its paradigm is the E. coli ntr system (Merrick & Edwards, 1995). This system is composed of five proteins: GlnD, an uridylyltransferase/uridylyl-removing enzyme (UTase/UR); GlnB, also called PII regulatory protein; GlnE, an adenyltransferase/adenyl-removing enzyme (ATase/AR), also called glutamate-ammonia- ligase adenylyltransferase; and the histidine protein kinase NtrB plus the response regulator NtrC, the members of a two-component regulatory system. The ntr system senses the intracellular ratio of glutamine/α-ketoglutarate (Figure 5). Under nitrogen-limiting conditions, the ratio of glutamine/α-ketoglutarate decreases, and PII is uridylylated by GlnD to form PII(UMP)3. This modified form of PII leads to the activation (via the adenylylating activity of GlnE) of the glutamine synthetase (GS). Under this condition, NtrB acts as a kinase on and activates NtrC, leading to the expression of the almost 100 NtrC-P dependent genes (including the glnA operon; refer the ‘‘Brucella nitrogen assimilation’’ section). Conversely, when nitrogen is in excess, PII is de-uridylylated, leading to the addition of adenylic groups on each GS monomer by GlnE and then to the inactivation of the glutamine synthetase. Under its unmodified form, PII interacts with NtrB, inhibiting its autophosphorylation and stimulating its phosphatase activity on NtrC, switching off the expression of its target genes (Figure 5). Not all nitrogen regulation occurs through PII proteins, and in addition, the coordination of nitrogen and carbon metabolism involves a complex array of metabolic interactions and of signal transduction systems including RpoN, also known as the ε54 factor, the phosphotransferase system(s) (PTS) and (p)ppGpp, the stringent response mediator.

Regulatory pathway controlling nitrogen metabolism in E. coli. GlnD controls uridylylation state of PII (also called GlnB) in response to glutamine availability. Dependant of its uridylation state, PII regulates glutamine synthetase activity by the adenylyltransferase GlnE and the expression by NtrB/NtrC two component systems. Solid lines and dashes lines indicate respectively nitrogen excess and nitrogen deficiency. Arrows indicate simulation effect. Exponential annotation indicate which activity is regulated: UT: Uridylytransferase; UR: Uridylyremoving; AT: adenylytransferase; AR: adenylyremoving; Ph: phosphatase; Ki: kinase.

Figure 5. Regulatory pathway controlling nitrogen metabolism in E. coli.

Nitrogen regulation in Brucella All genes mentioned hereunder are present and organized similarly in the genomes of Brucella that are available at the Biocyc website (http://biocyc.org/); as before, the B. melitensis 16M genome will be used as a reference. Figure 2 compares the conservation of the major players in the nitrogen regulation of several α-proteobacteria and in E. coli.

GlnD (BMEI1804) Transpositional mutants in glnD have been described as attenuated in both macrophages and HeLa cells (Köhler et al., 2002a; Foulongne et al., 2000; Wu et al., 2006). Interestingly, such as a number of bacteria, Brucellaceae carry glnD upstream of mviN, the latter encoding a membrane protein predicted to have ten or more transmembrane regions. Although frequently annotated as a virulence protein, mviN is not restricted to pathogens: it is an essential gene in S. meliloti (Rudnick et al., 2001) and E. coli and appears to be involved in peptidoglycan biosynthesis (Inoue et al., 2008). Although there is no direct evidence, genomic comparisons support the prediction that MviN (renamed MurJ) is a peptidoglycan lipid II flippase (Ruiz, 2008).

GlnB (BMEI0978) All Brucellaceae have only one PII (GlnB) protein, and it is encoded upstream of glnA, the gene for the ‘‘bona ﬁde’’ glutamine synthase (GS). This genetic organization is conserved in Rhizobiaceae, which have a second glnB-like
gene (glnK) upstream of the ammonium transporter amtB gene (Patriarca et al., 2002). GlnK is thought to regulate ammonium metabolism through an interaction withAmtB (Blauwkamp & Ninfa, 2003). As commented in the “What the genomes tell us” section, glnK is absent in the Brucellaceae sequenced so far, and this different operonic organization suggests that the regulation is likely different in Brucella. No Brucella glnB mutant has been described so far.

GlnE (BMEI1327)

In Agrobacterium spp., S. meliloti, R. etli and Ochrobactrum spp. but not in Caulobacter crescentus, glnE is located upstream and in reverse orientation of a gene encoding the cell cycle regulator PleC. Interestingly, in all Brucella species, an additional gene (wadA; formerly wa***) encoding an LPS core biosynthesis gene (González et al., 2008) is located between pleC and glnE. A transpositional mutant of glnE was attenuated in macrophages and in mice (Wu et al., 2006), and the level of the corresponding protein was shown to be highly upregulated at early times of infection in macrophages, when Brucella was still on its way to the replicative vacuole (Lamontagne et al., 2009). Similarly, GlnE of M. tuberculosis is essential for virulence due to its regulatory role on GS activity (Carroll et al., 2008).

NtrB NtrC (BMEI0865, BMEI0866)

This two-component system is known to play a critical role in the control of nitrogen metabolism in several bacteria. As opposed to enteric bacteria, the ntrBC genes of Brucella are not cotranscribed with glnA but with an ORF of the tRNA dihydrodouridine synthase family (IPR001269) that most likely forms the nifR3ntrBC operon. This genetic organization is conserved in Brucellaceae and Rhizobiaceae. The regulon controlled by NtrC in Brucella is presently unknown but is also predicted to bind RpoN (r54 factor; Dombrecht et al., 2002) and to be phosphorylated by the cytoplasmic NtrB kinase (via the uridylylated form of PII) at low concentrations of nitrogen.

An ntrC mutant of B. suis shows an increased metabolic activity in the presence of L-asparagine and L-alanine and decreased activity in the presence of other amino acids, such as glutamic, arginine and lysine, as well as several sugars (Dorrell et al., 1999). This mutant has a growth curve comparable to that of the wild-type in a medium containing peptones and 10% yeast extract. As the primary function of the ntr response is the assimilation of any of a number of nitrogen sources when nitrogen becomes limiting, it is unlikely that this response is needed in this rich medium. In contrast, the ntr system could be crucial in macrophages if, as reported (Klose & Mekalanos, 1997), they are present in a glutamine poor environment. However, the B. suis ntrC mutant was not attenuated in the macrophages. In mice, this mutant showed some attenuation 5 days (acute phase of splenic multiplication) but not 12 or 24 days (plateau or chronic phase) after infection, suggesting a change in the environment within the host cells during infection (Xavier et al., 2013). It is worth commenting that an ntrC mutant of S. typhimurium was not attenuated in macrophages or mice (Klose & Mekalanos, 1997); this difference underlines once more that the paradigm of the ntr system and of the nitrogen metabolism cannot be easily translated from enteric bacteria to Brucella. This is also exemplified by the existence in Brucella of a second two-component system (NtrY/NtrX) displaying considerable homology to NtrBC.

NtrY NtrX (BMEI0867, BMEI0868)

The NtrY/NtrX two-component system is encoded downstream of the ntrBC operon. This genomic organization was first identified in Azorhizobium caulinodans (Patriarca et al., 2002) and is conserved in most Rhizobiaceae. In addition, in all sequenced strains of Brucella spp., A. tumefaciens and S. meliloti, ntrY/ntrX is likely to form an operon with a gene encoding a potassium transporter homologous to E. coli TrkA (Carrica et al., 2012), which is reported to be controlled by PTSNtr (refer the “PTS” section) in this bacterium (Reaves & Rabinowitz, 2011). Its localization downstream of ntrBC, the pleiotropic defects in nitrogen metabolism displayed by an ntrY mutant of A. caulinodans, and the fact that the regulator NtrX has a RpoN binding site, strongly suggests that NtrY/NtrX represents a second two-component system linked to nitrogen metabolism. In addition, the ntrX operon can complement a nifR3ntrBC deletion mutant of Azospirillum brasilense for nitrate-dependent growth (Ishida et al., 2002). Although the exact link with the “classical” ntr system (i.e. NtrB/NtrC) and the signal sensed by the NtrY kinase was not identified, this result suggests a possible cross-talk between the NtrY/X and NtrB/C sensor/regulatory pairs (Ishida et al., 2002). However, due to the membrane localization of NtrY, the nature of its associated signal is presumed to be different from the intracellular glutamine/z-ketoglutarate levels detected by NtrB/C (Ishida et al., 2002). The link of this NtrY/X system with the nitrogen metabolism remains to be substantiated.

Very recently, it was found that the NtrY/X system of Brucella is involved in redox sensing through a hemo group contained in the PAS domain of the NtrY histidine kinase. Under low oxygen tension, NtrY activates its cognate response regulator NtrX by phosphorylation, which in turn increases the expression of the denitrification genes (refer the “Brucella and denitrification” section; Carrica et al., 2012). Interestingly, the ntrY mutant of B. suis was described as being attenuated in human macrophages more than a decade ago (Foulongne et al., 2000), and the double deleted ntrYX shows a slight growth defect in murine macrophages (Carrica et al., 2012). These data are consistent with the attenuation of the mutants in genes involved in denitrification (refer the “Brucella and denitrification” section). Nevertheless, a single ntrY mutant of B. abortus was not attenuated in mice (Carrica et al., 2013), suggesting a redundancy in the actors controlling Brucella adaptation to microaerobiosis. Interestingly, the expression of ntrY is under the control of another redox sensing two-component system (PrrBA) that co-regulates the denitrification genes with NtrY. The double mutant ntrY/prrB is severely attenuated in mice (Carrica et al., 2013).

Integration of nitrogen and carbon metabolism in Brucella

Ammonia assimilation requires energy, reducing power and precursor metabolites from the central carbon metabolism,
which needs to be coordinated with the metabolism of nitrogen. Hereunder we summarized what is known regarding *Brucella* of the following three metabolic integrators: RpoN, PTS and (p)ppGpp.

**RpoN (BMEI1789)**

RpoN (σ54) was initially discovered for its requirement for the expression of nitrogen metabolism genes. RpoN is structurally and functionally unrelated to the sigma factors of the σ70 family (Letesson & De Bolle, 2004) because it can bind the positions -12 and -24 of the promoters even in the absence of RNA polymerase. Nevertheless, for the initiation to proceed, the σ54–RNApol complex needs the additional presence of a transcriptional activator. There are only three σ54-dependent transcriptional regulators (NtrC, NtrY and BMEII0011) in *B. melitensis* compared to seven in *A. tumefaciens* and *S. melliloti* (Dombrecht et al., 2002). While the prediction of the RpoN regulon indicates that rhizobia appear to have recruited RpoN for symbiotic processes, the role of RpoN in *A. tumefaciens* regulon indicates that rhizobia appear to have recruited RpoN for symbiotic processes, the role of RpoN in *A. tumefaciens* and *B. melitensis* is unclear (Dombrecht et al., 2002). In *B. melitensis*, RpoN null mutants are not significantly affected in their virulence in cells or in mice (Delory et al., 2006).

The overall rpoN locus organization is conserved in α-Proteobacteria. Some aspects of this organization relevant to nitrogen metabolism are discussed in the next section.

**PTS**

The phosphoenolpyruvate–carbohydrate phosphotransferase system (PTS) is a phosphorelay catalyzing the uptake and concomitant phosphorylation of sugars using the PEP phosphoryl group. PTS proteins encompass: (i) the cytoplasmic enzyme I (EI) and the Hpr, neither of which show a sugar specificity; (ii) a variable number of enzyme II complexes (EIIA–EIIB), each one sugar specific; and (iii) the membrane-spanning EIIIC that constitutes the permease of the system. In addition to sugar uptake, PTS plays a key role in the regulation of many aspects of bacterial physiology, including carbon catabolite repression (CCR; Deutscher et al., 2006). Like other α-Proteobacteria, all *Brucella* spp. maintain all the PTS components except the sugar permeases, which stresses the importance of the regulatory aspects of the system.

A paralogue of this classical PTS was identified within the rpoN operon of *E. coli*, and for this reason, it was named nitrogen PTS (PTS^Ntr^). It is composed of three of the equivalent PTS proteins, EI^Ntr^, NPr and EIIA^Ntr^, but lacks the permease element (i.e. the EIIBC paralogue). It has been demonstrated recently that the phosphorylation status of the PTS^Ntr^ is reciprocally regulated by glutamine and α-ketoglutarate. These canonical signals of nitrogen availability are sensed by the N-terminal GAF sensory domain of the EI^Ntr^ and modulate the PTS^Ntr^ activity (Lee et al., 2013). Like many *Proteobacteria*, brucellae have genes to encode all the predicted PTS^Ntr^ proteins, and this system has been demonstrated functional (Dozot et al., 2010; Figure 6). Similar to its orthologues, *Brucella* EI^Ntr^ (BMEI0190) has a N-terminal GAF sensory domain (Dozot et al., 2010; Letesson & De Bolle, 2004) whose function and sensed signals are unknown. A *B. melitensis* mutant in this gene has been identified as attenuated in a cellular model (Dozot et al., 2010).

Contrary to the situation in *Enterobacteriaceae*, there is no *npr* homologue in the *rpoN* locus of *Brucella* or any other α-Proteobacteria. Nevertheless, downstream from *rpoN*, there is a gene encoding an RpoN modulating protein, and further downstream there is a gene (BMEI1786) encoding the orthologue of *E. coli* EIIA^Ntr^. A *B. suis* mutant in this gene (also called *pstN* or the nitrogen regulatory protein gene) is attenuated in macrophages (Köhler et al., 2002a).

A *B. melitensis* mutant in the NPr gene (BMEI 2031) has been identified as affecting virulence and survival in both macrophages and mice (Wu et al., 2006). Two additional PTS^Ntr^ genes are localized upstream of *npr*: one is a gene encoding an EIIA^Man^-like protein (BMEI2032), while the other is *nprK/P*, which encodes a kinase catalyzing the phosphorylation and dephosphorylation of a conserved serine residue in NPr (Dozot et al., 2010).

Interestingly, in all *Brucellaceae*, *Rhizobiaceae* and in *C. crescentus*, the genes encoding NPr, EIIA^Man^ and NprK/P are close to the genes encoding a two-component system...
involved in virulence or symbiosis (BvrR–BvrS in *Brucella* spp., ChvI–ChvG in *A. tumefaciens* and ChvI–ExoS in *S. meliloti*) and phosphoenolpyruvate carboxykinase, a key enzyme of gluconeogenesis (Charles & Nester, 1993; Cheng & Walker, 1998; Sola-Landa et al., 1998). The exact role of the PTSNtr in *z-Proteobacteria* is still unclear, but as originally proposed by Hu and Saier (Hu & Saier, 2002), the conservation of this genomic locus suggests a functional link between PTSNtr, HprK/P and the neighboring genes in regulating carbon/nitrogen metabolism in these bacteria. Several experimental data are in agreement with this prediction: (i) in *B. melitensis* the PTSNtr activity is regulated by fructose 1,6-bisphosphate, and EIIAMan physically interacts with the SucA subunit of the *z*-ketoglutarate dehydrogenase, a TCA cycle enzyme (Dozot et al., 2010; Poncet et al., 2009); (ii) in several *Rhizobiaceae*, this peculiar PTS system has been linked either to succinate-mediated catabolite repression, to reduced growth on dicarboxylates or to reduced oligopeptide or amino acid transport (Untiet et al., 2013), which is also remarkably related to K+ levels; and (iii) even more importantly, EfNtr of *S. meliloti* was recently described as integrating carbon and nitrogen metabolism by sensing both the phosphoenolpyruvate to pyruvate ratio and, via its GAF domain, glutamine (but not *z*-ketoglutarate as in *Enterobacteriaceae*; Goodwin & Gage, 2014).

**Rsh (BMEI1296) and the stringent response**

The stringent response network is activated when a bacterium encounters an amino acid or carbon starvation and culminates in a drastic modification of gene expression (inhibition of rRNA and tRNA synthesis, inhibition of DNA initiation, direct stimulation of biosynthetic or catabolic operons, etc.) that allows an optimized use of the resources remaining in the environment (Chatterji & Ojha, 2001).

Most γ- and δ-Proteobacteria (Gram-negative bacteria) possess the two genes encoding the RelA and SpoT homologues (Boutte & Crosson, 2013) that synthesize the alarmones (derivatives of GTP bearing 3′-pyrophosphate residues) collectively known as (p)ppGpp. These molecules mediate the global stringent response that likely functions through an interaction with the β subunit of the RNA polymerase, thereby affecting the sigma factor recruited by the latter. In addition, DksA, a small protein that binds RNA polymerase, potentiates the effects of ppGpp on transcription (Dalebroux et al., 2010). The *α*-Proteobacteria, such as Gram-positive bacteria, have only one of these proteins, named Rsh (for RelA SpoT Homolog). In *B. melitensis, B. suis* and *B. abortus*, this protein (encoded by BMEI1296) has been shown to be necessary for virulence in cellular or mouse models of infection (Dozot et al., 2006; Kim, 2005). These data seem to be in accordance with the proposition that the initial intracellular compartment in which *Brucella* traffics is poor in nutrients (refer the ‘‘Mutants and/or data from transcriptomic or proteomic experiments’’ and ‘‘Biosynthesis of amino acids by *Brucella*’’ sections) and could thus trigger the stringent response. Nevertheless, a comparative transcriptomic analysis of a *B. suis* rsh mutant and the wild-type strain in minimal medium (as a surrogate of the poor intramacrophagic compartment) failed to show an induction of any amino acid biosynthetic pathways except for methionine. The same mutant was unable to grow in Gerhardt’s minimal medium, and only methionine was able to restore a normal growth (Hanna et al., 2013). However, a ΔmetH mutant (blocked in methionine synthesis) was not attenuated. These results were interpreted to mean that methionine was available for *Brucella* multiplication in this model and that the attenuation of the rsh mutant was not due to amino acid starvation.

**Concluding remarks**

Despite the fact that the brucellae were among the first identified intracellular bacteria (Smith, 1919), we still have an imperfect understanding of the characteristics that enable these microorganisms to thrive within a variety of cells. It is true that research during the last decade has begun to unravel the paths through which the brucellae become stealthy invaders, influence antigen presentation and possibly modulate the signaling events of the adaptive immune response (Martirosyan et al., 2011). However, some aspects of the original observations made by *Brucella* researchers more than 50 years ago remain challenging and unexplained, in particular the remarkable ability of these bacteria to multiply to high numbers without apparently disturbing the physiology and even the cytokinesis of their host cells (Richardson, 1959). As discussed in the ‘‘*Bruccella* amino acid catabolism’’ section, while attenuated variants oxidize glutamate readily and cause a rapid destruction of monocytes, virulent strains do this slowly and are not deleterious even though the host cells become engorged. Based on this, the early researchers hypothesized that it is crucial for these bacteria to not interfere severely with the physiology of the host cell to remain intracellular, thus avoiding exposure to the inimical extracellular environment created by the adaptive immune response (Wilson & Dasinger, 1960). Our current understanding of these bacteria as stealthy pathogens is based on their peculiar structural makeup and on the presence of a type IV secretion system, VirB. Both are necessary to reach a safe intracellular niche before immunity develops (Barquero-Calvo et al., 2007) and to ravenously plunder this harbor would clearly be counterproductive, particularly when we consider the multiple and well-balanced functions of the endoplasmic reticulum from which the niche derives (Berridge, 2002). It seems thus that the insight of the early researchers was correct: a subtle but also efficient use of the resources within the cells is the necessary complement to the stealthy strategy employed at the onset of infection. Moreover, current evidence shows that, after trafficking from the endocytic compartment and proliferation in the endoplasmic reticulum-derived niche, the vacuole containing the bacteria is converted into a compartment with autophagic features from which the exit and invasion of the adjacent cells can occur once growth has stopped (Starr et al., 2012). An additional clue that may be of critical importance is the fact that the brucellae, such as many *α*-Proteobacteria, display polar growth and asymmetrical division (Van der Henst et al., 2013). In *C. crescentus*, another *α*-Proteobacteria member that is the paradigm of asymmetrical cell division, nitrogen limitation extends the life span of the migratory form.
(England et al., 2010). It is thus intriguing to consider how and to what extent these ancestral α-Proteobacteria characteristics are relevant in an intracellular parasite. Under all these perspectives, it is of primary interest to understand the regulatory systems and the environmental signals that explain how the brucella keeps a tight control of their nitrogen and carbon metabolism and how these are coordinated with the bacterial cell cycle.

**Declaration of interest**

The authors report no declarations of interest.

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