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Highlights

▶ The nature of the metabolic network is a fundamental aspect of pathogenic lifestyles. ▶ *Brucella* spp. are the intracellular pathogens responsible for chronic infections of mammals. ▶ Here we review new insights on the links between *Brucella* virulence and metabolism. ▶ Understanding of *Brucella* metabolic abilities will help to decipher its infectious strategies.

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Review

Brucella adaptation and survival at the crossroad of metabolism and virulence ☆

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ABSTRACT

"In vivo" bacterial nutrition, i.e. the nature of the metabolic network and substrate(s) used by bacteria within their host, is a fundamental aspect of pathogenic or symbiotic lifestyles. A typical example are the *Brucella* spp., facultative intracellular pathogens responsible for chronic infections of animals and humans. Their virulence relies on their ability to modulate immune response and the physiology of host cells, but the fine-tuning of their metabolism in the host during infection appears increasingly crucial. Here we review new insights on the links between *Brucella* virulence and metabolism, pointing out the need to investigate both aspects to decipher *Brucella* infectious strategies.

1. Introduction

In order to successfully colonize a host, symbiotic and pathogenic bacteria have to be able to occupy specific metabolic niches within their host. Indeed, it is becoming more and more obvious that the sensing of available carbon source(s) and the related metabolic adaptations are intimately linked to the coordinated expression of other virulence determinants, such as colonization factors [1,2]. However with the exception of some recent progress on model bacteria [5–7], the mechanistic basis for this coordination is still frustratingly poorly understood [3–5]. Here, we review the current on the links existing between metabolism and virulence of a particular intracellular pathogen: *Brucella*.

1.1. *Brucella*, a nasty Mr "Hides"

Brucella spp. are Gram-negative intracellular pathogens phylogenetically related to plant symbionts such as the Rhizobiaceae. Often referred as "nasty bugs" [8] because of their unusual virulence features, or as "Mr Hides", in reference to their stealthy ability to evade immune detection [9], they are major zoonotic pathogens, as they are able to induce chronic infections of both animals and humans [10,11]. In Latin America alone, the annual economic loss in animal production from brucellosis has been estimated to be more than $600.000.000 [12].

During the last few decades, efforts to solve the complex jigsaw puzzle of *Brucella* virulence have focused on "classical" virulence factors, or bacterial factors that interact directly with components of the host [13]. Despite an increasing knowledge of the molecular strategies used by this pathogen to interact with host cells during its infectious cycle [14,15], we are still far from understanding it. Moreover, a new piece of this puzzle, long forgotten, has come into view: bacterial metabolism.

2. *Brucella* virulence and metabolism: two sides of the same coin

The global picture emerging from what is known about *Brucella* virulence is an extremely efficient adaptation to shield itself from immune recognition and to manipulate key aspects of host cell physiology, for example apoptosis and vacuolar trafficking [8,9,14–16]. It has also become increasingly evident, though still poorly considered, that one of the keys to successful in vivo adaptation of a pathogen is its ability to fine-tune the metabolism to utilize specific nutrients encountered in each niche occupied by *Brucella* during the infectious cycle [4,17].

One aspect of the physiology of the *Brucellae* that is particularly poorly understood is the architecture and regulation of central metabolic pathways [18]. According to pioneering biochemical investigations [19], as well as more recent genomic data, hexoses can be catabolized through the pentose–phosphate (PP) pathway and an incomplete Embden–Meyerhof–Parnas glycolytic pathway (EMP), as *Brucellae* seem to lack a phosphofructokinase. However, in some cases, functions predicted from genomic analysis do not agree with results from biochemical analysis of metabolic function in vivo. For example, while genomic analysis indicated that *Brucellae* carry two genes predicted to encode enzymes of the Entner–Doudoroff (ED) pathway (gluconate-6-phosphate dehydratase and 2-keto-3-deoxygluconate aldolase), no gluconate-6-phosphate

☆ Dis-moi ce que tu manges, je te dirai ce que tues. Physiologie du goût (1825), Aphorisme IV. Citations de Anthelme Brillat-Savarin.

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dehydratase activity could be detected in vitro [19]. This is also true for the two enzymes of the glyoxylate shunt (isocitrate lyase and malate synthase), whose activity has never been demonstrated. Hexoses can be further metabolized through what appears to be a fully active tricarboxylic acid cycle (TCA) [19]. Nevertheless, it bears mentioning that hexoses are not the favoured carbon sources for the three best characterized *Brucella* species: *B. abortus*, *B. melitensis* and *B. suis*. Instead, these bacteria preferentially utilize a four carbon sugar alcohol (erythritol) [20], the catabolism of which yields one triose phosphate [21]. In summary, the actual picture of the central metabolic network of *Brucella* spp. appears to be: (i) active PP and TCA cycles, (ii) potentially active ED and glyoxylate pathways (iii) an interrupted EMP. It should be kept in mind that the above description reflects what is known about the most thoroughly investigated three main *Brucella* species. As new genomic sequences become available, species differences in this metabolic network will certainly emerge as illustrated, for example, by the pseudogenization in *B. ovis* of some genes of the erythritol catabolic and transport operons or of the phosphoenolpyruvate carboxykinase (*pckA*) gene involved in the first step of gluconeogenesis [22]. While these differences are likely to be of interest to understanding species differences between the *Brucellae*, due to the limited biochemical characterization of these additional species we will focus our review on three best characterized *Brucella* species.

The information above outlines the potential "architecture" of the central metabolic network of *Brucellae*. However, from this "blueprint", we can glean little about the functional metabolic pathways and nothing about the potential of *Brucellae* to adapt their metabolism to conditions in the host. However, the first clues on the nature of the in vivo metabolism of *Brucella* were provided by the identification of attenuated mutant strains.

2.1. Metabolic mutants are frequent among attenuated mutants

Previous studies, aiming to identify virulence factors in *B. abortus* or *B. melitensis* by screening for transpositional mutants attenuated in the cellular or the mouse model of infection, revealed a link between persistence of *Brucella* in its hosts and its metabolism [23,24]. Indeed, several systems for transport and degradation of carbohydrates appear to be essential for *Brucella* survival. Transporters whose predicted function is uptake of amino acids or peptides also appear to be required during infection. These findings suggest that carbohydrates, but also amino acids and peptides, could be available as energy and/or carbon sources at some points during the infectious process.

It can be expected that some of these carbon sources are likely metabolized through the PP pathway, since among the attenuated metabolic mutants, several are impaired in a gene encoding an enzyme of this pathway (see Fig. 1, boxes n°6). This is consistent with the fact that *Brucella* seems to lack a phosphofructokinase, for a "classical" glycolysis EMP [18]. The PP pathway is consequently suspected to be crucial for sugar degradation in addition to being essential for the generation of biomass precursors such as ribose required for de novo synthesis of purines and pyrimidines [25–27]. In addition, mutants in global regulators affecting metabolism are attenuated, emphasizing the fact that *Brucella* has to adapt its metabolic functions (including its central metabolism) for a successful infection. For example, *B. melitensis* and *B. suis rsh* mutants with an impaired stringent response are severely attenuated [28]. It has been recently shown in alpha-proteobacteria closely related to *Brucella* (namely *Sinorhizobium meliloti* and *Rhizobium etli*) that the stringent response to nitrogen or carbon limitation not only regulates expression of genes encoding biosynthetic or catabolic pathways (protein, amino acids, nucleotides, and lipids) but has also an impact on expression of genes encoding the functions of central metabolism (PP and EMP pathways as well as TCA cycle) [29,30]. Furthermore, three mutants in the *Brucella* Phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) are also impaired in their virulence [25] (see below). Similar mutants in *S.meliloti* were affected in their carbon metabolism and in their ability to cope with nutritional stress [31].

2.2. A profound and progressive adaptation of central metabolism occurs as Brucella enters and persists in its intracellular niche

Two recent studies further illustrate the central metabolic adaptation performed by *Brucella* during intracellular infection. In the first one, the proteome of *B. suis* was analyzed in J774 macrophages at 48 h post infection (PI) and compared to the proteome of *B. suis* at the early stationary phase in a rich medium [32]. The majority of the 44 differentially produced proteins are involved in the primary metabolism (metabolism strictly needed for survival) of *Brucella,* among which nine are related to the central metabolism (see Fig. 1, boxes 1). The results suggest that at 48 h PI *Brucella* had a restricted glycolytic activity and an increase in gluconeogenesis. Moreover isocitrate lyase (AceA) and malate synthase (AceB), two enzymes belonging to the glyoxylate shunt, were upregulated [32]. The glyoxylate shunt acts as an anaplerotic pathway for the Krebs cycle, providing succinate and malate from acetyl-CoA and isocitrate. Usually, a functional glyoxylate shunt allows bacteria to grow on fatty acids, which might thus become an important carbon source for *B. suis* during infection, as has been reported for *Mycobacterium tuberculosis* [33].

The second study adds a temporal dimension to the physiological adaptation. Using RAW 264.7 macrophages, Lamontagne et al. performed a proteome analysis on *B. abortus* at three time points: 3 h PI (when the bacteria are internalized but have not yet reached the replicative niche), 20 h PI (when they have escaped the initial microbicidal "burden" and started an active replication) and 44 h PI (when they reached the maximum of their intracellular number) [17]. Ninety proteins were differentially produced in *B. abortus* and most of them took part in primary metabolism, of which seven are involved in the central metabolism (see Fig. 1 for the 3 h time point boxes 7). The reduced production of enzymes of central carbon metabolism (TCA cycle, pyruvate and PP pathway), and of sugar uptake transport systems suggests that there is a limited sugar supply at the beginning of infection. At this time, amino acid catabolism feeding the TCA could be the privileged alternative to derive the needed precursors. At later time points, once in the ER derived compartment, the PP pathway would be active suggesting a re-supplying of sugars [17].

Thus, these in vivo experiments revealing dynamic metabolic adaptations during cellular infection, were particularly valuable, since they unmasked a metabolic flexibility that could not have been predicted using classical in vitro growth conditions.

2.3. Major virulence regulators act as metabolic regulators "and vice et versa"

The metabolic adaptations described above allow *Brucella* to withstand the wide array of environmental conditions existing within a host and its cells. In response to the conditions encountered at each specific stage of the infectious cycle, a tight and coordinated fine-tuning of gene expression is needed while unnecessary (or no longer needed) functions are accordingly switched off. Expression of virulence genes is usually governed by signaling pathways and regulatory mechanisms similar to those that control genes that are not specific to pathogenesis. These signaling pathways are often based on reversible phosphorylation of proteins (two component system or phosphoenolpyruvate dependent sugar

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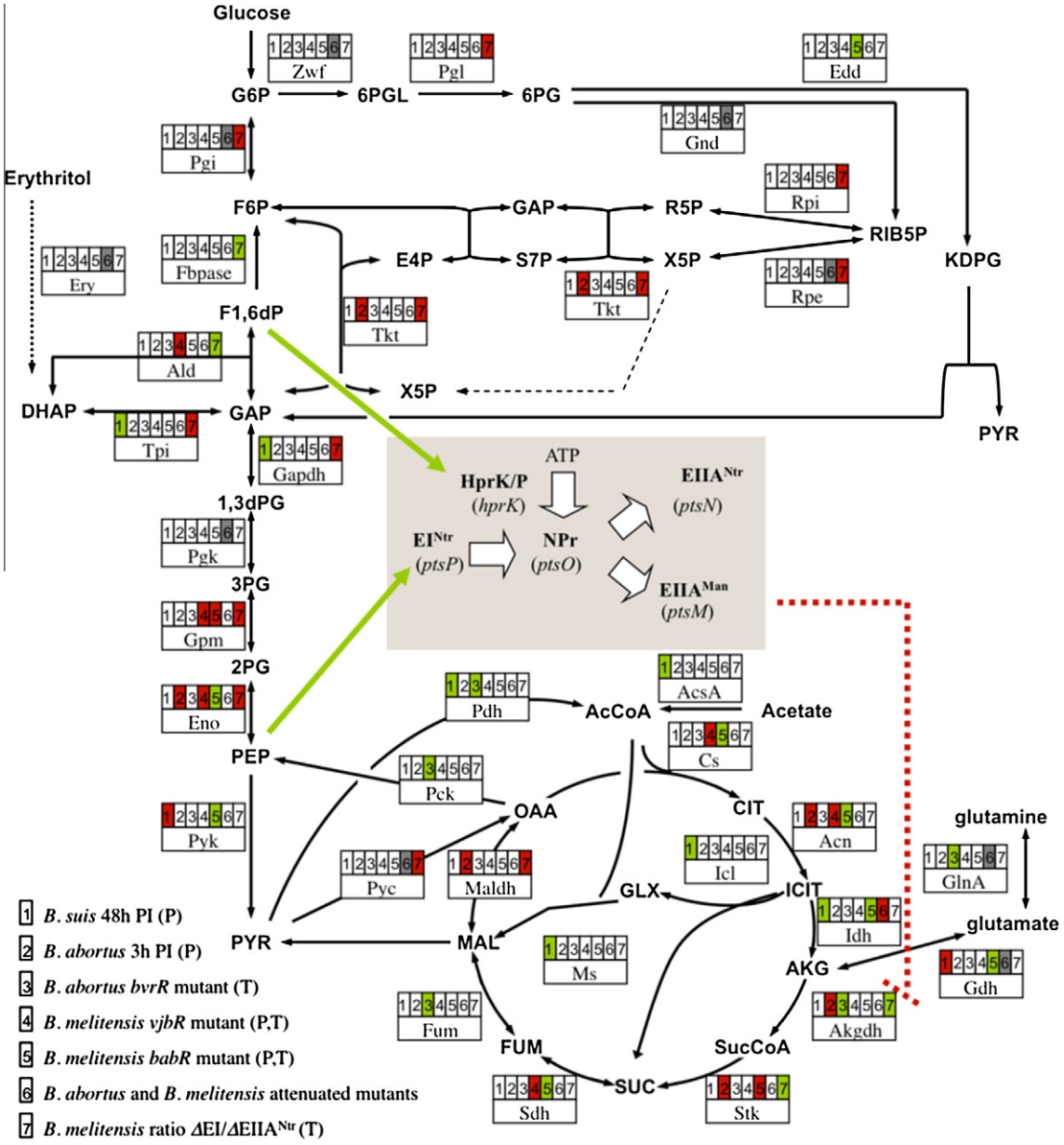

Fig. 1. Regulations of *Brucella* central metabolism in various conditions (see numbers in boxes). In green (boxes and arrows), up-regulations; in red (boxes and arrows), down-regulations; in grey, attenuated mutants. P: proteomic data, T: transcriptomic data. Pgi, phosphoglucoisomerase; Fbpase, fructose-1,6-biphosphatase; Ald, aldolase; Tpi, triose phosphate isomerase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gpm, phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase; Pck, PEP carboxykinase; Pyc, pyruvate carboxylase; Pdh, pyruvate dehydrogenase; AcsA, Acetyl- coenzyme A synthetase; Cs, citrate synthase; Acn, aconitase; Idh, isocitrate dehydrogenase; Akgdh, 2-oxoglutarate dehydrogenase; Stk, succinyl-CoA synthetase; Sdh, succinate dehydrogenase; Fum, fumarase; Maldh, malate dehydrogenase; Icl, isocitrate lyase; Ms, malate synthase; Zwf, glucose-6-phosphate dehydrogenase; Gnd, 6-phosphogluconate dehydrogenase; Rpi, ribose-5-phosphate isomerase; Rpe, ribose-5-phosphate epimerase; Tkt, transketolase; Edd, phosphogluconate dehydratase; GlnA, glutamine synthetase; Gdh, glutamate dehydrogenase. (1) *B. suis* 48h PI (proteomic data from [27]); (2) *B. abortus* 3h PI (proteomic data from [16]); (3) *B. abortus bvrR* mutant (transcriptomic data from [30]); (4) *B. melitensis DvjbR* mutant (transcriptomic and proteomic data from [37]); (5) *B. melitensis DbabR* mutant (transcriptomic and proteomic data from [37]); (6) *B. abortus* and *B. melitensis* attenuated mutants [21,27]; (7) *B. melitensis* ratio *D*EI/DEIIA^{Ntr} mutants (unpublished transcriptomic data).

phosphotransfer system) or specialized global regulators acting either at an individual cell or at a population level.

2.3.1. The critical BvrS/R two component system

The BvrS/R two component system (TCS) is a signaling pathway consisting of a membrane-bound histidine kinase (BvrS) and its corresponding response regulator (BvrR). Following the sensing of a (still unknown) specific environmental stimulus, BvrS autophosphorylates on a conserved histidine residue and mediates the transfer of the phosphoryl group to a conserved aspartate of BvrR. The latter coordinates the cellular response, through differential expression of target genes. The BvrS/R TCS is essential for virulence. Transpositional inactivation leads to defects in attachment, invasion, and intracellular replication [34]. A recent transcriptomic analysis revealed a clear impact of the *bvrR* mutation on the expression of genes related to carbohydrates, amino acids, fatty acids and nitrogen metabolism [35]. Among the genes up-regulated in the *bvrR* mutant are the phosphoenolpyruvate carboxykinase (*pckA*) encoding the first enzyme in gluconeogenesis, and four genes involved in TCA cycle and pyruvate metabolism (see Fig. 1, boxes 3).

Initially thought to regulate the homeostasis and structure of the *Brucella* cell envelope (Outer membrane proteins (Omp), lipoproteins, LPS, several periplasmic transporters), the BvrR/BvrS

TCS appears to affect a larger range of phenotypes related to metabolic functions that potentially mediate adaptation to an intracellular lifestyle [17,35].

Nevertheless, it should be mentioned that for the *bvrR* transposon mutant used for the studies discussed above, questions remain about the whether the transposon insertion led to a loss or gain of BvrR function, since attempts to create defined genomic disruptions or null mutations were unsuccessful, prompting its designation as essential gene. Similar observations was made for *Agrobacterium tumefaciens* and *S. meliloti* homologues of *bvrR* [36,37]. Mutation in these TCS prevents growth of the bacteria in complex media [38] and a null mutant can only be obtained on minimal media [38]. In addition, the homologue of *bvrR* in *S. meliloti* (*chvI*) is strictly needed for growth on more than 21 different carbon sources [38] and *the bvrR* mutant grows poorly on minimal medium [35], thus reinforcing the link between this TCS and *S. meliloti* metabolism. Whether the effects of *bvrR* transpositional mutation on the cell envelope (Omp and transporters) is the consequence or the cause of these growth defects remain to be investigated by identifying the direct targets of the regulator. The potential link of this TCS with the metabolism will be discussed further below in parallel with the Phosphotransferase System (PTS).

2.3.2. Quorum sensing and starvation sensing

Quorum sensing (QS) is a regulatory system that allows bacteria to coordinate gene expression at the population level according to the local bacterial cell density through the individual synthesis and sensing of diffusible signal molecules. Quorum sensing is also known to be involved in the regulation of *Brucella* virulence determinants mostly linked to the cell surface (Type IV secretion system, flagellum, outer membrane proteins and exopolysaccharide) [39–41]. Surprisingly, recent transcriptomic and proteomic analyses have put forward that inactivation of *vjbR* and *babR*, two QS regulators, has a strong impact on genes involved in metabolism and particularly on genes encoding enzymes of the TCA cycle and glycolysis (Fig. 1, boxes 4 and 5 respectively) [42,43]. Interestingly, VjbR and BabR regulate overlapping sets of target genes in an opposing manner, suggesting that QS could have a global reorganization effect on central metabolic processes. No growth delay for the *vjbR* and *babR* mutant strains could be observed though liquid or solid culture in rich media. However, differences in growth of these mutants were reported in defined media, depending on the available carbon source.

Placed into an intracellular context, in the vacuole, sensing a "Quorum" for *Brucella* could mean sensing limited diffusion due to space limitation. That corresponds to "starvation sensing". It can be suggested that QS is directly or indirectly involved in adjusting the metabolism of *Brucella*. Indeed, by slowing down *Brucella* basic metabolism, QS (through VjbR) would prevent multiplication until the ER-derived replicative compartment is reached. Subsequently, the BabR regulator could play a role in reactivating the basal metabolism. A similar proposal was made for the BvrS/R TCS [17,35]. Thus, both the BvrS/R TCS and the QS system could contribute to the adaptation of the metabolic network during the nutrient shift faced by *Brucella* all along its intracellular trafficking continuum. These two regulatory systems appear to be connected, since BvrR has an activating effect on *vjbR* transcription [35,44]. However, it is not yet known whether this activation is direct or whether it is mediated through other global starvation sensing mechanisms like the stringent response [28] and/or the PTS system [45].

2.3.3. The phosphoenolpyruvate phosphotransferase system (PTS) – a missing link

PTS systems are widespread among bacteria. Activated by phosphoenolpyruvate (PEP), this system usually consists of two cytoplasmic energy-coupling proteins (Enzyme I and HPr) as well as several carbohydrate-specific Enzymes II, which catalyze concomitant carbohydrate translocation and phosphorylation [46]. The phosphorylation status of PTS components reflects both the availability of carbohydrates and the energy conditions of the cell. In many bacteria, PTS and their associated proteins convert this information to signals, which are then transduced through different mechanisms (allosteric interactions, phosphorylation) and lead to phenomena including catabolite repression and inducer control [46,47]. The PTS provides bacteria with an integrated system that ensures optimal utilization of carbohydrates in complex environments, a feature that is particularly important in host–bacteria interaction [1,48,49]. In place of a classical PTS, some bacteria have evolved parallel systems that serve strictly regulatory functions. Among such systems are the so-called "Nitrogen PTS", which are thought to link carbon and nitrogen metabolisms but do not appear to catalyze substrate transport, as they lack the PTS permeases [50–53]. *Brucella* spp., *A. tumefaciens*, *S. meliloti* and other α-proteobacteria possess a Nitrogen PTS system, and their respective *pts* mutants were previously shown to be impaired for interaction with the host [25,54,55].

In all pathogenic or symbiotic α-proteobacteria, three *pts* genes (*hprK, ptsM* and *ptsO*) are located downstream of the conserved two-component system genes (*bvrS/R, exoS/chvI*) essential for infection or symbiosis [34,38]. This conserved genomic organization suggests a functional link between BvrS/R and the PTS [53,56]. Recently two papers substantiated this link by showing (i) that the above mentioned *pts* genes are co-transcribed with the *bvrS/R* genes [45] and (ii) that the *hprK* gene is downregulated in the *bvrR*:Tn5 mutant [35]. In addition, a proteomic study with a *B. abortus bvrR* mutant [57] revealed that BvrR regulates the 2-oxoglutarate dehydrogenase complex that converts 2-oxoglutarate into succinyl-CoA in the TCA cycle. The subunit SucA of the same enzyme was recently shown to interact with the PTS $EIIA^{Man}$-like protein encoded by *ptsM* itself located at the specific conserved locus previously mentioned [45]. Both *bvrR* and *pts* mutants have an unaltered growth in liquid rich media but display a restricted or abolished growth on minimal medium with defined carbon source(s) [35,45]. These findings support the hypothesis that the *Brucella* PTS senses the metabolic state of the cell (by sensing among others the ratio of PEP/pyruvate and the fructose 1,6-biphosphate) leading to a coordinated regulation of C and N metabolisms and as well as some key virulence genes (e.g. the *virB* operon [45], flagellar genes ...). This probably involves cross-talk with the two-component system BvrS/R. Interestingly, both the *bvrR* and one *pts* mutant (*ptsP*) seem to share the ability to regulate the expression of the QS regulator VjbR, which in turn regulates determinants of virulence and metabolism (see above).

3. What is NOT known about the central metabolism of *Brucella*: challenges for the ongoing century

Of course, this paper is not an exhaustive review of all the links connecting virulence and metabolism of *Brucella*. Our focus being mainly the central metabolism, we omitted some known links (*i.e.* the recent identification of the *virB* gene regulator HutC [58] or the role of the stringent response in regulating the crucial type IV secretion system [28]. In the near future other connections will likely be discovered. A major breakthrough will certainly come from the newly evolving field of RNA based regulation. Long considered only as informative macromolecules, small RNAs (sRNAs) are increasingly recognized as important regulators of gene expression allowing the rapid adaptation of cell growth in response to stress and changes in the environment. These sRNAs post-transcriptionally modulate gene expression, mostly through

base-pairing with target mRNAs, thereby regulating relative levels of translation or decay [59]. In addition, messenger RNAs themselves can act as direct sensors of the physical or metabolic state of the cell via their 5′-untranslated (5'-UTR) region that undergo structural changes upon metabolite binding (riboswitch). The conformational alteration of the mRNA structure affects the expression of the downstream transcript [60]. Altogether, these RNAs are widespread in bacteria and regulate metabolic pathways, carbon source utilization and the composition of the membrane [61]. Moreover, their direct or indirect involvement in the regulation of virulence genes and host-pathogen relationship is becoming more and more clear [62,63].

With regard to the impact of these sRNA on *Brucella* metabolism or virulence, this is almost "terra incognita". Nevertheless, owing to the recognized role of Hfq in facilitating the action of sRNA and the importance of this RNA binding protein in *Brucella* adaptation [64] the chances are high that *Brucella* RNA regulation will be brought to the center stage as has recently been the case for other intracellular bacterial pathogens such as *Listeria monocytogenes* [65,66] or *Legionella pneumophila* [67].

Nevertheless some basic questions, concerning the functioning of *Brucella* metabolism, still remain to be investigated:

What parts of the central metabolic network are functional, and under which conditions?
Why is erythritol a preferred carbon source for *Brucella*?
How is catabolite repression (if any) mediated in *Brucella*?
What are the carbon sources (sugars and/or amino-acids) that are available intracellularly?
How is the regulation of crucial virulence factors connected to central metabolic adaptation?
How does the PTS regulate the carbon fluxes in the central metabolism?
What is the link between the PTS and the BvrS/R TCS?
What is the link between these two regulatory systems and Quorum Sensing?
Is the metabolic network and/or its regulation responsible of the host specificity of *Brucella* strains?
And to a greater extent, how has the intracellular lifestyle of *Brucella* influenced the design of its metabolic network?

Undeniably, the way we are looking at bacterial physiology and host bacteria interactions is rapidly evolving in the «omics» era. In the near future, new approaches such as metabolomics [68] or ^{13}C-isotopologue-profiling analysis [2] will lead to an increased understanding of the *Brucella* metabolic plasticity both in vitro and during cellular infection. This will yield new insights on *Brucella* virulence and will, potentially, open new prophylactic avenues.

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