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Fun stories about *Brucella*: the ''furtive nasty bug''

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Abstract

Although *Brucella* is responsible for one of the major worldwide zoonosis, our understanding of its pathogenesis remains in its infancy. In this paper, we summarize some of the research in progress in our laboratory that we think could contribute to a better understanding of the *Brucella* molecular virulence mechanisms and their regulation.

Keywords: *Brucella*; Virulence; Quorum sensing; Flagella; CtrA

1. Introduction

Brucella was called ''nasty'' by Moreno and Moriyon in their commentary paper on the genomic sequence of *Brucella melitensis* published by DelVecchio and co-workers (DelVecchio et al., 2002; Moreno and Moriyon, 2002). *Brucella* is ''furtive'' as well. Unlike many other pathogenic bacteria, *Brucella* lacks obvious classical virulence factors such as capsules, fimbriae, flagella, exotoxins, exoenzymes including exoproteases, cytolysins, resistance forms, antigenic variation, plasmids and lysogenic phages. Further, *Brucella* is a facultative intracellular pathogen of both professional and non-professional phagocytic cells, hiding within the immune system from the immune system!

In the last decade, several technical improvements such as transposon mutagenesis, in vivo expression technology (IVET), differential fluorescence induction (DFI), and signature tagged mutagenesis (STM) have made it possible to analyze the molecular basis of microbial pathogenesis on a genome wide basis. In conjunction with the emerging

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discipline of cellular microbiology and its satellite techniques (confocal microscopy, etc.), the study of the host–pathogen relationships has progressed rapidly. The *Brucella* scientific community seized the opportunities offered by these powerful approaches to learn the secrets of the pathogenesis of their favorite bacterial pathogen.

The ever-increasing reports on the virulence determinants of *Brucella* have been reviewed recently (Boschiroli et al., 2001). Our team is very involved in the identification of virulence genes and in deciphering the virulence mechanisms of *Brucella* (Godfroid et al., 1998, 2000; Lestrate et al., 2000; Delrue et al., 2001). The purpose of this paper is not to make a complete survey of what has already been published by ourselves and others but, rather, to focus on three amazing and intriguing stories of *Brucella* that we discovered and are presently investigating further: communication by quorum sensing, flagellar apparatus without motility, and cell cycle regulation.

2. To communicate or not to communicate: the quorum sensing story

A novel communication circuit called quorum sensing (QS) that is involved in the regulation of virulence gene expression among diverse bacterial species and dependent on the number of cells was described recently (Fuqua et al., 2001). Quorum sensing describes a phenomenon in which the accumulation of a signal or actor molecule called a pheromone allows individual cells to perceive when a minimum bacteria number or quorum is reached. The signal allows for the initiation of a concerted response by the bacterial population. Several distinct families of pheromones have been identified (Holden et al., 1999; Pesci et al., 1999). In Gram-negative bacteria, *N*-acyl-L-homoserine lactone (HSLs) is the most widely studied family.

Quorum sensing was described first in *Vibrio fisheri*, a marine symbiotic bacterium. For this bacterium, quorum sensing regulates bioluminescence production by activation of two transcriptional units, *luxR* and *luxICDABEG* (operon *lux*). LuxR is a transcriptional activator of the *lux* operon. LuxI catalyzes HSLs production. The remaining genes encode the luciferase and its substrates. Light emission by *V. fisheri* occurs only at high cell-population density in response to the accumulation of secreted signaling molecule. At low density, *luxI* is transcribed at a low basal level and the concentration of HSL is low. When the population density raises, HSLs or the actor signal accumulates until a critical concentration is reached activating LuxR and inducing expression of the *lux* operon. This system allows for fast signal amplification (Fig. 1).

The QS mechanism described above is used by many Gram-negative bacteria for regulation of genes involved in symbiotic or pathogenic relationships with plant or animal hosts and is based on at least one protein pair homologous to LuxR/LuxI of *V. fisheri*. The importance of these communication systems to bacterial virulence is confirmed by the attenuation of bacterial pathogens by mutation of the quorum sensing locus as shown for both *Pseudomonas aeruginosa* and *Erwinia caratorova* (Pirhonen et al., 1993; Tang et al., 1996).

Insertional transposon mutants of *B. melitensis* 16M that are unable to replicate in HeLa cells have been isolated and characterized recently. In most of these mutants, the transposon disrupted one of the genes of the *virB* operon, leading to altered intracellular

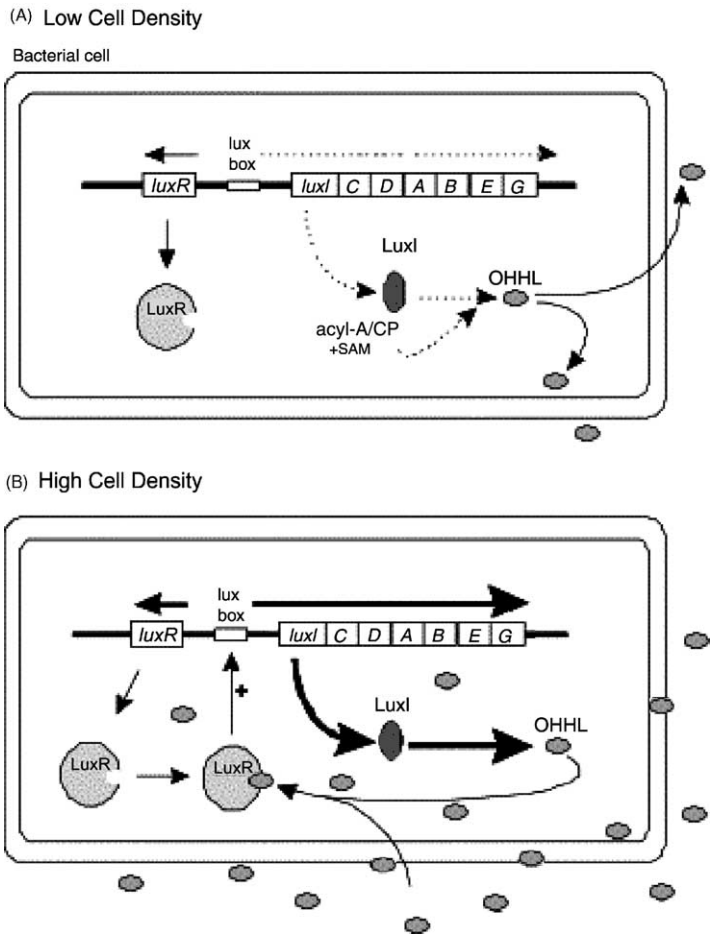

Fig. 1. The regulation of bioluminescence in *V. fischeri*: the quorum-sensing paradigm. (A) At low cell density, transcription of the genes for bioluminescence (*luxICDABEG*) is weak and insufficient for light emission due to low levels of OHHL. (B) At high cell density, a critical concentration of OHHL is reached. OHHL binds to LuxR and stimulates transcription of *luxICDABEG*, leading to rapid amplification of the OHHL signal and emission of light (Whitehead et al., 2001).

trafficking of the *Brucella* vacuole. The vacuole containing the mutant still avoids lysosomal fusion but seems unable to maturate into a replicative RER-like vacuole like the parental strain (Delrue et al., 2001). A recently isolated transposon mutant of *Brucella* has essentially the same phenotype. The transposon disrupts a homologue of a transcriptional regulator of the LuxR family involved in QS and was designated *vjrR* for vacuolar jacking *Brucella* regulator (*vjbR*, Delrue, unpublished data). The phenotype of this mutant led us to ask: is there a link between VirB expression and a putative *Brucella* QS system? And to ask more generally: who are the molecular actors of *Brucella* QS? And what are the biological functions of *Brucella* QS?

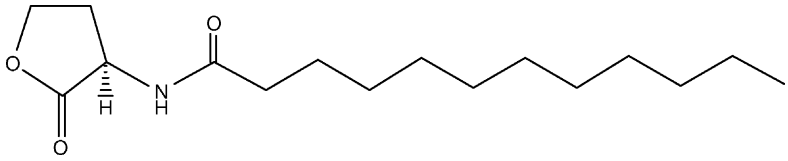

Fig. 2. The chemical structure of C12-HSL.

By the use of *E. coli* (pSB1075) and *E. coli* (pSB401) HSLs sensor strains (Winson et al., 1995), we have been able to identify a quorum sensing inducing molecule in a dichloromethane extract of spent culture supernatant from *B. melitensis* 16M. The pheromone was characterized by HPLC fractionation followed by mass spectrometry. The mass spectrum was found to be identical to that of synthetic *N*-dodecanoylhomoserine lactone (C12-HSL) (Fig. 2, Taminiau et al., 2002). A second inducing signal was also isolated, but we were unable to identify it by mass spectrometry. However, on thin layer chromatography assay (TLC) (Winson et al., 1995), this molecule migrates with a Rf value similar to that of 3-oxo-*N*-dodecanoylhomoserine lactone (3-oxo-C12-HSL), which is also produced by *P. aeruginosa*. This is the first report of the production of acyl-HSL by an intracellular pathogen.

Shortly after this identification, we learned from D. O'Callaghan and co-workers that transcription of the *virB* operon was dependent on the phase of the growth curve in E medium (Boschiroli et al., 2002). To determine whether this control was due to QS, we designed an experiment to test whether the addition of C12-HSL to *Brucella* cultures in early exponential phase would also trigger the decrease of *virB* mRNA level as observed at higher bacterial densities. Hybridization analysis revealed that, although it had no impact on general transcription, the addition of the pheromone diminished the amount of *virB* mRNA as compared to the control. This observation strengthens our belief that the pheromone has biological relevance and establishes a clear link between QS and the type IV secretion system (Taminiau et al., 2002).

In genetic screens to identify other molecular actors of *Brucella* QS, we isolated a sequence encoding a second *Brucella* homologue of QS response regulators of the LuxR family (BabR). We continually failed to identify a HSLs synthase (LuxI homologue). Moreover, the available genomic sequences of *B. melitensis* and *Brucella suis* have been scanned for the presence of known acyl-HSL synthase homologues without success. Thus, we suspect that the pheromones of *Brucella* are synthesized by a novel class of enzymes that we are seeking. Links between the *Brucella* HSLs, the two response regulators, control of *virB* operon expression, and effects on other biological properties of *Brucella* are currently under investigation.

3. To move or to secrete: the story of the flagellar gene clusters in *Brucella*

Eighty percent of the known bacterial species are flagellated. Assembly of a flagellum and the taxis system require around 50 genes and 1% of the energy of the cell (Macnab, 1996). Search for a favorable niche is the obvious function of flagellum based mobility but,

in pathogenic bacteria, this structure could also play a role in colonization or adhesion. The homology between subunits that form the protein transport apparatus of the flagellum and the contact dependent type III secretion apparatus led to the hypothesis that both apparatus share a common evolutionary origin (Harshey and Toguchi, 1996). Further, it has been demonstrated recently that the flagellar apparatus of *Yersinia enterocolitica*, in addition to having a dedicated role in flagellum biogenesis, is also involved in the transport of proteins involved in bacterial-host interactions (Young et al., 1999) and thus independent of mobility per se.

In the family of α-Proteobacteria, the flagellar systems that are the best described are from *Sinorhizobium*, *Agrobacterium* and *Caulobacter*. Flagellar genes are usually organized in one (*Sinorhizobium meliloti*) or several clusters (more than 10 in *Caulobacter crescentus*). There is a regulatory hierarchy of expression of these genes in four classes which reflect the temporal biogenesis of the flagella in the membrane: the class II genes encoding the basal body being first expressed and so on until the class IV genes corresponding to the filament. These genes are under the control in *C. crescentus* of the master class I gene product CtrA. The transcriptional regulator CtrA is a member of a complex phosphorelay system which coordinates flagellum expression with intracellular and environmental clues in *C. crescentus*. Homologues of CtrA are exclusively found among the α-Proteobacteria and could have similar functions in other flagellated bacteria of this family (e.g. differentiated bacteroids of *Rhizobium* are no longer flagellated; Moens and Vanderleyden, 1996).

Even though brucellae are described as non-motile, open reading frames (ORFs) encoding homologues of several flagellar-related proteins (FliF, FlgE, and FliC) were described in *Brucella abortus* (Halling, 1998). In related bacteria, these proteins are part of three different structural levels of a bona fide flagellum: the MS ring of the basal body (FliF), the hook (FlgE) and the flagellin filament (FliC). This suggested the presence of other flagellar gene homologues in *B. abortus*. Since flagellar-related genes are generally physically linked, we screened a *B. abortus* genomic library with a *flgE* probe and isolated a clone whose sequence demonstrates the presence, downstream of *flgE*, of eight other flagellar gene homologues (GenBank accession number GI12330679). Concurrently, S. Halling found five other ORFs with similarity to *flgC*, *fliE*, *flgG*, *flgA*, and *flgI* on a 4.3 kb DNA insert of *B. abortus* (Halling, personal communication).

The availability of the genome of *B. melitensis* 16M (DelVecchio et al., 2002) and *B. suis* data (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=b_suis) allowed us to complete the inventory of the flagellar genes. We identified 28 flagellar genes, distributed in three clusters on the small chromosome. All the structural genes for building a flagellum are present but no gene for chemotactic receptors or transducers were detected. Parts of these clusters showed a clear syntheny with other *Rhizobiaceae* (Kaneko et al., 2000a,b; Barnett et al., 2001a,b; Capela et al., 2001; Finan et al., 2001; Galibert et al., 2001). It is worth noting that *Brucella* shares with *Mesorhizobium loti* the following peculiar gene organization: the class II gene encoding the very first structure of the basal body; the MS ring (*fliF*) which is located downstream of and in the same orientation, as the class IV gene (*fliC*) encoding the last structure of the flagellar apparatus and the flagellin filament.

In addition, several ORFs strictly typical of α-Proteobacteria flagellar system are also present: *motC* and *motD* involved in the motility of *S. meliloti* (Platzer et al., 1997), *flaf* and

flbT involved in the building of filament in *C. crescentus* (Schoenlein et al., 1992; Mangan et al., 1999), at least two ORFs present in the flagellar cluster of different *Rhizobiaceae* without assigned function (Sourjik et al., 1998).

The absence of an identified chemotactic system precludes a role of these genes in a flagellum based mobility and leads to the following questions: Are the products of these genes involved in building a flagellar apparatus that are relevant for some aspects of the *Brucella* lifecycle or are they only evolutionary remnants? An STM screen of mutants in mice (Lestrate et al., 2000) helped to answer the question. One of the tagged transposon mutants of *B. melitensis* negatively selected in mice was disrupted in the *fliF* gene. Confirming data came from the work of Köhler on *B. suis* using differential fluorescence induction (DFI) with *gfp* as a reporter gene (Köhler et al., 1999). He showed that one of the *B. suis* promoter specifically induced in cell is the *fliF* promoter. It is interesting to note that *fliF* is homologous to *yscJ* of *Y. enterolitica* (Young et al., 1999). A systematic disruption of the flagellar genes is underway in our laboratory in order to get further insight into the biological function of the flagellar apparatus in *Brucella*.

4. Peculiarities of the cell cycle control in *Brucella*: the CtrA regulon story

The *ctrA* gene (cell cycle transcriptional regulator A) identified first in the asymmetrically dividing α-Proteobacterium *C. crescentus* (Quon et al., 1996) encodes an essential response regulator belonging to the OmpR family of two-component signal transduction proteins (Volz, 1993). In *C. crescentus*, CtrA is a global regulator that orchestrates many cell cycle events through the transcriptional control of about one fourth of the cell cycle-regulated genes (Laub et al., 2000): (1) CtrA represses DNA replication initiation through the regulation of *hemE* gene whose promoter overlaps the *C. crescentus* chromosomal origin of replication (Ori); (2) CtrA activates DNA methylation of the newly synthesized strand in both new chromosomes through the activation of *ccrM* expression just before cell division; (3) CtrA controls cell septation through the regulation of the *ftsZ* gene (cell division initiator); and (4) CtrA is required for the transcriptional regulation of many genes involved in flagella and pili biogenesis (for recent reviews (Jenal, 2000; Martin and Brun, 2000; D'Ari, 2001).

In the past few years, *ccrM* and/or *ctrA* homologues have been identified in many α-Proteobacteria including *S. meliloti*, *Agrobacterium tumefaciens*, *Rhodobacter capsulatus* and *B. abortus* (Stephens et al., 1996; Wright et al., 1997; Lang and Beatty, 2000; Lestrate et al., 2000; Robertson et al., 2000;Barnett et al., 2001a,b; Kahng and Shapiro, 2001; Bellefontaine et al., 2002). The conservation of both *ctrA* and *ccrM* in these organisms, displaying very distinct lifecycles and ecology (free-living, symbiotic or parasitic bacteria, symmetric or asymmetric cell division, motile or non-motile, single or multiple replicas) raises the question of their functions.

The first insight into the function of the CtrA regulon in *Brucella* was provided by Robertson et al. (2000). It was shown in vitro binding studies that *C. crescentus* CtrA (CcCtrA) binds to the *B. abortus ccrM* promoter. This result suggests that, in *B. abortus*, *ccrM* transcription is also CtrA-regulated and that the CtrA recognition motif might be conserved between *Caulobacter* and *Brucella*. In addition, Robertson and collaborators

have shown that overexpression of *B. abortus ccrM* (Ba*ccrM*) impairs normal intracellular replication in murine macrophages and that *B. abortus ccrM* (Ba*ccrM*) is essential for viability. These observations support the hypothesis that CtrA might also be a central regulator in *B. abortus*. At the same time, one of the *B. melitensis* mutants isolated by STM in our laboratory (strain G7; Lestrate et al., 2000) was shown to contain a transposon located just downstream of *ccrM*, suggesting that this locus might be necessary for normal survival in mice.

More recently, we have shown that *ctrA* is expressed in *B. abortus* (BaCtrA) in liquid culture and in the cellular infection model. It encodes a protein of 26 kDa and was detected by Western blot in five other *Brucella* reference strains (Bellefontaine et al., 2002). Our data support that this gene is the functional homolog of the well characterized *C. crescentus ctrA* gene: it has more than 80% identity at the protein level, cross-reacting antibodies, same phosphorylation site (D51) and similar 9-mer DNA binding motif (5′-TTAAN$_7$-TTAAC-3′). In addition, we have shown that BaCtrA binds to its own promoter as well as to the *ccrM* promoter, suggesting autoregulation and DNA methylation control in *B. abortus*, as already demonstrated for *C. crescentus ctrA* (Domian et al., 1999; Reisenauer et al., 1999). The cell division defect (branched and elongated cells) observed in *B. abortus* when overexpressing *ctrA* is yet another piece of evidence that BaCtrA has a similar biological role to its *C. crescentus* homolog. Finally, analyses of the recent availability of *B. melitensis* 16M (DelVecchio et al., 2002) and *B. suis* 1330 (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=b_suis) genomes allowed us to confirm that all the factors involved in the CtrA activating phosphorelay in *C. crescentus* (*pleC*, *divJ*, *cckA*, *divL* and *divK*—already cloned in *B. abortus*) are also present in other brucellae as well as a third histidine kinase of the PleC/DivJ family.

We found several shared characteristics between the CtrA regulons in *Brucella* and *Caulobacter*. Their regulated generic processes remain the same (activating phosphorelay, cell division, chromosome segregation and replication). However, we have preliminary evidence that the target genes are somewhat different between these two bacteria. While *divK* is CtrA-regulated in *C. crescentus*, we propose that, in *Brucella*, CtrA controls its own phosphorelay through the regulation of *pleC*, *divJ* and *divL* genes, instead of *divK*. The proposal is made on the basis of the absence of CtrA motifs in the 5′ non-coding regions of *divK* and on their presence in *pleC*, *divJ* and *divL* in *B. abortus*. Furthermore, in footprinting assays, no CtrA protection was observed in the *B. abortus divK* promoter while *pleC* bound CtrA. It is well established in *C. crescentus* that the downstream *ftsZ*, *hemE* [*Cori*], class II flagellar genes, but not *ccrM*, are CtrA regulated. Our results suggest that, in *B. abortus*, CtrA does not directly regulate *ftsZ* nor does it initiate replication by binding to the *ori*. In addition, we did not find any CtrA boxes upstream class II flagellar genes in *B. melitensis*. Although flagellar genes are present in brucellae (Halling, 1998) and some of them have been shown to be specifically induced in the macrophage and to be required for the normal infection pathway, there is no evidence yet that brucellae build flagellum. In order to find CtrA targets that would be specific to brucellae, we searched for the 9-mer consensus motif TTAA-N$_7$-TTAAC in the *B. melitensis* genome and found more than 30 putative CtrA binding sites including *rpoD* (sigma 70 RNA polymerase subunit), *minC* (FtsZ inhibitor), *ftsE* (cell division and/or salt transport), two *ftsK* genes (septation and chromosome dimer resolution), many genes whose products might in someway be involved with cell wall structure and

recycling (OMP's, murein hydrolysis), genes of unknown function conserved among *Rhizobiaceae*. Footprinting experiments of the *B. abortus rpoD*, *minC* and *ftsE* genes support and validate the in silico approach (Bellefontaine et al., 2002).

In conclusion, our data support that both the regulation of the CtrA activating pathway and cell division are under CtrA control in *B. abortus*. Thus, the CtrA target genes from *Brucella* are distinct from those described in *C. crescentus*. In addition, while CtrA regulates the initiation of replication in *C. crescentus*, we propose that it controls segregation of the two chromosomes in *Brucella* through the regulation of both *ftsK* genes. Regarding host–parasite interaction, CtrA might also be involved in the regulation of cell wall and outer membrane components. There is a similar distribution of CtrA boxes upstream *M. loti*, *A. tumefaciens* and *S. meliloti* homologs to some of the newly identified CtrA targets in *Brucella*. This also strongly suggests that the whole regulation network involving CtrA is very similar among these *Rhizobiaceae* that interact with an eucaryotic host and is in contrast to that from the free-living *C. crescentus* (Fig. 3).

In the future, to gain insights to the biological role of the CtrA regulon in *Brucella*, the relevance of data obtained mainly in silico and in vitro will be assessed in vivo by deletion and complementation experiments, gene fusions and reporter analyses. These results, combined with the increasing number of bacterial genomic data available, will likely contribute to our understanding of the CtrA paradigm established firstly in *C. crescentus* and how this regulon allowed adaptation to various lifestyles among the α-Proteobacteria.

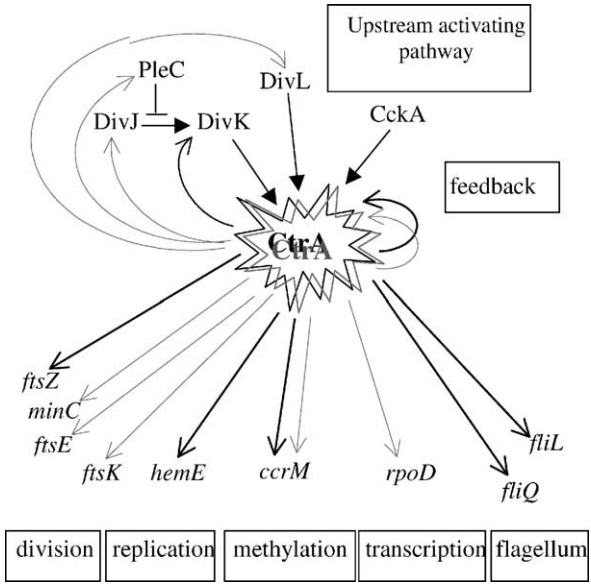

Fig. 3. CtrA regulons in *C. crescentus* (black lines) and *B. melitensis* (gray lines). Bold arrow heads represent CcCtrA activation by phosphate transfer. Thin arrow heads represent transcriptional control (positive or negative) on target genes.

5. Conclusion

We are still far from understanding the regulation and the fine tuning of the molecular mechanisms of *Brucella* and, finally, how this bacteria causes brucellosis. Not all virulence factors are known and, for those that are known, the cellular targets they interact with or interfere with still need to be identified. One of the ways will be to identify the common mechanisms shared with plant pathogens and endosymbionts of the α-Proteobacteria because genes involved in the virulence of *A. tumefaciens* and *Rhizobium* symbiosis are required for *Brucella* pathogenesis as well (Inon de Iannino et al., 1998; Sola-Landa et al., 1998; LeVier et al., 2000; Briones et al., 2001). Some of these genes are also under investigation in our laboratory. They encode the ability to synthesize or to induce the synthesis of opines by *Agrobacterium* or rhizopine by *Rhizobium*. Rhizopine (L-3-*O*-methyl-scyllo-inosamine (3-*O*-MSI) is specifically synthesized by *Rhizobium* in the plant nodule. The synthesis of 3-*O*-MSI is under the control of the *mos* operon and the *mosA* gene is strictly required for the synthesis of 3-*O*-MSI. Rhizopine is a specific growth substrate for strains of *Rhizobium* carrying the *moc* operon and is thought to give a specific advantage to moc^+ strains in the rhizosphere (Murphy et al., 1993, 1988). In our lab, we found that a *Brucella* STM mutant in a gene homologous to *mosA* is attenuated in cellular and animal models (Lestrate, unpublished data). Analysis of the recently released genome of *B. melitensis* (DelVecchio et al., 2002) allowed us to find genes similar to the *moc* operon of *Rhizobium*. Further, it has been shown that a gene similar to *mocC* is specifically induced in macrophages after 24 h of infection (Eskra et al., 2001). The biological relevance of these data is still under investigation. Just as in the case of determining the genetic basis of virulence mechanisms of *Brucella*, we will take advantage of the conceptual breakthroughs resulting from the availability of whole genome data not only for the *Brucella* species but also for *Agrobacterium*, *Sinorhizobium* and *Mesorhizobium*. The data will open the way to comparative genome analysis and to related topics ending in 'ome' (proteome, transcriptome, interactome, metabolome) that will continue to unravel the strategies and mysteries of the ''furtive'' as well as the ''nasty'' bug called *Brucella*. There is still a lot of excitement remaining for researchers in the analysis of the *Brucella* pathogenesis.

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