Commentary

Brucella melitensis: A nasty bug with hidden credentials for virulence

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On September 23, 1905, a cargo carrying 60 goats from Malta arrived in New York. The herd was kept in quarantine because of several deaths that occurred during the journey. Crewmen, an agent from the U.S. Bureau of Animal Industry, which was responsible for the shipment, and a woman who drank milk that "escaped" from the quarantine station displayed the characteristic symptoms of "Mediterranean fever." Lieutenant Colonel David Bruce, a physician of the Royal Army, who discovered "*Micrococcus melitensis* " in 1887 in infected British soldiers residing in Malta, had forewarned the U.S. sanitary authorities about the risk of "Mediterranean fever" by importing goats from Malta. In November 1906, after isolation of "*M. melitensis*," the goats were destroyed. Almost 100 years after this episode, the genome sequence of *Brucella melitensis* (renamed after David Bruce) has been resolved by DelVecchio *et al.* (1), bringing new light to the understanding of the biology of this pathogen. The disease, known as brucellosis, is found in all continents, affecting mainly low-income countries; in addition, it constitutes a contemporary concern because *Brucella* strains are potential agents of biological warfare.

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The six recognized *Brucella* species, named according to their host preference, affect economically important livestock, and several undesignated strains infect marine mammals. Abortion is the main outcome of the infection in pregnant animals, resulting from complex, not well understood interactions between the placental tissues, the intracellular brucellae, and the fetus. *Brucella* invades professional and nonprofessional phagocytes and replicates within compartments resembling the endoplasmic reticulum after evading fusion with lysosomes (2). The brucellae are exceedingly well adapted to this niche (see Fig. 1, which is published as supporting information on the PNAS web site, www.pnas.org) and do not survive for protracted periods of time outside the host. Their textbook description, "facultative intracellular parasites," does not give credit to their true behavior, which is better described as that of a facultatively extracellular intracellular parasite. Therefore, understanding the pathogenicity of brucellae is relevant not only because this pathogenicity represents a major infectious disease but also because it will shed light on basic aspects of intracellular parasites and of cellular immunity. One of the striking features that distinguishes *Brucella* organisms is that they do not display obvious virulence factors such as capsules, fimbriae, flagella, exotoxins, exoproteases, or other exoenzymes, cytolysins, resistance forms, antigenic variation, plasmids, or lysogenic phages. Thus identification of classical virulence factors has been elusive. It is in this context that genomics and comparative phylogenetic analyses are yielding data that improve our understanding of *Brucella* pathobiology (1, 3–5) and are leading us to a refinement of classical concepts about virulence.

The brucellae are α-*Proteobacteria*, phylogenetically related to other cell-associated parasites of plants and animals as well as to free living bacteria (3). Their closest relatives (*Ochrobactrum* sp.) are bacteria of the rhizosphera that behave as opportunistic pathogens of humans. Chromosomal sequences of a number of α-*Proteobacteria* have been released, facilitating phylogenetic, biochemical, and biological comparisons (see Table 1, which is published as supporting information on the PNAS web site). The genome analyses of three *Brucella* species have confirmed the absence of functional sequences for most of the "classical" virulence factors, pathogenic islands, as well as the lack of a complete set of genes to mount, types I, II, and III secretion systems. On the other hand, some potential sequences for virulence were discovered. For instance, *Brucella* recruits actin and activates small GTPases during its internalization to cells (6), but the molecules involved in these events remain unknown. The revelation of putative genes coding for adhesins, invasins, and *vir*G-like genes for attachment and actin recruitment calls for the generation of null mutants in these sequences. Whether some of the presumed hemolysins and proteases could be produced during intracellular parasitism and transferred by alternative secretion systems such as type IV or V, incomplete type III, or flagellar type secretion systems, remains speculative. Among these, the type IV secretion system plays a relevant role during *Brucella* intracellular trafficking (7) (see Table 2, which is published as supporting information on the PNAS web site), presuming by this the translocation of bacterial factors inside cells. Other proteins such as the putative outer membrane TolC, which is required for hemolysin secretion in enteric bacteria, may also serve for parasitism (8). *Legionella* hemolysins form pores in the vacuolar and cellular membranes soon after bacterial replication ceases (9), suggesting that a similar phenomenon could take place with other intravacuolar parasites, including *Brucella*. Concomitantly to this, *Brucella* inhibits apoptosis (10) and replicates within cells without interfering with mitosis (Fig. 1).

Most features related to virulence seem to be concentrated or to act at the *Brucella* surface (Table 2). The *Brucella* LPS gathers a remarkable set of properties. Some are ancestral, such as its very low biological activity, a favorable attribute for not activating intracellular killing mechanisms through cytokine networks (3). Others are idiosyncratic (the resistance to bactericidal peptides). A few may have been

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acquired horizontally (the O-chain). Most other factors depict ancestral systems present in plant and animal cell-associated relatives, with departures reflecting adaptation to the new environment (Table 2, www.pnas.org). Comparison with the various *α-Proteobacteria* chromosomes reveals that most genes known to be critical for cycle progression, translation machinery, stress responses, membrane lipids, basic heterotrophic metabolism, and energy conversion have been retained in *Brucella* organisms. Some of these genes seem to be in the interface with virulence (Table 2), stressing the fine adjustments between essential functions and parasitism. In contrast, genetic cassettes for autothrophy, antibiotic resistance, or for mounting the required structures for living outside host cells (e.g., flagella) are absent, cryptical, or truncated. Similarly, the absence of plasmids and lysogenic phages in the intracellular *α-Proteobacteria* of animals corresponds to their confined environment, as these bacteria do not require additional genetic systems to confront variable external conditions, in contrast to their free-living and plant-associated relatives (11).

Commensurate with these features are the intermediate values regarding the genome size and the G + C content of *Brucella* in comparison with its free-living/plant-associated and obligate intracellular *α-Proteobacteria* relatives (Table 1, www.pnas.org). The presence of two chromosomes with the same G + C content and almost identical proportion of potential coding regions (1,028 and 1,035, respectively) in relation to the chromosomal sizes, as well as the equilibrated distribution of housekeeping genes, reveal that both replicons have a long coexistence. Indeed, the closest *Brucella* relative, the free-living and opportunistic *Ochrobactrum intermedium*, possesses two chromosomes (12), suggesting that the ancestor of these two genera already exhibited two megareplicons (11). Thus, it is tempting to speculate that the smaller chromosome of the *Brucella*/*Ochrobactrum* ancestor evolved from a megaplasmid. Indeed, certain clusters, such as the arginine and ornithine cyclodeamidase genes and the *vir*B operon, all located in chromosome II, are homologous to genes located in the same order in the *Ti* plasmid of *Agrobacterium tumefaciens.* Translocation of housekeeping genes to the ancestral megaplasmid, promoted by an extensive number of insertion elements and transposases, could have transformed this megareplicon into a chromosome. Although this explanation takes into account the ancestor/descendant rules, an alternative hypothesis has been offered (13).

Because *Brucella* is so well adapted to intracellular life, it is expected that this behavior would be reflected at all levels of its biology. It may come as a surprise that a bacterium generally described as nutritionally fastidious is endowed, with exceptions, with all major biosynthetic pathways. However, it has been known for a long time that the growth requirements of smooth *Brucella* are not excessive because, in chemically defined media containing mineral salts and glutamate or glucose, many strains require only niacin and thiamin (14). This property is largely consistent with the genome analysis of *B. melitensis* (1). Niacin dependence is the phenotype of *nadA-C* mutants of prototrophic bacteria and, therefore, the absence of quinolinate synthetase (*nadA*), and nicotinate-nucleotide pyrophosphorylase (*nadC*) genes was not unexpected. On the other hand, the presence of the genes predicted to be necessary for thiamin synthesis contrasts with the requirement of this vitamin, a point that needs reexamination. The conflict may lie in the fact that some steps en route to the thiazole unit and the regulation of the pathways remain to be elucidated (15). Thus, comparative analyses of the *B. melitensis* genome may help in understanding basic aspects of thiamin metabolism. Strain 16 M (1) has been reported to require also cysteine or methionine (16) and differs from other strains in this and possibly other minor requirements. Consistent with the ability of most brucellae to grow with sulfate or thiosulfate as the only sulfur source (14, 16), the reductive assimilatory pathway and related permease are predicted to be in *B. melitensis* 16 M, and the difference between this and other strains may lie in the activity of the *O*-acetylserine sulfhydrylase. This and other minor strain differences are now amenable to investigation.

Critical events take place in the *Brucella* cell envelope (Table 2), and it is noteworthy that, in contrast to its closest phylogenetic neighbors, the *Brucella* outer membrane is permeable to hydrophobic compounds (5). Usually, impermeability to hydrophobic molecules is complemented by efflux pumps, whose presence and/or efficiency in *Brucella* could thus theoretically be questioned. The predicted presence of efflux pumps and outer membrane export channels that, like the AcrAB/TolC system, are characteristically active on a wide range of bulky hydrophobic compounds (17), illustrates how the genetic data raise intriguing questions. For example, it may be asked whether the *Brucella* efflux pump genes are expressed *in vitro*, become activated only in the host, or are just "fossil" sequences. These pumps have been detected in various *Brucella* phylogenetic relatives, and some of them control virulence factors. Also, these pumps may serve to export moderately hydrophobic metabolites, such as the autoinducers of quorum-sensing systems (18). Similar intriguing questions are raised by the conservation of genes predicted to code for heavy-metal pumps, as these are characteristic of soil microorganisms or microorganisms that cycle between animal hosts and the environment. Phagocytes control the level of iron within phagosomes and endosomes. Keeping iron under control is necessary for intracellular parasites, not only because it is an essential nutrient and a component of critical detoxifying enzymes, but also because free iron catalyzes production of harmful hydroxyl free radical. In this regard, the report that *B. melitensis* 16 M carries enterobactin (an iron chelator derived from 2,3-dihydroxybenzoyl-serine) synthetase gene is also striking. *B. melitensis* has been shown to release 2,3-dihydroxybenzoate but no complex catechols under conditions fully inducing enterobactin synthesis in control bacteria. *In vitro*, 2,3-dihydroxybenzoate promotes iron uptake by *Brucellis abortus*, and its addition to macrophage cultures prevents killing of this bacterium, but its role in infection is unclear (19). Also interesting is the presence in *B. melitensis* of all of the genes that putatively code for the Entner–Doudoroff pathway enzymes. This pathway occurs in other *α-Proteobacteria* but has not been detected in *Brucella* (3). Although 6-phospho-2-keto-3-deoxygluconate aldolase activity exists at least in *B. abortus* US19 vaccine, 6-phosphogluconate dehydratase activity has not been found, and cell-free extracts yield the same amount of pyruvate from glucose as from ribose-5-phosphate (20). This observation suggests that 6-phosphogluconate dehydrogenase coupled to the pentose shunt is in fact the major route of pyruvate generation. Are these strain or species peculiarities or more general features of *Brucella* species? US19 is unable to use erythritol and may be atypical in other pathways, but its pattern of $^{14}CO_2$ release from glucose labeled at different positions is closely similar to that of *B. melitensis* 16 M (21). US19 is relatively attenuated but still infectious in humans. Anecdotal, but illustrative of a daily problem in *Brucella* research, the authors of the classical metabolic studies stated that they chose to work with US19, "as a model to minimize the hazard of infection" (20). It seems, therefore, that genomics will bring not only a wealth of information but also safer research! A British relative of US19 was used

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to elucidate the erythritol catabolic pathway (22) (fully confirmed by the genetic studies), the glucose uptake systems (23), and the components of the electron transport chain (24). The latter studies showed several primary dehydrogenases (including lactate and erythritol-1-phosphate dehydrogenases), a "branched" terminal section suggestive of the ability to adapt to low oxygen tension, and a functional nitrate reductase that should allow anoxybiontic growth. These observations are extended by genomic analyses that also suggest a dissimilatory sulfate system. Obviously, the usefulness of these alternative electron acceptors in the biological niche of *Brucella* is intriguing.

Many aspects of *Brucella* biology remain to be understood, and their investigation will provide both basic knowledge and new approaches to cure and prevent brucellosis. Indeed, the fact that *Brucella* is a monophyletic genus, the various species still display distinct virulence and host preference. The report by DelVecchio *et al.* (1) is expected to be followed shortly by similar data on other *Brucella*, which will further expand the possibility of performing comparative analyses. These data will also help us understand how these pathogens emerged during evolution by bringing into light their long-hidden virulence credentials.

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