Identification of *Brucella suis* Genes Affecting Intracellular Survival in an In Vitro Human Macrophage Infection Model by Signature-Tagged Transposon Mutagenesis

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Bacteria of the genus *Brucella* are facultative intracellular pathogens which have developed the capacity to survive and multiply in professional and nonprofessional phagocytes. The genetic basis of this aspect of *Brucella* virulence is still poorly understood. To identify new virulence factors, we have adapted signature-tagged transposon mutagenesis, which has been used essentially in animal models, to an in vitro human macrophage infection model. A library of 1,152 *Brucella suis* 1330 tagged mini-Tn5 Km2 mutants, in 12 pools, was screened for intracellular survival and multiplication in vitamin D₃-differentiated THP1 cells. Eighteen mutants were identified, and their attenuation was confirmed in THP1 macrophages and HeLa cells. For each avirulent mutant, a genomic fragment containing the transposon was cloned. The genomic DNA sequence flanking the transposon allowed us to assign functions to all of the inactivated genes. Transposon integration had occurred in 14 different genes, some of which were known virulence genes involved in intracellular survival or biosynthesis of smooth lipopolysaccharide (the *virB* operon and *manB*), thus validating the model. Other genes identified encoded factors involved in the regulation of gene expression and enzymes involved in biosynthetic or metabolic pathways. Possible roles in the virulence of *Brucella* for the different factors identified are discussed.

Transposon mutagenesis is the most frequently used approach in the identification of genes involved in the virulence of bacterial pathogens (28). Classical transposon mutagenesis strategies are limited by the fact that each mutant must be tested individually to identify an attenuated phenotype. For example, Fields et al. tested 10,000 *Salmonella enterica* serovar Typhimurium Tn10 mutants individually to obtain less than 100 attenuated mutants (21). The use of modified transposons which generate active fusions with envelope proteins often essential for bacterial virulence, such as TnphoA or TnblaM, has been adopted in an attempt to increase the chances of identifying virulence factors (24, 42). Recently, a modification of the classical transposon mutagenesis technique, called signature-tagged transposon mutagenesis (STM), has been described in which the transposon in each mutant carries a unique 80-bp DNA tag which has a variable central portion and constant flanking regions (29). The unique tag allows the detection of a given mutant within a complex pool of mutants by hybridization with a probe obtained by PCR with primers based on the constant regions. Animals are infected with a pool of mutants and, at an appropriate time after infection, the surviving bacteria are recovered. The tags in the recovered bacteria are amplified and labeled by PCR and then used to probe an array of all the tags present in the inoculum. Mutants with attenuated virulence are identified as those whose tags are absent (not amplified) from the bacteria recovered from the infected animal. This system was originally used to identify genes involved in the virulence of serovar Typhimurium, including the *Salmonella* pathogenicity island 2 (29, 52). *Brucella*

is a small gram-negative, facultative intracellular pathogen that infects animals and humans, inducing abortion in pregnant susceptible hosts and producing chronic infections with recurrent bacteremia (31). The physiopathology of brucellosis remains poorly understood. *Brucella* spp. infect and multiply within both professional and nonprofessional phagocytes. As with many intracellular pathogens, *Brucella* seems to use phagocytes as vehicles to disseminate within the organism, allowing it to colonize organs of the reticuloendothelial system and the trophoblasts in the placenta (49). *Brucella* invades cells, blocks phagosome-lysosome fusion, and replicates within a novel intracellular membrane-bound compartment (14, 45). Very little is known about the genetic basis of *Brucella* virulence; stress response proteins (20, 33, 35) and smooth lipopolysaccharide (LPS) (25) have been shown to be required for virulence in in vitro and animal models. Recently, a two-component system, BvrAS (57), and a type IV secretion system, VirB (42), have been identified as essential virulence factors. We have used STM to identify new *Brucella* virulence factors. STM technology has so far been applied essentially to animal virulence models (8, 28). In this study, we report a further application of STM, using an in vitro cell culture model to identify genes encoding factors required for the survival and multiplication of *Brucella* in human macrophages.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetic manipulations. The *Brucella* strains used in this study were derived from *Brucella suis* 1330 NaI^r, a nalidixic-acid-resistant mutant of *B. suis* 1330 (ATCC 23444^T), and were grown in low-salt 2YT medium (10 g of tryptone/liter, 10 g of yeast extract/liter, and 5 g of NaCl/liter, with 16 g of agar/liter when required). *Escherichia coli* SM10 λ pir (40) and JM109 (Pharmacia) were grown in Luria broth (10 g of tryptone/liter, 5 g of yeast extract/liter, and 10 g of NaCl/liter, with 16 g of agar/liter when required). When appropriate, antibiotics were added to the following final concentrations: kanamycin 25 μ g/ml; nalidixic acid, 25 μ g/ml; polymyxin E, 100 IU/ml; and ampicillin, 100 μ g/ml. A pool of tagged mini-Tn5 Km2 transposons in pUT was

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a generous gift from David Holden, Royal Postgraduate Medical School, London, United Kingdom (29). Transposon mutagenesis of *Brucella* by conjugation was done as described previously (39).

DNA manipulation. DNA manipulation was performed following standard techniques (50). Restriction enzymes and oligonucleotide primers were purchased from Eurogentec. *Taq* DNA polymerase, deoxynucleoside triphosphates, and digoxigenin-dUTP were purchased from Roche. Pulsed-field gel electrophoresis and Southern blotting were done as described previously (39).

Infection, intracellular survival assay, and screening of tagged mutant bank. The human monocyte-like cell line THP1 was obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 2 mM glutamine, 10% fetal calf serum, and penicillin and streptomycin (100 µg/ml) at 37°C in 5% CO₂. THP1 cells were allowed to differentiate into macrophages for 72 h in the presence of 10⁻⁷ M 1,25-dihydroxyvitamin D (a generous gift of C. Damais, Paris, France). The virulence of individual mutants was determined as described previously (42). Briefly, 24-well plates were seeded with 5 × 10⁵ THP1 cells. After differentiation, the cells were infected at a multiplicity of infection (MOI) of 20 with stationary-phase bacteria for 30 min (three wells per mutant). Extracellular bacteria were removed by washing the cells twice with phosphate-buffered saline and addition of medium containing gentamicin (30 µg/ml). At different times postinfection, the cells were lysed and the number of recovered bacteria was determined by plate counts of suitable dilutions. HeLa cells were grown as semiconfluent 24-h monolayers in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% fetal calf serum, and penicillin and streptomycin (100 µg/ml) in 24-well culture dishes. The monolayers were infected at an MOI of 100, and the bacteria were brought rapidly into contact with the cells by centrifugation (300 × g for 15 min), incubated at 37°C for 2 h, and then treated as for the macrophages. Bacteria were recovered and enumerated at 48 h postinfection.

Identification of attenuated mutants. To identify attenuated mutants, 10⁷ THP1 cells were differentiated in a 25-cm² culture flask and infected for 30 min with pools of 96 tagged mutants at an MOI of 20 (input pool). At 48 h postinfection, intracellular bacteria were recovered by lysing the cells and plating on 2YT agar supplemented with kanamycin. After an overnight incubation at 37°C, the plate was washed and an aliquot of recovered bacteria was used as the output pool.

PCR, digoxigenin labeling, and hybridization. Aliquots of bacteria from the input and output pools were lysed by boiling them for 1 h in 200 µl of water, and 5 µl of the lysate was used as a template for a first round of PCR using primers P3 and P4 as described by Hensel et al. (29). The 80-bp PCR product was gel purified from a 3% low-melting-point agarose gel (Nusieve), and 5 µl of the remelted agarose was used as a template for a second-round PCR to generate labeled probes with a PCR mixture containing 0.1 mM digoxigenin-dUTP (Roche).

A set of 96 plasmids containing tagged mini-Tn5 Km2 transposons (see Results) were dispatched in 96-well microtiter dishes and blotted with a replicator to a Nytran-N nylon membrane filter (Schleicher & Schuell). The filters were hybridized overnight at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide and washed twice at room temperature in 2× SSC–0.1% sodium dodecyl sulfate and twice at 68°C in 0.1× SSC–0.1% sodium dodecyl sulfate. Hybridization was detected with a chemiluminescence substrate (CSPD; Roche) on Biomax film (Kodak) with a 10-min exposure at 37°C. The membranes were stripped by alkali treatment as recommended by the manufacturer.

Analysis of transposon insertion sites. Chromosomal DNA was prepared from the attenuated mutants, digested with *EcoRV* (no site in mini-Tn5 Km2), and ligated into pUC18-*SmaI*-BAP (Pharmacia). The ligations were used to transform *E. coli* JM109, selecting for ampicillin and kanamycin resistance. Plasmid DNA was extracted, and the chromosomal DNA sequence flanking the transposon was obtained commercially (Genome Express, Grenoble, France) using primers P6 and P7 (29). GenBank sequence database searching was performed with the BLASTX and BLASTN search algorithms (<http://www.ncbi.nlm.nih.gov/blast/>).

RESULTS

Construction of a tagged *B. suis* mutant library. A prerequisite for successful STM screening is that the different tags be equally recognized by the probes derived from the pools. A major limitation of the STM screen is that the different tags do not amplify evenly (7, 29). To overcome this problem, we preselected 96 transposons with tags which amplified and labeled efficiently and did not cross-hybridize, as described by Chiang et al. (8). Briefly, the initial pUT::transposon pool was electroporated into *E. coli* SM10 *λpir*, and 384 transformants were grouped in four sets of 96. The four pools were screened by plasmid dot blot hybridization with their corresponding digoxigenin-dUTP-labeled probes. Plasmids containing trans-

posons with tags which amplified and labeled poorly or showed nonspecific hybridization were excluded. A set of 96 plasmids containing transposons with tags which amplified and labeled evenly and did not cross-hybridize were retained, and SM10 *λpir* strains containing these tagged transposons were transferred to a 96-well master plate. These 96 strains were used to perform 96 separate conjugations with *B. suis* 1330 Nal^r, and the resulting *Brucella* transconjugants were arrayed, based on the tags they carried, to the corresponding well of the 96-well plate. Since the 96 tags were invariable in the pools, we could use plasmid DNA to prepare multiple filters in advance which were used for all the hybridizations performed. A second advantage was that the dot blot analysis with plasmid DNA was simpler and more sensitive than that with colony blots and allowed the use of nonradioactive probes. To verify that transformants resulted from random insertion of a single transposon, the chromosomal DNAs of 14 clones selected at random were extracted and subjected to *EcoRV* digestion (no site in the transposon). Southern analysis with a mini-Tn5 Km2 probe showed that the insertion sites of the clones were different (data not shown).

Identification of attenuated mini-Tn5 insertion mutants. A bank of 1,152 mutants in pools of 96 was used to infect differentiated THP1 macrophages in 25-cm² flasks as described in Materials and Methods. An aliquot of the inoculum was kept to prepare the input probe. At 48 h postinfection, the cells were washed, lysed, and plated on 2YT agar. After 24 h of incubation, the plate was washed and the bacteria were harvested to prepare the output probe. In preliminary experiments, the output probe was prepared in parallel directly from the cell lysate; this gave identical results but a weaker signal. In the original STM method, the number of clones that can be screened successfully as a pool is restricted by the number of clones which can establish an infection simultaneously in the animal. This raised a problem of reproducibility and made it necessary to reduce the complexity of the pool (7, 29). In our in vitro model, this problem is not encountered, since infection of 10⁷ differentiated macrophages at an MOI of 20 means that up to 2.5 × 10⁵ cells can be infected by a given clone. As the multiplication of the wild-type strain in macrophages is almost 1,000-fold (15, 16, 33), our recovered pool was always representative of the infection events.

Filters of the plasmids containing the 96 tagged transposons were first hybridized with the output probe, stripped, and then hybridized with the input probe for comparison. Initially, we considered as attenuated the clones which did not give any signal with the output probe but had a clear signal with the input probe (Fig. 1). With these criteria, only five potential mutants were identified. We thought that this might be because the PCR amplification of the output pool was too efficient and tags from small numbers of residual attenuated mutants were amplified. To circumvent this problem, the cell lysate was diluted 100-fold before being plated on 2YT agar. However, the results were identical. To identify more attenuated mutants, 32 mutant strains which gave a weaker signal with the output probe than with the input probe were selected. Individual testing for virulence in THP1 cells confirmed attenuation in 18 of these mutants, with bacterial counts at 48 h between 10- and more than 1,000-fold lower than that of the wild type (Fig. 2). Similar results were obtained with nonphagocytic HeLa cells (Table 1).

Characterization of attenuated strains identified by STM. Chromosomal DNA was prepared from the 18 mutants, and Southern blotting with a mini-Tn5 Km2 probe showed that each clone contained a single transposon insertion. Pulsed-field gel electrophoresis and Southern blotting allowed us to

FIG. 1. Identification of attenuated mutants by differential hybridization. A dot blot of plasmids carrying the 96 tagged mini-Tn5 Km2 transposons was hybridized with probes prepared from the output and input pools from plate IX. In this pool clone H9 (arrows) was attenuated (*lysR*-like). The apparent attenuation of clones A4, G3, and E5 was not confirmed by individual testing. Clones C4 and E6 did not grow, and well H7 was the negative control (no inoculum).

identify the *SpeI* fragment containing the transposon and to assign a position on the physical maps of the two chromosomes (Table 1). The *EcoRV* genomic fragment containing a transposon of each of the mutants was cloned in pUC18, and the DNA sequence of the region flanking the transposon was determined. BLASTX searches of GenBank allowed us to identify the genes disrupted by the transposon (Table 1), which can be assigned to three classes.

The first class corresponds to virulence determinants previously identified in *Brucella*. We found three mutations in the recently described *virB* operon (*virB2*, *virB4*, and *virB8*) and

three mutations in an LPS biosynthesis gene (*manB*) coding for a phosphomannomutase.

The second group includes genes involved in the regulation of gene expression. One insertion was found in a gene encoding a transcriptional regulator belonging to the LysR family. Several insertions were found in genes encoding proteins involved in the regulation of nitrogen metabolism; a homologue of *ntfY* from *Azorhizobium caulinodans*, the sensor of a novel two-component system (44), and three insertions in a *glnD*-like gene, which can be linked to this category, since it encodes a uridylyl transferase which is the primary sensor of nitrogen.

FIG. 2. Growth curves of wild type and four mini-Tn5 Km2 mutants of *B. suis* in THP1 macrophages. Differentiated THP1 cells were infected as described in Materials and Methods. The four representative mini-Tn5 Km2 mutants shown here are IXH9 (*lysR*-like), IVA10 (*virB4*), IIVF11 (*gpt*-like), and IIVH4 (*pgi*-like). The data points are the means of three wells with the standard deviations. This data set is from one of three independent experiments with similar results. The 48-h-postinfection points for all 18 mutants are detailed in Table 1.)

TABLE 1. *B. suis* genes identified by STM

Mutant	MiniTn5 insertion site ^a	% Amino acid identity (homology)	Function	Chromosomal mapping ^c	THP1 $\Delta\log\text{CFU}^b$	HeLa $\Delta\log\text{CFU}^b$
IIC5	<i>virB</i> ₂ (Bsu)	100 (100)	Type IV secretion system/virulence	210 (S)	>3	2.2
IIIH12	<i>ntfY</i> (Aca)	47 (64)	Sensor of a two-component system/nitrogen metabolism	79 (L)	1.2	1.1
IIIF2, IVG11, VIIA12	<i>glnD</i> (Rro)	70 (84)	Uridyl transferase/nitrogen metabolism	200/210	1.8	1.3
IIIF11	<i>gpt</i> (Vch)	53 (79)	Guanine phosphoribosyl transferase/nucleotide biosynthesis	335 (L)	1.3	1.2
IVH4	<i>pgi</i> (Sco)	44 (53)	Phosphoglucose isomerase/glucose metabolism	420 (L)	>3	2.3
IVA10	<i>virB</i> ₄ (Bsu)	100 (100)	Type IV secretion system/virulence	210 (S)	>3	2.5
VF7, VIG4, VIIIF4	<i>manB</i> (Bab)	98 (98)	Phosphomannomutase/LPS biosynthesis	100/140	>3	2
VIIA2	<i>aroC</i> (Sty)	67 (79)	Chorismate synthase/aromatic amino acid and PABA biosynthesis	420 (L)	1.5	1.7
VIIC11	<i>virB</i> ₈ (Bsu)	100 (100)	Type IV secretion system/virulence	210 (S)	2.3	2
IXH9	LTTR-like (Abr)	53 (67)	LysR family transcriptional regulator/unknown	305 (L)	2.5	1.9
XA2	<i>carAB</i> (Stm)	62 (79)	Carbamoyl phosphate synthase/arginine and pyrimidine metabolism	420 (L)	1.3	1.4
XIIB10	<i>rpsA</i> (Sme)	74 (76)	Ribosomal protein S1	420 (L)	>3	ND
XIIC6	<i>pyc</i> (Ret)	79 (91)	Pyruvate carboxylase/glucose metabolism	305/315 (L)	1.8	ND
XIIE3	<i>leuA</i> (Sme)	78 (87)	Isopropylmalate synthase/leucine biosynthesis	200/210	2	1.2

^a Determined by comparison of sequences with GenBank database using BLASTX over a minimum of 500 bp. Abr, Aca, Bab, Bsu, Ret, Rro, Sco, Sme, Stm, Sty, and Vch denote *Azospirillum brasilense*, *A. caulinodans*, *B. abortus*, *B. suis*, *Rhizobium etli*, *Rhodococcus rodochrous*, *Streptomyces coelicolor*, *Sinorhizobium meliloti*, serovar Typhimurium, serovar Typhi, and *V. cholerae*.

^b Difference in recovered CFU between the mutants and the wild type expressed as $\log\text{CFU}$ at 48 h postinfection. This value is a mean of three assays where the standard deviation was in a range of 0.1 to 0.2 log units. ND, not done.

^c Size (in kilobases) of the *SpeI* fragment; S, small chromosome, L, large chromosome (39).

A third group of genes encodes enzymes involved in metabolic pathways, such as glucose metabolism (pyruvate carboxylase and phosphoglucose isomerase) or amino acid biosynthesis (isopropylmalate synthase, carbamoyl phosphate synthase, and chorismate synthase). We also isolated a mutant with an insertion in a *gpt*-like gene which codes for hypoxanthine-guanine phosphoribosyl transferase, an enzyme of the purine nucleotide biosynthesis pathway.

One attenuated mutant, with an insertion in a gene encoding a ribosomal protein, had a reduced growth rate in rich bacteriological medium. None of the other attenuated mutants had detectable growth defects.

DISCUSSION

Survival and multiplication in macrophages are keys to the virulence of *Brucella*. The genetics of virulence mechanisms remains poorly understood, and so far, reports of the mechanisms by which *Brucella* enters and multiplies within macrophages are only descriptive. In comparison with other intracellular pathogens which have devised successful strategies to survive and multiply within phagocytic cells (22, 23), few real virulence genes have been characterized in *Brucella*.

The STM technique is a powerful method that allows a large number of mutants to be screened for attenuation. It was initially used to identify new virulence genes in serovar Typhimurium (29, 52) and recently in *Staphylococcus aureus* (11, 37, 51), *Vibrio cholerae* (7), *Streptococcus pneumoniae* (46), *Yersinia enterocolitica* (13), *Proteus mirabilis* (62), and *Legionella*

pneumophila (19). In these bacteria, many virulence factors had already been identified, making it possible to validate the STM approach by the isolation of insertions in known virulence genes. The purpose of this study was to assess the usefulness of the STM technique for identifying virulence genes of *Brucella* in an in vitro macrophage infection model. Recently, an adhesin mediating *Candida glabrata* adherence to HEP-2 cells was identified through an STM screen (10). Our study is the first application of STM to a tissue culture infection model for the identification of factors involved in the intracellular survival of a bacterium. The isolation of attenuated mutants in the *virB* operon and in *manB*, two known *Brucella* virulence factors, validates the approach.

Smooth LPS is classically described as an important factor in the virulence of *Brucella*, since rough mutants are usually attenuated or nonvirulent (25). The O antigen of *Brucella* is a homopolymer of 4,6-dideoxy-4-formamido- α -D-mannopyranose (*N*-formyl perosamine). We identified three independent highly attenuated mutants with insertions in the *manB* gene encoding a phosphomannomutase. Slide agglutination tests with specific antisera and acriflavine showed that these mutants were rough (2). Phosphomannomutase is an enzyme involved in the synthesis of perosamine, converting mannose-6-phosphate to mannose-1-phosphate (59), and therefore *manB* mutants are unable to synthesize O antigen. Allen et al., (1) isolated rough transposon mutants of *Brucella abortus* with decreased virulence in mice, including a *manB* mutant. These results confirm the role of this enzyme in the biosynthesis of smooth LPS and its role in the intracellular survival of *Brucella*

in human macrophages and in mice. Attenuated rough mutants including *manB* mutants were also identified by STM in serovar Typhimurium, *V. cholerae*, and *Y. enterocolitica* (7, 13, 29). However, it should be noted that *Brucella ovis* and *Brucella canis*, which are naturally rough, are fully virulent in their natural hosts, showing that smooth LPS is not the only factor necessary for the virulence of *Brucella* (56).

The ability to modulate gene expression to adapt to the intracellular environment is a key component of bacterial virulence. Two mutants were identified with insertions in regulatory systems. The most attenuated was a member of the LysR transcriptional regulator (LTTR) family. This observation is of great interest, since LTTRs are, together with the two-component systems, the most common type of positive regulator in prokaryotes (54). LTTRs activate divergent transcription of linked or unlinked target genes or regulons in response to coinducers. LTTRs play an important role in the regulation of virulence genes in many bacteria. The best studied is in serovar Typhimurium, where SpvR controls the expression of virulence plasmid-encoded genes upon entry into stationary phase (4, 53). Other members of this family involved in virulence are ChvO in *Agrobacterium tumefaciens* (17), PtxR in *Pseudomonas aeruginosa* (27), and IrgB in *V. cholerae* (26). Two-component systems allow bacteria to make rapid adaptive responses to changes in their environment and play a major role in the virulence of many pathogens (23, 30, 38). In previous studies we have identified and mutated genes encoding the FeuP and NtrC response regulators but found that they played little or no role in virulence (15, 16). So far, the only two-component system shown to play a major role in *Brucella* virulence is BvrAS, a system related to ChvI-ExoS in *Rhizobium meliloti* and ChvI-ChvG in *A. tumefaciens* which is required for virulence in HeLa cells and murine macrophages (57). In this study, we isolated a mutant with a mutation in a gene homologous to an *ntrY*-like gene of *A. caulinodans*. The NtrY protein is a sensor of an *ntr*-related regulon which may be part of the *glnALG* operon (44). This mutant has a weakly attenuated phenotype (reduction of 1.2 log units versus the wild type at 48 h postinfection) which could be explained by a pleiotropic effect on the *ntr* regulon, since the *ntrC* mutant did not show such a phenotype.

Glutamine is a central metabolite, since it is a major donor of amino groups in the amino acid and purine biosynthesis pathways. Klose and Mekalanos have reported that simultaneous inactivation of *glnA* and *ntrC* reduced the virulence of serovar Typhimurium (32). In their STM approach, Polissi et al. have shown that insertions in genes encoding enzymes involved in glutamine metabolism induced a virulence defect in *S. pneumoniae* (46). In this study, the mutation of the *glnD*-like gene, which codes for a uridylyl transferase, supports the hypothesis that the concentration of glutamine in host cells is critical for the intracellular survival of *Brucella*. This enzyme has been previously described as the primary sensor of nitrogen and glutamine status in the nitrogen regulation cascade (41).

The nutritional environment of the host's cells imposes a requirement for de novo biosynthesis of various amino acids, cofactors, and nucleotides in many pathogens. In *Brucella*, nutrient and amino acid biosynthesis pathways are also critical for virulence, since auxotrophic mutants for leucine, arginine, or aromatic amino acids are attenuated. Bacteria synthesize all their aromatic metabolites through a pathway leading to chorismic acid, which is absent in mammalian cells. Chorismate is a branching point with pathways leading to aromatic amino acids, *para*-amino benzoic acid (PABA), and, hence, folic acid, vitamin K, and 2,3-dihydroxybenzoic acid; mutations in this

pathway attenuate many bacterial pathogens. We identified a mutant with a mutation in *aroC* encoding chorismate synthase; *aroC* mutations have been shown to attenuate both serovar Typhimurium and *Salmonella enterica* serovar Typhi (18, 36), as well as *Shigella flexneri* (6). The reason for the attenuation may be a lack of PABA, with its effects on protein synthesis (58), or it may be due to an inability to synthesize 2,3-dihydroxybenzoic acid, which has been shown to be the main siderophore produced by *Brucella* (34).

Two insertions in genes involved in glucose metabolism (*pgi* and *pyc*) were identified. Phosphoglucose isomerase converts glucose-6-phosphate to fructose-6-phosphate. Fructose-6-phosphate is the first step of the Embden-Meyerhof pathway, which is the major source of pyruvate in most bacteria. In *Brucella*, fructose-6-phosphate cannot enter this pathway, since the glucose metabolism follows the hexosemonophosphate pathway (48). In *Brucella*, fructose-6-phosphate is converted to mannose phosphate, which is the initial step of the polyoside biosynthesis pathway. The mutation of the *pgi* gene could also affect the biosynthesis of the bacterial peptidoglycan. A more tempting hypothesis is that the insertion in *pgi* could have a pleiotropic effect on the expression of many genes, since in *E. coli* and serovar Typhimurium a mutation in *pgi* was shown to affect DNA supercoiling by mimicking carbon starvation (3).

An insertion in the *gpt*-like gene, which encodes hypoxanthine-guanine phosphotransferase, an enzyme involved in nucleotide biosynthesis, gives rise to an attenuated phenotype. This could be related to results previously described in other studies where many auxotrophic mutations for purine or nucleotide biosynthesis lead to attenuated strains (43). On the other hand, previous reports showed that *Brucella* releases compounds, such as adenine or GMP, which inhibit phagocyte functions (5, 9). We can hypothesize that the attenuation after *gpt* mutation could be at least partly explained by this mechanism.

Three mutants had insertions in different genes of the recently described VirB type IV secretion system of *Brucella* (42). In *A. tumefaciens*, the VirB system is involved in DNA transfer of the Ti plasmid (55). The Ptl system of *Bordetella pertussis* secretes the pertussis toxin (61). The Dot/Icm type IV system of *L. pneumophila* is thought to export the macromolecules which affect the maturation of the phagosome, allowing the bacteria to develop intracellularly (60). In *Helicobacter pylori*, the proteins encoded by genes of the *cag* pathogenicity island translocate an effector molecule which activates the NF- κ B signaling pathway, stimulating the production of interleukin-8 and a cascade of modifications to the host cell cytoskeleton (12). The identification of the effector molecule exported by the *Brucella* type IV secretion system is now a priority.

Our screen was designed to identify factors involved in the survival and multiplication of *Brucella* in human macrophages. Far fewer attenuated mutants were identified than in STM studies in animal models (1.5 versus 4%). This may be related to the nature of the macrophage model: in such a model, we explore only one specific aspect of the virulence of *Brucella*. Factors such as those essential for adhesion to and invasion of nonphagocytic cells or serum resistance would not necessarily be identified. It should be noted that a similar frequency of attenuated mutants was found in a previous attempt to identify attenuated mutants in HeLa cells (42) and that the mutants obtained were also attenuated in macrophages. In this study, all the mutants were also attenuated in both macrophages and HeLa cells; this is a reflection of the fact that most of the attenuated mutants had metabolic defects rather than lesions in "classical" virulence factors, a situation encountered in all of

the STM screens described so far. It should also be considered that *Brucella* appears to block phagosome-lysosome fusion in both phagocytes and HeLa cells (45, 47) and therefore the mechanisms of intracellular survival may be similar for both cell types. It is interesting that many classes of genes were not identified or were poorly represented; for example, we identified only one two-component system and no type III secretion systems. Another surprising point is that, despite the small number of gene sequences available for *Brucella*, we were able to assign a function to all 18 of the genes. The loci we identified in this study seem to spread out independently of the two chromosomes of *Brucella*. We do not consider that we have identified all of the factors required for *Brucella* virulence; the screen of 1,152 mutants is not exhaustive, and this mutagenesis is far from saturating the 3.2-Mb *Brucella* genome. Furthermore, the transposon does not appear to integrate randomly into the genome: we observed three independent insertions in the *manB* gene and three in the *glnD*-like gene. Finally, it is conceivable that genes involved in intracellular survival belong to a complex network and that mutation of one of these genes could be compensated for by numerous salvage pathways.

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