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Molecular Host-Pathogen Interaction in Brucellosis: Current Understanding and Future Approaches to Vaccine Development for Mice and Humans

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INTRODUCTION

Brucellosis is one of the five common bacterial zoonoses in the world caused by organisms belonging to the genus *Brucella*, gram-negative, non-spore-forming, facultative, intracellular bacteria (39). The genus *Brucella* consist of seven species according to antigenic variation and primary host: *Brucella melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats), and *B. maris* (marine mammals) (38, 80). *B. abortus* induces spontaneous abortion in cattle and causes serious economic loss to dairy farms. Currently, *B. abortus* RB51 or *B. melitensis* REV.1 strains are used to immunize cattle and to immunize goats and sheep, respectively (14, 17, 39, 83). No other vaccines are available for other animals, and a human *Brucella* vaccine does not exist.

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Phylogenetically, *Brucella* is classified within the α2 subdivision of *Proteobacteria*, which includes *Agrobacterium*, *Rickettsia*, *Rhodobacter*, and *Rhizobium* (109). The *Brucella* genome consists of two circular chromosomes without plasmids, suggesting a remarkable difference compared to the single chromosome of many bacteria (105, 106). The recent completion of *B. melitensis* (GenBank NC 003317 and NC 003318) (44), *B. suis* (GenBank NC 002969), and *B. abortus* (142) genome sequence projects has provided tremendous information for understanding the mechanisms of *Brucella* pathogenicity. *B. melitensis* contains 3,197 open reading frames and, as found with certain other α *Proteobacteria*, genes associated with DNA replication, transcription, translation metabolism, and cell wall biosynthesis are present on both chromosomes. Although *Brucella* is a serious pathogen, type I, II, and III secretion systems and pathogenicity islands were absent. However, *B. melitensis* did contain genes for flagellum-specific type III and IV secretion systems. Future studies to define the role of these secretion systems in *Brucella* intracellular survival will be necessary before we can understand the mechanisms of *Brucella* pathogenesis. Currently, these investigators (44) are evaluating the proteomic expression of *Brucella* to identify the bacterial proteins expressed in vitro. At present, the genomic sequence of *B. suis* is still being edited and annotated while the *B. abortus* sequence is still being completed. The availability of the genomic sequences of *B. melitensis*, *B. suis*, and *B. abortus* will provide an opportunity to identify the virulence mechanisms of *Brucella* at the molecular level that have previously proven highly elusive. Presently, six groups of bacteriophages (which are all closely related DNA phages of the same morphological group, *Pedoviridae*) are identified as lytic for *Brucella* (38). However, the presence of bacteriocins specific for *Brucella* or any evidence of gene transfer mediated by bacteriophages has not been confirmed.

Brucella infection occurs through inhalation or ingestion of organisms via the nasal, oral, and pharyngeal cavities (22). Following penetration of the mucosal epithelium, the bacteria are transported, either free or within phagocytic cells, to the regional lymph nodes. The spread and multiplication of *Brucella* in lymph nodes, spleen, liver, bone marrow, mammary glands, and sex organs occurs via macrophages. In general, *B. melitensis*, *B. abortus*, and *B. suis* can infect humans and the pathological manifestations of brucellosis in humans are meningitis, endocarditis, spondylitis, and arthritis. Due to the epidemic potential, the absence of a human vaccine, the drawbacks of current vaccine strains in terms of safety, and the efficiency of aerosol infection, this airborne pathogen is classified as a biosafety level 3 pathogen and considered to be a potential bioterrorist agent (86).

Bacteriologic culture and serological agglutination tests are widely used for the diagnosis of infected humans and animals. Human brucellosis patients are treated with combinations of antibiotics such as rifampin and doxycycline or streptomycin and doxycycline (22). Historically, 2% of untreated *B. melitensis*-infected patients die (103), and *Brucella*-induced deaths are still reported (31). Therefore, to eradicate human brucellosis, the control of brucellosis in agricultural animals is crucial because of the zoonotic aspects of this infectious disease. Currently, mice are used as an animal model for brucellosis and molecular genetic tools for *Brucella* are available, providing the efficient experimental tools to investigate the bacterial pathogenesis and host immune response for the development of vaccine strains.

BRUCELLA PATHOGENICITY

The origins of the diverse clinical manifestations in *Brucella*-infected human beings and animals have not been clearly elucidated. However, there is no doubt that the augmentation of *Brucella* replication in the host is ascribed to these symptoms. This increase of *Brucella* numbers in the host is mainly due to their ability to avoid the killing mechanisms and proliferate within macrophages like other intracellular pathogens. For example, *Brucella* organisms not only resist killing by neutrophils following phagocytosis (26, 133) but also replicate inside macrophages (85) and nonprofessional phagocytes (47). Additionally, survival in macrophages considered to be responsible for the establishment of chronic infections allows the bacteria to escape the extracellular mechanisms of host defense such as complement and antibodies. Therefore, to understand the pathogenesis of *Brucella*, the mechanisms that allow the intracellular survival of this pathogen have to be elucidated.

Phagosomes, a Unique Niche for *Brucella*

To be a successful infectious agent, *Brucella* requires four steps, like other pathogenic intracellular bacteria: adherence, invasion, establishment, and dissemination within the host (57, 99). Opsonized or nonopsonized *Brucella* organisms can infect macrophages, suggesting that antibody or complement-mediated phagocytosis as well as the direct *Brucella*-host cell contact allows adherence and invasion. In macrophage phagosomes, *Brucella* cells survive and multiply, inhibiting phagosome-lysosome fusion through rapid acidification of the phagosome following uptake. Thus, phagosomes of the macrophage are the intracellular niche for this pathogen (91). Finally, the accumulated bacteria are disseminated to other host cells. Thus, the most critical step for understanding the pathogenic mechanism of this intracellular bacterium is how *Brucella* organisms cope with phagocytic mechanisms of the host.

Brucella can infect phagocytic and nonphagocytic cells in vivo and in vitro. Infected nonphagocytic cells such as trophoblasts of the pregnant ruminant in vivo (5) as well as epithelioid HeLa (128) and fibroblast Vero (47) cell lines in vitro contain virulent *Brucella* organisms in the autophagosome and endoplasmic reticulum (ER). Also, the ER is regarded as a location for *Brucella* multiplication. Infected phagocytic cells such as murine J774 cells contain *Brucella* in the phagosomes with a pH of 4 to 4.5, while autophagosomes or the ER of murine J774 do not harbor *Brucella* (130). However, the presence of *Brucella* in both phagocytic and nonphagocytic cells inhibits or delays phagosome and lysosome fusion (7, 128). The specific mechanism used to hamper phagolysosome fusion has not been elucidated clearly, but the soluble extract of virulent *B. abortus* strains excluding lipopolysaccharide (LPS) is considered to be involved in this phenomenon (62). Studies using *Brucella* mutants of a two-component regulatory system (*bvrR-bvrS*) or a type IV secretion system (*virB*) in a nonphagocytic HeLa cell model suggest the involvement of specific *Brucella*

TABLE 1. Deletion or insertion of *Brucella* genes resulting in attenuated bacteria

Gene	Function	Species	Virulence		References
			In vitro	In vivo	
pgm	Phosphoglucomutase (LPS synthesis)	*B. abortus*	Virulent	Attenuated	156
rfbE	Perosamine synthetase (LPS synthesis)	*B. melitensis*	Virulent	Attenuated	65
pmm	Phosphomannomutase (LPS synthesis)	*B. abortus*	Attenuated	Attenuated	2
wboA	Glycosyltransferase (LPS synthesis)	*B. abortus*	ND[a]	Attenuated	104
cydB	Cytochrome *bd* oxidase (respiratory system)	*B. abortus*	Attenuated	Attenuated	54
cgs	Cyclic β-1,2 glucan synthetase	*B. abortus*	Attenuated (only S19)	Attenuated (only S19)	23, 77
sod	Cu-Zn SOD	*B. abortus*	Virulent	Attenuated (only S2308)	154
dnaK	Heat shock protein	*B. suis*	Attenuated	ND[a]	93
lon	Protease	*B. abortus*	Attenuated	Virulent	135
bacA	Cytoplasmic membrane transport protein	*B. abortus*	Attenuated	Attenuated	98
hfq	Host factor-1, RNA-binding protein regulating stationary-phase stress resistance	*B. abortus*	Attenuated	Attenuated	136
virB	Type IV secretion system	*B. suis, B. abortus*	Attenuated	Attenuated	59, 75, 116, 146
bvrRS	Two-component regulatory protein	*B. abortus*	Attenuated	Attenuated	147
aroC	Chorismate synthase (aromatic compound synthesis)	*B. suis, B. abortus*	Attenuated	Attenuated	59, 60, 75
purE	De novo purine synthesis pathway	*B. melitensis*	Attenuated	Attenuated	41, 49, 76

[a] ND, not determined.

factors in the maturation of phagosomes. For example, *Brucella* defective in the *bvrRS* two-component regulatory system are not directed to the ER-like compartment but, rather, are present within phagosomes unable to inhibit fusion with lysosomes (147). Also, *virB* mutants deficient in a type IV secretion system are unable to reach the replicative niche and reside in a membrane-bound vacuole expressing the late endosomal marker, LAMP1, and the Sec61b protein from the ER membrane (35, 43).

Current Understanding of *Brucella* Pathogenesis

The recent availability of genetic techniques to manipulate the *Brucella* genome permits identification of virulence factors of *Brucella* at the molecular level. Previous studies to identify attenuation relied on culture passage of isolated strains or collection of diverse field strains following phenotypic characterization based on LPS structure (rough or smooth form) or injection of animal models (162). Since the early 1990s, molecular manipulation of the *Brucella* genome has permitted the characterization and identification of virulence genes by producing specific or random mutants searching for avirulent phenotypes (69, 131). The initial reverse genetic characterization of virulence factors relied on cloning, sequencing, mutagenesis, and infection of mutants in vitro or in vivo. This approach did not elucidate many virulence factors of *Brucella*. Many putative virulence factors that had been characterized from other intracellular bacterial pathogens turned out to be insignificant in this particular pathogen, suggesting that a unique host-pathogen interaction is established in brucellosis. Mini-Tn*5* transposon mutagenesis was introduced to study *Brucella* pathogenesis. Even though this transposon mutagenesis requires an intensive screening step to obtain phenotypic mutants, this method was more productive in identifying a virulence factor than was the single-gene targeting approach. Characterized virulence factors following the generation of individual mutants are listed in Table 1.

Lipopolysaccharide. *Brucella* has two forms, smooth and rough (38). In general, rough strains, containing less or no O polysaccharide (OPS), are less virulent than smooth strains and less resistant to complement attack. However, occasionally, naturally virulent strains of *B. ovis* and *B. canis* are rough stains. There are two aspects to the identification of *Brucella* LPS as a virulence factor. First, *Brucella* possesses less immunogenic LPS than enterobacterial LPS. Nonpyrogenic *Brucella* LPS does not activate the alternative complement pathway to any significant level and is a very weak mitogen for murine B cells (143). Furthermore, at least 10-fold more *Brucella* LPS is required for lethality and interferon (IFN) production than with enterobacterial endotoxin (87). Thus, the lower biological activity induced by *Brucella* LPS might be one of the factors contributing to the survival of these pathogens in phagocytic cells. Second, OPS-deficient *Brucella* mutants are sensitive to complement-mediated lysis and polymyxin B. For example, *B. abortus* phosphomannomutase (*pmm*) transposon mutants were attenuated in vivo and in vitro and were sensitive to complement-mediated killing as well as to polymyxin B, a defensin-like molecule (2). *B. abortus* glycosyltransferase (*wboA*) deletion mutants were more attenuated than the parental strain, S2308, but more persistent than RB51 in vivo (104). *B. abortus* phosphoglucomutase (*pgm*) insertional mutants were attenuated in vivo but not in vitro (156). *B. melitensis* perosamine synthase (*rfbE*) deletion mutants were attenuated in mice but not in macrophages (65). All of the above OPS-deficient mutants were attenuated in vivo, but there were some survival differences in macrophages, indicating that complement-mediated lysis or a lectin pathway may play a role in controlling extracellular *Brucella*; however, the level of intracellular infection and replication was not reduced.

Cyclic β(1–2) glucan. Cyclic β(1–2) glucan is an osmoregulated periplasmic polysaccharide of the plant pathogens *Agrobacterium tumefaciens* and *Rhizobium meliloti*, which are involved in nodule invasion or crown gall tumor induction in plants (19). *Brucella* spp. classified within the α2 subdivision of *Proteobacteria*, which includes *Agrobacterium* and *Rhizobium*, also contain cyclic β(1–2) glucan (24). In *Brucella*, this sugar is produced by cyclic β(1–2) glucan synthetase encoded by *cgs* (23) but is not osmotically regulated (24). The *cgs* deletion mutants made from *B. abortus* S19 (vaccine strain) were attenuated in vivo and in vitro, but minimal attenuation was noted in *cgs* mutants made from S2308 (field strain) (23), indicating that strain differences may exist. Also, a protection assay using S19 *cgs* mutants showed similar protection to that for S19, but more immunoglobulin G2a (IgG2a) was induced.

bacA. A highly conserved *B. abortus* homologue of the *R. meliloti bacA* gene, which encodes a putative cytoplasmic membrane transport protein required for symbiosis, was identified (98). *B. abortus bacA* deletion mutants were attenuated in vivo and in vitro, suggesting that the bacterium-plant symbiosis could be an alternative model to study *Brucella* pathogenesis.

bvrR-bvrS. The *B. abortus* two-component regulatory system consisting of the *Brucella* virulence-related regulatory (BvrR) and sensory (BvrS) proteins is highly similar to the two-component regulatory systems ChvG-ChvI of *A. tumefaciens* and ExoS-ChvI of *R. meliloti*, which are equivalent to *Salmonella* PhoP-PhoQ and *Bordetella bronchiseptica* BvgA-BvgS systems (147). Transposon mutants of *bvrR* and *bvrS* were originally identified by their sensitivity to polymyxin B but were also sensitive to other bactericidal polycations such as melittin and poly-L-lysine, suggesting that a change of the outer membrane occurred in these mutants. Insertional *bvrR* or *bvrS* mutants possessed reduced multiplication and minimal persistence in mouse spleens in vivo and displayed reduced invasiveness and replication in macrophages and HeLa cells in vitro, suggesting the inability to inhibit lysosme fusion. Other putative two-component regulatory systems involved in *Brucella* virulence, such as *ntrY* (59) and *vsrB* (97), have been discovered by signature-tagged transposon mutagenesis (STM), but specific phenotypes have not been delineated yet.

virB. *B. suis virB* genes, encoding a type IV secretion system homologous to those encoded by *A. tumefaciens virB* and *B. pertussis ptl* genes, were originally identified from attenuated transposon mutants *in vitro* (116); later, the *B. abortus* S2308 genome project confirmed the intact *virB* operon (146). The *A. tumefaciens virB* operon encodes a pilus-like structure necessary for secretion of transfer DNA, and the *B. pertussis ptl* operon encodes the apparatus that allows the secretion of pertussis toxin, suggesting that *Brucella* may secrete regulatory DNA or protein for intracellular survival. VirB10 is an inner membrane-bound protein possessing a C-terminal periplasmic domain with homology in all type IV secretion systems. *Brucella virB10* polar and nonpolar mutants had the same penetration effect as the wild type but were unable to replicate in phagocytic and nonphagocytic cells in vitro. Also, these mutants were attenuated in vivo, suggesting that *virB* is a virulence factor. In fact, phagosomes containing *virB10* polar mutants fused with lysosomes and were directed to a degradation pathway (35, 43). In contrast, *virB10* nonpolar mutants were capable of avoiding interactions with the endocytic pathway but were unable to reach the ER to establish their intracellular replication niche in HeLa cells. These results suggest that VirB proteins are involved in the maturation of *Brucella*-containing vacuoles.

cydB. *B. abortus cydB* transposon mutants, attenuated in vitro and in vivo, are defective in cytochrome *bd* oxidase, a high-oxygen-affinity terminal oxidase used by *Escherichia coli* under microaerobic conditions (54). These mutants were sensitive to hydrogen peroxide. Overexpression of the oxidative radical-scavenging enzyme Cu-Zn superoxide dismutase (SOD) or catalase restored resistance to hydrogen peroxide similar to that of wild-type *Brucella*, suggesting that *cyd* mutants are susceptible to reactive oxygen intermediates (ROIs) in macrophages. However, the specific mechanisms of ROI susceptibility in *cydB* mutants have not been investigated.

purE* and *aroC. Phagosomes of macrophages contain limited nutrition for intracellular bacteria (58). Thus, auxotrophic mutations in essential metabolic pathways generate derivative strains with reduced virulence (74). For example, *Salmonella* mutants that require aromatic compounds (*aroA*) or adenine (*purA*) for growth were attenuated. In this context, *B. melitensis* purine-auxotrophic *purE* deletion mutants defective in the de novo pathway leading to inosine biosynthesis were generated and were attenuated in vitro (49) and in vivo (41). *B. suis* aromatic compound auxotrophic *aroC*::Tn*5* mutants were originally identified by STM in vitro (59), and then in vivo attenuation was confirmed (60). The *aroC* gene encodes chorismate synthase, which catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate (EPSP) to chorismic acid. Chorismate is a branching point from which separate pathways lead to the aromatic amino acids (i) *para*-aminobenzoic acid and to hence folic acid, vitamin K, ubiquinone, and the electron transport systems, and (ii) dihydroxybenzoic acid, which is the first step in the biosynthesis of the siderophore enterochelin. Thus, the *aroC* mutation causes serious metabolic problems in bacteria. In fact, these auxotrophic mutants are useful for developing genetically defined live, attenuated vaccine strains of bacterial pathogens. *B. melitensis purE* mutants are being tested as a vaccine strain (33, 121).

Heat shock proteins. Bacterial pathogens that maintain long-term residence within host phagocytes probably express a variety of genes to help them adapt to the harsh environmental conditions of pH, nutrition deprivation, ROIs, and reactive nitrogen intermediates (RNIs) as well as lysosomal enzymes encountered within the phagosome (10). Prominent among these responses is the induction of heat shock proteins, suggesting that considerable protein misfolding and damage occurs within this compartment (101, 132). However, the role of these proteins in *Brucella* pathogenesis was uncertain. *B. abortus lon* transposon mutants were attenuated in BALB/c resident peritoneal macrophages but persistent in BALB/c mice except for a minor attenuation at 1 week postinfection, suggesting that Lon protease is important for *Brucella* survival during early infection (135). *B. suis dnaK* insertional mutants, defective in a member of the Hsp70 family, were attenuated in the human macrophagic cell line, U937 (93). *B. abortus htrA* deletion mutants, deficient in a serine protease called high-temperature-requirement A protein, have been considered to be attenuated in vitro and in vivo (53, 126), but a recent report suggests that *htrA* mutants were in fact *htrA cycL* double de-

letion mutants (50). An additional report suggests that a *B. melitensis* authentic *htrA* deletion mutant was not attenuated in goats, suggesting that HtrA is not involved in *Brucella* pathogenesis (139). Also, the *B. suis clpA* deletion mutant was not attenuated in vitro or in vivo (51). Taken together, these reports suggest that not all heat shock proteins are critical to *Brucella* pathogenesis or that redundant function of heat shock proteins will compromise the functional deficiency caused by the loss of one heat shock protein.

hfq. RNA chaperone host factor 1 (HF-1) is an abundant protein that participates in numerous regulatory pathways in *E. coli* (112). This protein acts posttranscriptionally to regulate the expression of the alternative sigma factor, RpoS, required for maintenance of the stationary-phase growth state. *B. abortus hfq* deletion mutants were sensitive to H_2O_2 and acid stress in stationary phase and were attenuated in BALB/c resident peritoneal macrophages as well as BALB/c mice (136), suggesting that HF-1 contributes to resistance of killing by host macrophages.

sod. SOD forms part of the antioxidant defense system that protects bacteria from the toxic effects of ROIs by converting superoxide radicals into hydrogen peroxide and oxygen (63), suggesting that it plays a crucial role in *Brucella* intracellular survival. *B. abortus* S19 *sod* deletion mutants were not attenuated in vitro or in vivo (154). However, *B. abortus* S2308 *sod* deletion mutants were attenuated in BALB/c mice but virulent in HeLa and J774 cell lines, suggesting a role for SOD in activated macrophages in vivo.

BRUCELLA IMMUNITY

Innate Immunity

Innate immunity is composed of nonspecific immune response in the early stage of infection before adaptive immunity, mediated by clonal selection of specific lymphocytes, has become established. Thus, the ultimate roles of the innate immune system in *Brucella* infection in vivo are to reduce the initial number of infected bacteria without memorization and to provide the environment for generating Th1 immune responses in the host.

Complement. Complement is a system of plasma proteins that interact with bound antibodies or bacterial surfaces to opsonize or directly kill the pathogens by forming a membrane attack complex with gram-negative bacteria (137). Currently, three pathways of complement activation mechanism have been elucidated: (i) the classical pathway, mediated by antigen-antibody complexes; (ii) the alternative pathway, induced by certain structures on the surface of microorganisms in an antibody-independent manner; and (iii) the lectin pathway, activated by the binding of mannose-binding lectin to carbohydrates on microbial surfaces. Because the major surface component of *Brucella* is an LPS, the interaction between *Brucella* and complement components will be mediated by this molecule. In general, smooth strains of *B. abortus* are more resistant than OPS-deficient rough strains to serum bactericidal activity (37) because *B. abortus* LPS obtained from smooth strains does not activate the alternative pathway (72). Thus, the action of the classical pathway mediated by IgM and low concentrations of IgG has been considered to be the dominant bactericidal action of serum against *B. abortus* mediated in the early stage of infection. Recent results using *B. abortus* and *B. melitensis wboA* mutants lacking OPS suggest that both the classical pathway and the lectin pathway are involved in complement deposition and complement-mediated killing of *Brucella* (56). However, at the later stage of *Brucella* infection in cattle, the increased concentration of IgG1 and IgG2 antibodies appeared to block killing of extracellular *Brucella* (37), even though opsonization still occurred, suggesting concomitant extension of disease. This contradictory role for antibody and complement in *Brucella* immunity has been explained by prozone effects (73).

Neutrophils. Neutrophils are short-life-cycle cells constituting 50 to 70% of human blood cells; their major function is phagocytosis. Thus, neutrophils are probably the first immune-associated cell in human infection to encounter *Brucella*. Rapid phagocytosis of virulent and attenuated *Brucella* strains by neutrophils occurs only following opsonization with normal human serum (164), suggesting that opsonization is a prerequisite for phagocytosis. However, the survival of *Brucella* in neutrophils during early infection has been observed (133), suggesting that the transportation of *Brucella* to lymphoid tissues can be mediated by neutrophils and that antimicrobial resistance mechanisms against hypohalide, lactoferrin, bacterial permeability-increasing factor, serprocidins, phospholipase A_2, cathelicidin, lysozyme, and defensins, as well as ROIs and RNIs, may exist in *Brucella* (115). For example, *B. abortus* releases GMP and adenine and inhibits the myeloperoxidase-H_2O_2-halide antibacterial systems in bovine neutrophils by hampering degranulation (27). Also, viable *Brucella* organisms, but not cell wall fractions of virulent *Brucella* strains, inhibit the respiratory oxidative burst by neutrophils (27, 95).

NK cells. Aqueous ether extract of *B. abortus* strain 456, known as Bru-Pel, stimulated NK cytotoxicity against YAC-1 cells (163). The cytotoxicity of human NK cells was enhanced when NK cells were treated with interleukin-12 (IL-12) obtained from macrophages treated with heat-killed *B. abortus* in vitro (165), suggesting a significant role of NK cells in brucellosis. In addition, human brucellosis patients have impaired NK cytotoxicity against YAC-1 cells that can be overcome by in vitro incubation with IL-2 (141), suggesting that the dysfunction of NK cells promotes the chronicity of infection. In contrast, mice with NK cell depletion as a result of monoclonal antibody anti-NK1.1 or polyclonal antibody anti-asialo-GM_1 antiserum did not harbor the increased splenic and hepatic *B. abortus* CFU during the first week after infection (55), suggesting that the role of NK cells in mice is minor.

Macrophages. The bactericidal functions contained in macrophages are ROIs and RNIs, which are induced by gamma interferon (IFN-γ) and tumor necrosis factor (TNF-α). Treatment of murine macrophages with methylene blue, an electron carrier, and ROI inhibitors increased and decreased the number of intracellular *B. abortus* organisms, respectively (82). However, the RNI inhibitor N^G-monomethyl-L-arginine could not block the anti-*Brucella* activities in macrophages, suggesting that ROIs play a greater role than RNIs in *Brucella* killing in vitro. This result is compatible with a previous report that phagocytosis of *B. suis* by mouse macrophages induced superoxide production (64). In addition, iron-loaded macrophages activated with IFN-γ had an increased capacity to kill intracel-

TABLE 2. *Brucella* infection in gene KO or immunocompromised mice

Mouse strain	Function	Deficient phenotypes	Species	Result of infection	Reference(s)
IRF-1$^{-/-}$	IRF-1	IL-12, IL-15, IL-18, iNOS, CD8$^+$ T cells, NK cells	*B. abortus*	Acute death	89
IRF-2$^{-/-}$	IRF-2	IL-12, NK cells	*B. abortus*	Resistant	89
ICSBP$^{-/-}$	ICSBP	IL-12, ROIs	*B. abortus*	Susceptible	89
NOS2$^{-/-}$	iNOS	Nitric oxide in macrophages	*B. abortus*	Delayed control	89
gp91$^{phox-/-}$	NADPH oxidase component	NADPH oxidase-dependent ROIs	*B. abortus*	Delayed control	89
IL-12p40$^{-/-}$	IL-12 component	IL-12	*B. abortus*	Susceptible	89
IFN-$\gamma^{-/-}$	IFN-γ	IFN-γ	*B. abortus*	Death (C57BL/6 and BALB/c)	113
TNFR$^{-/-}$	TNF receptors 1 (p55) and 2 (p75)	TNF-α signaling	*B. abortus*	Susceptible	168
NF-IL-6$^{-/-}$	Nuclear factor IL-6	G-CSF	*B. abortus*	Susceptible in vitro	127
β2-m$^{-/-}$	MHC class I β_2-microglobulin	CD8$^+$ T cells	*B. abortus*	Susceptible to S19, resistant to S2308	113, 119
A$\beta^{-/-}$	MHC class II Aβ chain	CD4$^+$ T cells	*B. abortus*	Resistant	119
Rag-1$^{-/-}$	Recombination activation gene 1	Matured B and T cells	*B. melitensis*	Susceptible	79
SCID	Lacks mature B and T cells	Matured B and T cells	*B. abortus*	Resistant	Morfitt et al.[a]
Nude	Thymic stroma, matured T cells	Lacks matured T cells	*B. abortus*	Susceptible (persistence)	32
Perforin$^{-/-}$	CTL killing	Absence of perforin-mediated killing	*B. abortus*	Resistant	113

[a] D. C. Morfitt, N. F. Cheville, and A. E. Jensen, Proc. Conf. Res. Workers Anim. Dis. **72:**2, 1991.

lular brucellae and frequently eliminated all intracellular brucellae due to the ability of iron to catalyze the Haber-Weiss reaction, confirming an important function of ROIs (9, 81). However, *B. abortus*-infected NOS2 as well as gp91^{phox} knockout (KO) mice showed delayed control of infection, suggesting that both RNIs and ROIs play a role in controlling *Brucella* infection in the early stages of infection in vivo (89) (Table 2). In addition, the infected NOS2/gp91^{phox} double-KO mice showed a delayed control of infection, suggesting that inducible nitric oxide synthase (iNOS) and NADPH oxidase-independent killing mechanisms exist in mice. A recent understanding of IFN signaling suggests that IFN-regulatory factors (IRFs), a family of second transcriptional factors, mediate diverse phenotypes induced by IFNs. Among the nine IRFs, the expression of IRF-1, IRF-2, ICSBP (IRF-8), and IRF-9 is induced by IFN-γ, and IRF-1-, IRF-2-, and ICSBP-deficient mice were infected with *B. abortus* (Table 2) (89). Infected IRF-1 KO mice died within 2 weeks due to hepatic damage, while IRF-2 KO mice were more resistant to infection than were wild-type mice. The role of IRF-2 involves downregulation of the genes activated by IRF-1, supporting the significance of IRF-1 in brucellosis. Also, ICSBP KO mice were susceptible to infection. These results suggest that IRF-1 and ICSBP are involved in the *Brucella*-controlling mechanisms induced by IFN-γ.

Nramp1. Murine susceptibility to *Leishmania*, *Salmonella*, and *Mycobacterium* was influenced by one gene, located on chromosome 1 (12). A reverso-genetics approach identified a candidate gene, *Nramp1*, which was expressed only in macrophagelike cells. A single nonconservative amino acid substitution in this transporter was found to correlate with the susceptible genotype in 27 inbred mouse strains. In murine brucellosis, resistance to the attenuated *B. abortus* strain 19 occurs in the *Lsh*/*Ity*/*Bcg*-susceptible mouse strains such as C57BL/10 and BALB/c while susceptibility occurs in the *Lsh*/*Ity*/*Bcg*-resistant CBA/H mice (71), suggesting that resistance to *Brucella* seems to be more complex or associated with different genes. However, bovine *Nramp1* stable transfectants of RAW264.7 cells control *B. abortus* infection better than do parental cell lines in vitro (11), suggesting that natural heritable resistance plays a role in bovine brucellosis.

Adaptive Immunity

Adaptive immune responses are critical for providing the memory function that is the key player in vaccination. Functions of the adaptive immune response in brucellosis can be classified into three mechanisms. First, IFN-γ produced by CD4$^+$, CD8$^+$, and $\gamma\delta$ T cells activates the bactericidal function in macrophages to hamper the intracellular survival of *Brucella*. Second, cytotoxicity of CD8$^+$ and $\gamma\delta$T cells kills the infected macrophages. Third, Th1-type antibody isotypes such as IgG2a and IgG3 opsonize the pathogen to facilitate phagocytosis.

$\alpha\beta$ CD4$^+$ and CD8$^+$ T cells. The major role of T cells in *Brucella* immunity is secretion of IFN-γ for the activation of bactericidal function in macrophages and cytotoxic T-lymphocyte activity as well as IgG2a and IgG3 isotype switching. The significance of CD4$^+$ and/or CD8$^+$ T cells in *Brucella* immunity has been controversial. Adoptive transferred BALB/c mice with CD4$^+$, CD8$^+$, and whole T-cell populations obtained from mice immunized for 6 weeks contained fewer splenic *Brucella* CFU than did untreated mice after infection (6), suggesting that both T-cell populations are important in *Brucella* immunity. However, recent experiments with A$\beta^{-/-}$ and β2-m$^{-/-}$ mice infected with *B. abortus* strain 19 suggest that major histocompatibility complex (MHC) class I-deficient mice, which have no CD8$^+$ T cells, control the infection more slowly than do wild-type mice while MHC class II-deficient mice, defective in CD4$^+$ T cells, control the infection similarly to

wild-type mice (119), suggesting that $CD8^+$ T cells play a critical role. This result is compatible with the previous report that anti-Lyt-2- and complement-treated spleen mononuclear cells could not provide protection (124), suggesting that $CD8^+$ T cells comprised the predominant protective population. Others, however, have suggested that $\beta2\text{-}m^{-/-}$ mice infected with *B. abortus* strain 2308 controlled infection and that $CD8^+$ cytotoxic T lymphocytes were not important after the first week of infection with a virulent strain of *B. abortus* (113). The differences in the results may be related to the strain of *B. abortus* used in the $\beta2\text{-}m^{-/-}$ mice. Additional experiments with MHC class I-deficient mice as well as wild-type mice suggest that the production of Th-1 type cytokines such as IFN-γ and IL-2 by $CD8^+$ T cells has an important function in controlling brucellosis (119). The importance of IFN-γ in resolution of *Brucella* infection was supported by studies with BALB/c and C57BL/6 IFN-$\gamma^{-/-}$ mice infected with *B. abortus* strain 2308 since these infected mice died in approximately 6 weeks (113). However, the major T-cell population is $CD4^+$ T cells in terms of number, and this cell population secretes IFN-γ. Thus, the role of $CD4^+$ T cells in brucellosis should not be ignored.

γδ T cells. Human patients infected with *B. melitensis* contain increased numbers of γδ T cells possessing the Vγ9Vδ2 T-cell receptor (TCR) in peripheral blood (13, 123). Vγ9Vδ2 T cells activated by nonpeptide antigens control the increase in the number of intracellular *Brucella* organisms by secreting TNF-α and IFN-γ to activate macrophage bactericidal function and by lysing the infected cells through cytotoxicity in vitro (122). Interestingly, in cattle younger than 1 year, the major T-cell population is not αβ T cells but γδ T cells, suggesting that the role of γδ T cells could be more significant in *Brucella*-infected calves. However, the role of these γδ T cells in vivo has not been characterized.

B cells. Many serum passive-transfer experiments suggest the significance of humoral immunity in murine brucellosis. For example, passive transfer of sera containing anti-LPS antibodies to mice could protect against challenge with virulent *B. abortus* (6, 107, 161). Also, passive transfer of *B. abortus* OPS-specific monoclonal antibody (IgG2a) could reduce bacterial infection in mice or protect mice from *B. abortus* infection (106, 108, 125). IgG2a and IgG3 are dominant antibody isotypes detected from the infected mice, similar to the natural host, suggesting that a Th1 immune response to *Brucella* infection occurred (52). Opsonization, probably coupled with enhancement of intracellular killing, is regarded as the principal protective role of antibody against *Brucella* infection. Despite numerous reports of a role for humoral immunity in resistance to brucellosis, the ability of antibody to protect the host appears controversial. For instance, the *B. abortus* RB51 strain lacking OPS still provides the best protection as a vaccine strain, indicating that immune protection is possible without OPS-specific antibody (145). Also in bovine brucellosis, the high concentration of IgG during active infection prevents extracellular bacterial lysis mediated by complement and promotes bacterial phagocytosis, enhancing the intracellular location of bacteria and the extension of disease (73).

Cytokines. Cytokines play two critical roles in immune responses: (i) to mediate innate and adaptive immunity and (ii) to direct the immune response among immune-associated cells. Cytokines, regarded as key players in brucellosis, are IL-12, IFN-γ, and TNF-α. IL-12 is a key cytokine produced by B cells and macrophages and leads Th1 immune responses in the host that will ultimately induce the secretion of IFN-γ from T cells. The significance of IL-12 in brucellosis has been well described (89, 167). However, the mechanisms of IL-12 induction in those cells have not been elucidated. Th1-polarized T cells produce IFN-γ, which activates the bactericidal function of macrophages, the host cells of *Brucella* spp., and this phagocytic function can be maximized by the treatment of TNF-α produced by macrophages and NK cells (166, 168, 170). In fact, live *B. suis* can inhibit TNF-α production in human macrophages by secreting proteins, suggesting another *Brucella* strategy for intracellular survival (28). An elevated IL-6 concentration in serum has been detected in *Brucella*-infected humans and antigen-treated mice (1, 169). $CD4^+$ T cells produce IL-6, but the role of IL-6 in brucellosis has not been clearly delineated (144). Additionally, other cytokines are involved in *Brucella* immunity in mice. The detection of fewer CFU in spleens in mice given recombinant IL-1 suggests an important role of IL-1 in murine brucellosis (171). The role of IL-1 in murine brucellosis was speculated to induce colony-stimulating factor (CSF) and increase the numbers of neutrophils and macrophages in the spleen. In fact, mice given CSF for the first 14 days of infection experienced an influx of macrophages into the spleen and liver, and fewer CFU were detected in the spleen but not in the liver, supporting the previous speculation about the role of IL-1 (48). Also, the addition of granulocyte CSF (G-CSF) to macrophages obtained from NF–IL-6 KO mice restores both endocytosis and the morphology of endosomes as well as bactericidal activity in macrophages (127), suggesting the importance of G-CSF in phagocytosis.

Vaccine Strategy

Live or dead vaccines. Immunization with killed *B. abortus* S2308 provided better protection than immunization with live S19 at 1 week postinoculation (107). However, at 4 weeks postinoculation mice immunized with killed *Brucella* could not provide protection while mice immunized with live *B. abortus* S19 provided log 2 protection, suggesting that a live vaccine is superior to a killed vaccine. Additional vaccination results using *B. abortus* S2308, S19, and RB51 suggest that the persistence of the *Brucella* vaccine strain is critical to maximum protection (68, 149, 151, 157). Compared to subunit and DNA vaccines, the attenuated vaccine contains all the immunogenic components that can be involved in protection, making this vaccine type more efficient. Currently, many B- and T-cell antigens have been identified. B-cell antigens (Table 3) have been used as a diagnostic marker, but the antibodies produced by these antigens also can be involved in opsonization in the host. T-cell antigens (Table 4) that have been characterized by cell proliferation assays are $CD4^+$ T-cell antigens presented to the TCR through MHC class II. These activated $CD4^+$ T cells produce a significant amount of IFN-γ. However, C57BL/6 mice infected with *B. abortus* S19 produced cytotoxic T-lymphocyte activity (119), suggesting that antigens presented to the TCR through MHC class I exist. No endogenous peptides presented by MHC class I molecules have been identified.

Subunit and DNA vaccines. Cell-mediated immunity is the dominant immune response required for protection against

TABLE 3. *Brucella* antigens that induce a B-cell response

Antigen	Function	Species	Serum host	Protection	Reference(s)
Omp19	Outer membrane protein	*B. abortus*	Mouse, goat, dog, human	ND[a]	94
Omp25	Outer membrane protein	*B. ovis*	Mouse	Yes	18
Omp28	Outer membrane protein	*B. melitensis*	Human	ND	102
Omp31	Outer membrane protein	*B. ovis*	Ram	Yes	88
Omp89	Outer membrane protein	*B. abortus*	Cattle	ND	100
CP24	Ribosome-releasing factor	*B. melitensis*	Sheep	ND	159
HtrA	Heat shock protein	*B. abortus, B. melitensis*, *B. canis*	Mouse, cattle, goat, dog	ND	138
18-kDa protein	Lumazine synthase	*B. ovis, B. canis*	Human, cattle	ND	8, 66, 67
BCSP31	Unknown	*Brucella* spp. except *B. ovis*	Rabbit	ND	20
DnaK	Heat shock protein	*B. melitensis*, *B. ovis*	Mouse, sheep	ND	155, 160
BP26 (CP28)	Periplasmic or cytoplasmic protein	*B. abortus, B. melitensis*	Cattle, human, ram	ND	42, 140
17-kDa protein	Unknown	*B. abortus*, *B. melitensis*	Sheep, cattle	ND	70
22.9-kDa protein	Unknown	*B. abortus*	Unknown	Yes[b]	30
32.2-kDa protein	Unknown	*B. abortus*	Unknown	No	30
20-kDa protein (antigen A-2)	Unknown	*B. melitensis*	Unknown	ND	148, 173
Dihydrolipoamide succinyltransferase	Dihydrolipoamide succinyltransferase	*B. ovis*	Mouse	ND	155
31-kDa protein	Unknown	*B. ovis*	Unknown	ND	155
Malate dehydrogenase	Malate dehydrogenase	*B. ovis*	Unknown	ND	155
Succinyl coenzyme A synthetase alpha subunit	Succinyl coenzyme A synthetase alpha subunit	*B. ovis*	Unknown	ND	155
ABC[a]-type transporter	ABC-type transporter	*B. ovis*	Unknown	ND	155
Leu/Ile/Val-binding-protein precursor	Leu/Ile/Val-binding-protein precursor	*B. ovis*	Unknown	ND	155
ClpP	Stress protein	*B. ovis*	Unknown	ND	155
NikA	Nickel transport	*B. ovis*	Unknown	ND	155

[a] ND, not determined; ABC, ATP-binding cassette.
[b] With Freund's adjuvant.

brucellosis. Thus, the concept of a subunit vaccine in brucellosis is based on the generation of memory Th1 cells by immunization with T-cell antigen. The initial requirement for a subunit vaccine approach is to identify T-cell antigens (Table 4). So far, P39, bacterioferritin, and L7/L12 proteins have been purified and tested as subunit vaccines with adjuvants (3, 120). Mice immunized with these proteins had a certain level of protection when challenged. Alternatively, DNA vaccination has been tried by using genes encoding the L7/L12 proteins, and this method also conferred some degree of protection (96). However, will subunit or DNA immunizations provide long-term protection to the hosts? Perhaps the injection of recombinant proteins made with many T-cell epitopes or DNA encoding recombinant T-cell epitopes may provide better protection than a single-epitope subunit vaccine. However, it is not clear whether such vaccination approaches will be effective for long-term protection. No specific experiment has been performed to answer this question.

TABLE 4. *Brucella* antigens that induce a T-cell response

Antigen	Function	Species	Host	Protection	Reference(s)
P39	Periplasmic binding protein	*B. melitensis*	Guinea pig, cattle, mouse	Yes, with CpG	3, 45, 46
Bacterioferritin	Iron-binding protein	*B. melitensis*	Guinea pig, cattle, mouse	Yes, with CpG	3, 46
GroEL	Heat shock protein	*B. abortus*	Mouse, guinea pig	ND[a]	117, 118, 120, 150
GroES	Heat shock protein	*B. abortus*	Mouse, guinea pig	ND	117, 120
YajC	Transmembrane protein	*B. abortus*	Mouse	ND	158
UvrA	DNA repair enzyme	*B. abortus*	Cattle, mouse, guinea pig	ND	117, 120, 172
L7/L12	Ribosomal proteins	*B. abortus*	Mouse	Yes, with Freund's adjuvant	117, 120
BA14K	Unknown	*B. abortus*	Mouse	ND	34
SOD (BCSP20)	Cu-Zn SOD	*B. abortus*	Cattle, mouse	Yes, but synthetic peptide	21, 152, 153
31 kDa	Unknown	*B. abortus*	Cattle	ND	25

[a] ND, not determined; CpG, unmethylated synthetic CpG oligonucleotides.

FUTURE TRENDS

The 21st century is the period of postgenomics. For brucellosis research, the completion of the human and murine genome projects will provide an opportunity for screening genome-wide responses to *Brucella* infection in the host. In addition, the *B. melitensis* and *B. abortus* genome projects should provide useful information for performing reverse-genetic approaches to elucidate virulence factors. Thus, based on "gain of function" and "loss of function," various genetic approaches in the host and pathogen are being explored. Also, diverse computer-aided fluorescence- or luminescence-based detection systems will be utilized to monitor the location of *Brucella* in vitro and in vivo.

Genome-Wide Screening, Gene Arrays and Proteomics

Gene arrays and proteomics are novel computer-aided screening systems to detect changes at the transcriptional and/or translational level in the host and/or pathogen (29). These complementary screening systems are very powerful in detecting the small changes in gene transcription or translation and providing patterns of gene expression to improve our knowledge of highly complex networks that cross-communicate in the cells. However, there are two problems with these techniques. First, there is not a good correlation between mRNA and protein levels (4), implying that many transcriptional changes are not biologically relevant. Second, there is a requirement to substantiate the biological relevance of the genes that indicated transcriptional and translational changes in host-pathogen interaction models. The application of gene KOs within the pathogen and host will provide an approach to investigate the significance of the changes observed in gene arrays and proteomics. Taken together, even with the shortcomings of genome-wide screening systems, the patterns of gene expression will facilitate the elucidation of the interplay between host and pathogen.

Introduction of Green Fluorescent Protein-encoded *Brucella* Strains

Cellular microbiology is an emerging discipline that is being used to investigate the effect of microbes on cell biology (40, 129). For instance, many intracellular bacteria can survive inside specialized compartments or in the cytoplasm, suggesting that these bacteria have unique invasion mechanisms. In fact, the dissection of bacterial pathogenesis provides information for understanding the dynamics of the host cell cytoskeleton, intracellular membrane, and signal transduction events. In *Brucella* research, the movement of *Brucella* inside of phagocytic and nonphagocytic cells is being investigated with green fluorescent protein (GFP)-expressing *Brucella* strains (114, 134). This fluorescence-based technique is a useful tool to detect the cellular location of *Brucella* in combination with diverse monoclonal antibodies to define the molecular organelles. In addition, GFP is quite stable and can be used for immunostaining of *Brucella* on the tissue sections with GFP-specific monoclonal antibodies.

Novel Methods To Identify *Brucella* Virulence Factors

The most recent significant breakthrough in *Brucella* pathogenesis research is the application of STM, where mutants were tested in phagocytic and nonphagocytic cells (59) as well as acute (97) and chronic (51) stages of infection in mice. Many *Brucella* attenuated mutants have been identified and are being characterized individually (Table 5). In addition, differential fluorescence induction (92) has identified several promoters induced specifically within macrophage cell lines. However, individual mutants must be generated to establish a role for these genes in *Brucella* virulence. In addition, the recent completion of the *Brucella* genome projects should provide useful information about *Brucella* physiology and pathogenesis (142, 146), codifying the renaissance of reverse genetics.

In Vivo Imaging System

The virulence of *Brucella* has been assessed in vivo by enumerating the residual CFU in splenic or hepatic tissues in a time-dependent manner. However, the current development of photonic detection systems promises to monitor the kinetics of bacteria in vivo without killing the infected mice (36, 61). Basically, *luxABCDE Brucella* can be used to infect mice and the infected mice can be analyzed using an intensified charge-coupled device camera and analytical software. This simple equipment will alleviate the ethical problems caused by killing mice, accelerate the detection of attenuation in *Brucella* strains, and detect the location of *Brucella* in vivo in a time-dependent manner.

Dominant Negative Cells and Gene Knockout Mice

The loss of function is a dominant dogma in genetics to analyze the function of a gene. The two major approaches to generate loss-of-function phenotypes are the use of dominant negative cell lines and the use of gene KO mice. Diverse KO mice have been used in *Brucella* research for elucidating the function of respective immune components (Table 2). However, dominant negative cell lines have not been used in brucellosis research, unlike other intracellular bacterial pathogen research (78, 111). The generation of dominant negative cells requires a relatively short time compared to the production of gene KO mice. Besides, dominant negative cell lines will be useful for in vitro experiments in cases where the gene KO is lethal to the mice.

Gene-to-Gene or Function-to-Function Interactions

During the last several decades in brucellosis research, three different kinds of combinatorial experiments were widely performed. First, to understand the general immune responses in the host, wild-type mice were infected with wild-type *Brucella* strains. Second, to find a virulence factor or to detect the attenuation in *Brucella*, mutant *Brucella* strains were used to infect wild-type mice. Third, to understand the role of specific immune components in brucellosis, gene KO mice were infected with wild-type *Brucella* strains. However, gene KO mouse infection with mutant *Brucella* strains had not been performed in brucellosis research until the introduction of IRF-$1^{-/-}$ mice. The virulence of *B. abortus* in IRF-$1^{-/-}$ mice

TABLE 5. *Brucella* genes identified by STM

Gene	Function	Species	Virulence		References
			In vitro	In vivo	
ntrY	Two-component system (nitrogen metabolism)	*B. suis*	Attenuated	ND[a]	59
glnD	Uridylyl transferase (nitrogen metabolism)	*B. suis*	Attenuated	ND	59
pgi	Phosphoglucose isomerase (glucose metabolism)	*B. suis*	Attenuated	ND	59
manB	Phosphomannomutase (LPS synthesis)	*B. suis*	Attenuated	ND	59
gpt	Guanine phosphoribosyltransferase (nucleotide synthesis)	*B. suis*	Attenuated	ND	59
LTTR-like	LysR family transcriptional regulator	*B. suis*	Attenuated	ND	59
carAB	Carbamoyl phosphate synthase (arginine and pyrimidine metabolism)	*B. suis*	Attenuated	ND	59
rpsA	Ribosomal protein S1	*B. suis*	Attenuated	ND	59
pyc	Pyruvate carboxylase (glucose metabolism)	*B. suis*	Attenuated	ND	59
leuA	Isopropylmalate synthase (leucine synthesis)	*B. suis*	Attenuated	ND	59
cysK	*O*-Acetylserine (thiol)-lyase (amino acid metabolism)	*B. melitensis*	Attenuated	Attenuated	97
cysI	Sulfite reductase (amino acid metabolism)	*B. melitensis*	Attenuated	Attenuated	97
metH	Homocysteine-*N*5-methyltetrahydrofolate transmethylase (amino acid metabolism)	*B. melitensis*	Attenuated	Attenuated	97
mgtB	Mg^{2+} pump (transporter)	*B. melitensis*	Attenuated	Attenuated	97
rbsC	Ribose permease (transporter)	*B. melitensis*	Attenuated	Attenuated	97
ugpA	Glycerol-3-phosphate permease (transporter)	*B. melitensis*	Attenuated	Attenuated	97
ccrM	Adenine DNA methyltransferase (DNA metabolism)	*B. melitensis*	Attenuated	Attenuated	97
dnaJ	Heat shock protein	*B. melitensis*	Attenuated	Attenuated	97
mtgA	Peptidoglycan transglycosylase (membrane metabolism)	*B. melitensis*	Attenuated	Attenuated	97
vsrB	Two-component system	*B. melitensis*	Attenuated	Attenuated	97
fliF	Flagellar MS ring	*B. melitensis*	Attenuated	Attenuated	97
gcvB	Glycine dehydrogenase	*B. abortus*	ND	Attenuated	75
gluP	Glucose/galactose permease	*B. abortus*	ND	Attenuated	75
wbkA	LPS synthesis	*B. abrotus*	ND	Attenuated	75
gltD	Glutamate synthase	*B. abortus*	ND	Attenuated	75

[a] ND, not determined.

can be assessed by either the death or survival of infected mice (89). The rapidity of death of IRF-$1^{-/-}$ mice and the hepatic damage correlated with virulence when mice were infected with 5×10^5 CFU (90), suggesting that the hepatic damage contributes to the death of IRF-$1^{-/-}$ mice. This is one example of a combinatorial experiment. Similarly, STM experiments can be performed directly with specific gene KO mice instead of wild-type mice. Each KO mouse provides a different selective pressure to the mutants, and different sets of mutants are selected compared to wild-type mice. Thus, a unique opportunity exists to identify particular bacterial genes that cope with specific immune components. Alternatively, in vitro STM can be performed using specific KO cell lines such as dominant negative cell lines or cells obtained from individual gene KO mice. By using a specific dominant negative cell line infected with *Brucella*, host responses could be analyzed with DNA chips or proteomics. Also, gene KO mice could be infected with *Brucella* and then the responses of a purified cell population could be detected by using DNA chips or proteomics. Not only wild-type *Brucella* but also diverse genetically manipulated *Brucella* mutants can be used for these kinds of combinatorial experiments.

CONCLUSION

Novel *Brucella* vaccine strains have to be made based on an insightful understanding of bacterial pathogenesis and host immunity. The rapid progress in brucellosis research was possible due to the application of STM, GFP-encoded *Brucella* strains, and gene KO mice. Characteristics of a *Brucella* vaccine, based on current knowledge, would be a live, attenuated organism without OPS because (i) a live, attenuated *Brucella* strain can persist in the host to stimulate Th1 immune responses and provide the antigens to stimulate B and T cells and (ii) the absence of OPS can serve as a diagnostic marker to discern infected and vaccinated animals. Therefore, the generation of live, attenuated strains based on host-pathogen interactions is being explored in combination with rapid and effective methods to detect attenuation in *Brucella* strains. In conjunction with genome-wide screening techniques, application of in vivo imaging of bacteria and IRF-1 KO mice will facilitate the detection of attenuation and ultimately accelerate the development of *Brucella* vaccine strains.

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