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# Molecular Characterization of the *sucB* Gene Encoding the Immunogenic Dihydrolipoamide Succinyltransferase Protein of *Bartonella vinsonii* subsp. *berkhoffii* and *Bartonella quintana*

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**Members of the genus *Bartonella* have historically been connected with human disease, such as cat scratch disease, trench fever, and Carrion's disease, and recently have been recognized as emerging pathogens causing other clinical manifestations in humans. However, because little is known about the antigens that elicit antibody production in response to *Bartonella* infections, this project was undertaken to identify and molecularly characterize these immunogens. Immunologic screening of a *Bartonella vinsonii* subsp. *berkhoffii* genomic expression library with anti-*Bartonella* antibodies led to the identification of the *sucB* gene, which encodes the enzyme dihydrolipoamide succinyltransferase. Antiserum from a mouse experimentally infected with live *Bartonella* was reactive against recombinant SucB, indicating the mounting of an anti-SucB response following infection. Antigenic cross-reactivity was observed with antiserum against other *Bartonella* spp. Antibodies against *Coxiella burnetti*, *Francisella tularensis*, and *Rickettsia typhi* also reacted with our recombinant *Bartonella* SucB. Potential SucB antigenic cross-reactivity presents a challenge to the development of serodiagnostic tests for other intracellular pathogens that cause diseases such as Q fever, rickettsioses, brucelloses, tularemia, and other bartonelloses.**

There is an increasing awareness of *Bartonella* spp. as causative agents of emerging diseases of human and veterinary importance. The genus *Bartonella* is comprised of several species of human and animal pathogens causing various zoonotic-related diseases (4, 9). In humans, *B. bacilliformis* causes Carrion's disease, seen mainly in the Andes mountain region of South America. The acute form of the illness is a severe hemolytic anemia, with the chronic form characterized by vascular proliferative lesions of the skin (21). *B. henselae* is responsible for cat scratch disease (CSD) and bacillary angiomatosis (28, 41). *B. quintana* is also associated with bacillary angiomatosis (28) but is more widely recognized as the causative agent of trench fever (26). Human endocarditis cases have been described involving *B. henselae*, *B. quintana*, *B. elizabethae* (16), and *B. vinsonii* subsp. *berkhoffii* (42). *B. vinsonii* subsp. *arupensis* has recently been described as causing a human febrile bacteremia (45), and *B. grahamii* has been associated with human neuroretinitis (24).

*Bartonella* infections are associated with arthropod vector transmission. *B. bacilliformis* is transmitted by sand flies (21), and *B. henselae* has been demonstrated in cat fleas (18, 22), with the human body louse (*Pediculus humanus*) instrumental in the transmission of *B. quintana* (43). Domestic cats are considered a reservoir host for *B. henselae* (14, 27), but some *Bartonella* are carried asymptomatically in a variety of wild rodents worldwide (5, 7, 31). Additionally, there is serological and molecular evidence of California coyotes serving as reservoir hosts (10, 13), and PCR data have implicated *Ixodid* ticks in harboring *Bartonella* (11, 12).

The current state of diagnostics for the determination of infection is underdeveloped, but serology by the indirect fluorescence assay and enzyme immunoassay against whole cells are the predominant methodologies being applied, mostly for CSD (15, 41). However, problems with cross-reactivity among *Bartonella* species and variable sensitivities and specificities observed among laboratories have led to caution when interpreting the serologic-based results (1, 6, 19, 23, 32). Furthermore, diagnostic assays for bartonelloses caused by organisms other than *B. henselae* or *B. quintana* are underdeveloped.

Little is known regarding antigens that induce an antibody response following *Bartonella* infection. Several immunogenic proteins associated with *Bartonella* infections have been noted by Western blot banding patterns (20, 35, 36–39), but only the *B. henselae* 17-kDa antigen and HtrA stress response protein and the *B. bacilliformis* Bb65 antigen have been characterized (2, 3, 25). *Bartonella*-specific monoclonal antibodies have been described, but the molecular identities of the corresponding antigens have not yet been elucidated (33, 34). The goal of this project was to identify immunogens associated with *Bartonella* infections. As a first step, we screened *Bartonella* genomic libraries with polyclonal antiserum against whole-cell lysates of various *Bartonella* isolates. In this report, we describe an immunoreactive *Bartonella* gene product as being dihydrolipoamide succinyltransferase expressed by the *sucB* gene, which is part of the α-ketoglutarate dehydrogenase complex that has been described in several prokaryotes.

**Identification of the *sucB* gene from genomic libraries.** *Bartonella* strains used in this study for DNA manipulations, immunoblotting, and antibody production are listed in Table 1. *B.*

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TABLE 1. *Bartonella* strains used in this study

| Strain | Description |
|---|---|
| *B. quintana* Fuller ATCC VR-358 | Human isolate |
| *B. vinsonii* subsp. *berkhoffii* ATCC 51672 | Dog isolate |
| *B. vinsonii* subsp. *vinsonii* ATCC VR-152 | Vole isolate |
| *B. henselae* Houston | Type strain |
| *B. elizabethae* ATCC 49927 | Type strain |
| A1, strain Sh6397ga | Cotton rat (*Sigmodon hispidus*) isolate (31) |
| B1, strain Sh6396ga | Cotton rat (*S. hispidus*) isolate (31) |
| C1, strain Sh6537ga | Cotton rat (*S. hispidus*) isolate (31) |
| *Neotoma albigula* strain Na18985nm | Wood rat isolate; New Mexico |
| *Peromyscus maniculatus* strain Pm15590co | Deer mouse (*P. maniculatus*) isolate; Colorado |
| *Spermophilus beecheyi* strain Sb944nv | California ground squirrel (*S. beecheyi*) isolate; Nevada |

*quintana* and *B. vinsonii* subsp. *berkhoffii* were cultivated on brain heart infusion agar medium supplemented with 5% rabbit blood (BBL Becton Dickinson Microbiology Systems, Cockeysville, Md.) and harvested as previously described (31). Genomic DNA was purified from thawed cell suspensions by a phenol-chloroform extraction procedure followed by ethanol precipitation according to standard procedures. For genomic cloning, purified *Bartonella* DNA was subjected to partial *Sau*3AI restriction enzyme digestion and was ligated into the ZapExpress *Bam*HI-predigested bacteriophage lambda cloning vector (Stratagene, La Jolla, Calif.) with subsequent packaging of the ligated DNA with the GigaPack III Gold packaging extract (Stratagene) as directed by the manufacturer. Recombinant lambda plaques were plated, titrated, and amplified according to the manufacturer's instruction manual.

Thirty-five immunopositive plaques from the *B. vinsonii* subsp. *berkhoffii* genomic library were recognized by a pool of mouse polyclonal antibodies raised against heat-killed *Bartonella* sp. cotton rat isolates A1, strain Sh6397ga, B1, strain Sh6396ga, and C1, strain Sh6537ga, and selected for further analysis. Western blotting of the recombinant products revealed one clone that consistently produced a stable protein that was strongly reactive to the screening antibody. The insert of this clone was 3.36 kb, and DNA sequence analysis revealed three open reading frames. According to the GenBank database, these genes were homologs of three components of the α-ketoglutarate dehydrogenase operon complex present in several prokaryotes: *sucA*, *sucB*, and *lpdA*, which encode the α-ketoglutarate dehydrogenase (E1o), dihydolipoamide succinyltransferase (E2o), and dihydrolipoamide dehydrogenase enzymes, respectively. The *sucA* and *lpdA* genes are truncated within this insert, and *sucB* is represented in its entirety. The *sucB* coding sequence consists of 1,233 bp with a calculated molecular mass of 43.8 kDa from the deduced amino acid sequence. The *sucB* gene coding sequence was subcloned into a plasmid expression vector and transformed into *Escherichia coli*, and the gene product was synthesized. The recombinant SucB immunoblotted positively with the anti-*Bartonella* antibodies used to screen the library (Fig. 1A, lanes 1 to 3).

The *sucB* gene was amplified by PCR from *B. quintana* DNA using primers derived from the *B. vinsonii* subsp. *berkhoffii* sequence, which encompassed the entire coding sequence of the gene. The *B. quintana sucB* gene was determined to have 87.8% amino acid sequence identity to the SucB of *B. vinsonii* subsp. *berkhoffii*. A BLAST search of the protein database with SucB found the closest matches were to *Mesorhizobium loti*, *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, and *Brucella melitensis*. Not surprisingly, these organisms are closely related phylogenetically to *Bartonella*. The amino acid sequence identity comparisons are shown in Table 2.

**Expression of *sucB* and antibody reactivities.** The coding sequence of the *B. vinsonii* subsp. *berkhoffii sucB* gene was amplified by PCR using the primers BvSucB-F (5′ ATGACT ACTGAAATCCGTGTTCC 3′) and BvSucB-R (5′ CAAGTC AAGAACAAGGCGTTC 3′) under the following conditions: 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, a 200 μM concentration of each deoxynucleoside triphosphate, a 0.5 μM concentration of each primer, and 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Branchburg, N.J.). Approximately 10 ng of the original recombinant plasmid containing the *sucB* gene was amplified using the thermocycler parameters 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s for 35 cycles. Purified *sucB* amplicon was subcloned into the expression vector pBAD/thio TOPO (Invitrogen) and transformed into *E. coli* strain TOP10 (Invitrogen). A colony containing the *sucB* gene was inoculated into Luria-Bertani broth and incubated at 37°C until growth reached mid-log phase, whereby protein expression was induced by the addition of 0.02% arabinose. The culture was incubated another 2 to 4 h, and then the cells were harvested, pelleted, and frozen at −20°C until needed. Recombinant SucB was purified from *E. coli* proteins by His-tag affinity chromatography using His-Bind Quick 300 cartridges (Novagen, Madison, Wis.) following lysis of the *E. coli* cells by suspension in B-PER bacterial protein extraction reagent (Pierce, Rockland, Ill.) with the addition of the protease inhibitor Pefabloc SC (Boehringer GmbH, Mannheim, Germany).

SucB reactivity was tested against polyclonal antibodies raised against various *Bartonella* species and strains. Cross-reactivity was observed against all anti-*Bartonella* antibodies tested (Fig. 1A). Although reactivity against anti-*B. quintana* and anti-*B. henselae* was weaker, this may simply reflect the strength of the antiserum used. Four samples of preimmunized mouse serum showed no reactivity to SucB (Fig. 1B, lanes 11 to 14). Significantly, there was robust reactivity with the recombinant SucB against antibodies from an experimentally infected mouse injected with live *Bartonella* isolated from *P. maniculatus* and boosted with an *S. beecheyi* strain (Fig. 1A, lane 10). This result demonstrated that an antibody response is mounted against SucB in response to a *Bartonella* infection, as

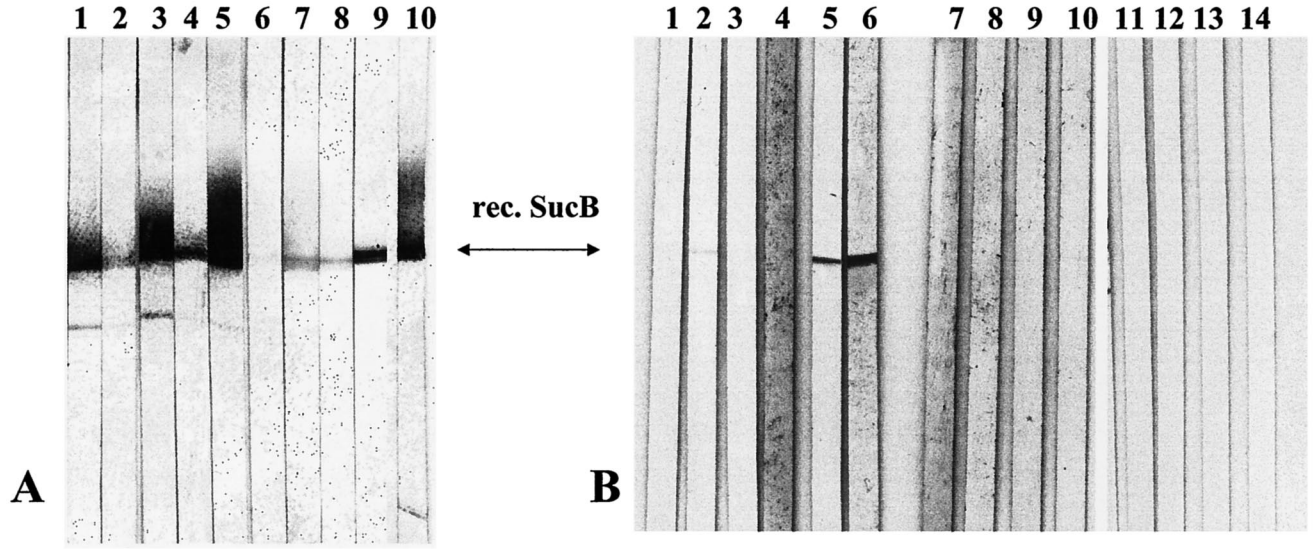


FIG. 1. Immunoblots of recombinant *B. vinsonii* subsp. *berkhoffii* SucB reacted with various polyclonal antibodies. (A) Mouse anti-*Bartonella* diluted 1:200 raised against the following isolates: cotton rat strain A1 (lane 1); cotton rat strain B1 (lane 2); cotton rat strain C1 (lane 3); *B. vinsonii* subsp. *berkhoffii* (lane 4); *B. vinsonii* subsp. *vinsonii* (lane 5); *B. quintana* (lane 6); *B. elizabethae* (lane 7); *B. henselae* (lane 8); *N. albigula* (wood rat) strain Na18985 nm (lane 9); BALB/c mouse experimentally infected with *Bartonella* strain Pm15590co and boosted with *Bartonella* ground squirrel strain Sb944nv (lane 10). (B) Polyclonal antiserum samples against the following various pathogens diluted 1:500: anti-*L. pneumophila* (lane 1); anti-*R. typhi* (lane 2); anti-*R. prowazekii* (lane 3); anti-*R. rickettsii* (lane 4); anti-*C. burnetti* (lane 5); anti-*F. tularensis* (lane 6); anti-*Y. pestis* (lane 7); anti-*B. burgdorferi* (lane 8); anti-*Leptospira* spp. (lane 9); anti-*T. pallidum* (lane 10); mouse preimmunized negative control serum (lanes 11 to 14).

well as showing the strong cross-reactivity between *B. vinsonii* subsp. *berkhoffii* and rodent-isolated *Bartonella*.

Antibodies against other bacterial pathogens were tested for cross-reactivity against SucB. Polyclonal antisera against various bacterial pathogens were obtained from the following sources: rabbit anti-*Legionella pneumophila* from Yousef Abu Kwaik (University of Kentucky); rabbit anti-*Coxiella burnetti* phase II from Bob Heinzen (University of Wyoming); rabbit anti-*Rickettsia rickettsii*, *Rickettsia typhi*, and *Rickettsia prowazekii* generated at the Rocky Mountain Laboratories, Hamilton, Mont.; rabbit anti-*Francisella tularensis* and human anti-*Yersinia pestis* from the Diagnostic and Reference Section, Division of Vector-Borne Infectious Diseases (DVBID), Centers for Disease Control and Prevention (CDC); human anti-*Borrelia burgdorferi*, *Leptospira* spp., and *Treponema pallidum* from the Molecular Bacteriology Section, DVBID, CDC. SucB seroreactivity was seen with antisera specific to *C. burnetti* and *F. tularensis*, while weaker reactivity was observed against *R. typhi* (Fig. 1B). No cross-reactivity was observed with antibodies against the other microbes tested. Anti-*Brucella* spp. antibodies were unavailable for testing.

Recent serological testing of patients with a febrile illness of unexplained origin from New Mexico by this laboratory had suggested possible infections with rodent-associated *Bartonella* (M. Y. Kosoy et al., Abstr. Am. Soc. Rickettsiology-Bartonella Emerg. Pathogen Group 2001 Joint Conf., abstr. 108, 2001; F. Koster et al., Abstr. Am. Soc. Rickettsiology-Bartonella Emerg. Pathogen Group 2001 Joint Conf., abstr. 133, 2001). This observation and the discovery that rodent species in the western United States harbor *Bartonella* led us to investigate whether these organisms could be the causative agents of illnesses in humans having exposure to wild rodents. Concomitantly, this laboratory recently discovered that *Bartonella* isolates obtained from ground squirrels in Nevada had *gltA* (citrate synthase), 16S rRNA, and *groEL* gene sequences identical to those of *B. washoensis* isolated from a cardiac patient from the same area, providing evidence of *Bartonella* rodent-to-human transmission (30). In conjunction with these obser-

TABLE 2. SucB amino acid identity between related organisms

| Organism | Identity (%) | | | | | |
|---|---|---|---|---|---|---|
| | *B. vinsonii* subsp. *berkhoffii* | *B. quintana* | *B. melitensis* | *A. tumefaciens* | *M. loti* | *S. meliloti* |
| *B. vinsonii* subsp. *berkhoffii* | 100 | 87.8 | 72.4 | 70.2 | 72.0 | 69.6 |
| *B. quintana* | | 100 | 71.6 | 69.3 | 71.5 | 68.0 |
| *B. melitensis* | | | 100 | 81.7 | 80.2 | 78.0 |
| *A. tumefaciens* | | | | 100 | 77.3 | 83.2 |
| *M. loti* | | | | | 100 | 79.6 |
| *S. meliloti* | | | | | | 100 |

vations, we sought to expand our understanding of *Bartonella* infection-associated immunogens, particularly those putatively causing non-CSD illnesses, by screening genomic libraries using antibodies generated against rodent-isolated *Bartonella*.

*B. vinsonii* subsp. *berkhoffii* and *B. quintana* were initially chosen as genomic library representatives for *Bartonella*, as we were interested in investigating antigens from other species besides *B. henselae*. Although *B. vinsonii* subsp. *berkhoffii* is associated with infection in dogs (8, 29), coyotes and ticks have been implicated as reservoir and vector hosts, respectively, in the western United States (11, 13), and there has been one documented human case infection (42). In addition, a related organism, *B. vinsonii* subsp. *arupensis*, was recently isolated from a human patient in Wyoming (45).

The genomic libraries were screened initially with antibodies specific to rodent isolates. The rationale was to recognize any putative gene products reactive against antibodies to rodent-borne *Bartonella* and subsequently to use comparative genomics to determine the extent of cross-reactive, homologous genes between genus and species. Although in this study we screened genomic libraries from only two *Bartonella* species, we have purified genomic DNA from several *Bartonella* strains and generated libraries to other rodent *Bartonella* isolates for future genetic comparisons. We have indeed been successful in amplifying *sucB* by PCR from several *Bartonella* spp. and isolates, indicating the presence of this gene as expected (data not shown).

At the time this study began, *Bartonella* isolates from clinically defined human cases in the western United States were not available and, accordingly, neither were antiserum samples from culture-confirmed patients. In addition, we could not utilize antiserum from the rodent reservoir hosts from which the *Bartonella* organisms were isolated, as these naturally infected animals do not seem to mount a detectable antibody response (31). Antigens identified by polyclonal antibodies prepared against killed whole-cell lysates do not necessarily correlate with immunogens associated with *Bartonella* infections; however, this approach does identify candidate antigens that can be assayed for their reactivity against antiserum raised in an infected host. Indeed, SucB proved to be an infection-associated immunogen, as it was detected by antiserum from the experimentally infected mouse shown in Fig. 1A.

Molecular analysis of the expression library clone indicated that the gene encoding the protein reactive against anti-*Bartonella* antibodies was *sucB*, which encodes dihydrolipoamide succinyltransferase (E2o), an enzyme that is one part of three components forming the α-ketoglutarate dehydrogenase complex found in several eukaryotes and prokaryotes. This enzyme complex is also composed of α-ketoglutarate dehydrogenase (E1o) and dihydrolipoamide dehydrogenase (E3) and is encoded by the genes *sucA* and *lpd*, respectively. The DNA sequence of the *B. vinsonii* subsp. *berkhoffii* cloned insert showed a similar gene arrangement as that described in *Rhodobacter capsulatus* and *E. coli* (17, 44).

To our knowledge, only three other immunoreactive *Bartonella* antigens have been molecularly characterized. They are the 17-kDa antigen of *B. henselae* that elicits a strong humoral response in patients with CSD (2), the *B. henselae* HtrA stress response protein (3), and a GroEL class of heat shock protein from *B. bacilliformis* termed Bb65 (25). Other *Bartonella* antigens have been identified by Western blot banding patterns, but they have not been cloned and sequenced. Several groups have shown that anti-*Bartonella* serum samples are reactive on immunoblots with proteins of molecular masses of 45 to 50 kDa. These bands may correspond to the SucB antigen (20, 35, 39).

Antigenic cross-reactivity was seen when recombinant SucB was assayed with antibodies raised against various *Bartonella* strains. Significantly, the SucB was reactive against antiserum from a mouse experimentally infected with live *Bartonella*. The antigenic cross-reactivity was evidenced by the fact that this antibody was directed against *Bartonella* obtained from deer mice (*P. maniculatus*) and California ground squirrels (*S. beecheyi*). This result suggested that SucB can be a broad indicator of infection against different *Bartonella* species. Importantly, antigenic cross-reactivity was observed when SucB was immunoblotted against antibodies to *C. burnetti* and *F. tularensis*, with slight reactivity against anti-*R. typhi*. SucB may therefore be one of the antigens responsible for the serological cross-reactions that have been noted in Western blotting and indirect fluorescence assays by other researchers (32, 39).

SucB has been shown to be an immunogenic protein during infections by two other intracellular pathogens, *B. melitensis*, which causes ovine and caprine brucellosis and can be transmitted to humans (46), and *C. burnetti*, the causative agent of Q fever (40). Noting the immunogenicity of SucB from these organisms that are close phylogenetic relatives of *Bartonella* and the cross-reactivity observed in serological assays between them, one must be cautious when correlating antibody reactivity against SucB with a *Bartonella* infection and vice versa. Also confounding is that the clinical manifestations of bartonelloses, Q fever, and brucelloses are similar and could be confused. However, a broadly cross-reactive antigen such as SucB could potentially be used as an identifier of these diseases, with more specific diagnostic tools to differentiate the infectious agents. Moreover, as serologic diagnostic assays for these diseases are explored and become more developed, researchers should be aware of the potential cross-reactivity of *Bartonella* SucB with antibodies against other organisms.

**Nucleotide sequence accession numbers.** The DNA sequence of the *B. vinsonii* subsp. *berkhoffii* clone, which includes the sequence encoding the *sucB* gene and the partial sequence encoding the *sucA* and *lpdA* genes, has been submitted to GenBank under accession number AY160679. The DNA sequence of the *B. quintana sucB* gene has been submitted to GenBank under accession number AY160680.

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