Cloning, Nucleotide Sequence, and Expression of the *Brucella melitensis* *sucB* Gene Coding for an Immunogenic Dihydrolipoamide Succinyltransferase Homologous Protein

MICHEL S. ZYGMUNT,* MARÍA A. DÍAZ,† ANA P. TEIXEIRA-GOMES, AND AXEL CLOECKAERT‡

Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France

Received 12 February 2001/Returned for modification 16 May 2001/Accepted 18 July 2001

The *Brucella melitensis* *sucB* gene encoding the dihydrolipoamide succinyltransferase (E2o) enzyme (previously identified as an immunogenic protein in infected sheep) was cloned and sequenced. The amino acid sequence predicted from the cloned gene revealed 88.8 and 51.2% identity to the dihydrolipoamide succinyltransferase SucB protein from *Brucella abortus* and *Escherichia coli*, respectively. Sera from naturally infected sheep showed antibody reactivity against the recombinant SucB protein.

Brucella spp. are gram-negative, facultative intracellular bacterial pathogens that cause brucellosis, an infectious disease affecting animals and humans. *Brucella melitensis* is the most important species involved in ovine and caprine brucellosis, which is characterized by abortion, low production, and infertility in infected animals. *B. melitensis* is also the most pathogenic species for humans.

One of the principal aims in brucellosis research is the identification of *Brucella* antigens eliciting humoral and/or cell-mediated responses, which might be of interest for the development of diagnostic tests or subcellular vaccines that avoid the drawbacks of those currently used. *B. melitensis* Rev 1, a live attenuated vaccine strain that is currently used in sheep and goats, has been successful in disease eradication and control programs in some countries (1). However, there have been significant problems associated with its use. The most important among them are the residual virulence of Rev1 for humans and the development of agglutinating antibodies in animals vaccinated as adults which are indistinguishable from those elicited by natural infection (8). The construction of new brucellosis vaccines and associated diagnostic tests lacking these undesirable properties would be of great interest to veterinary medicine.

A number of immunogenic proteins have been previously identified by immunoblotting, such as the BP26 protein, and are currently being considered for the development of new diagnostic tests for ovine brucellosis (3, 6, 7, 10). Recently, two-dimensional electrophoresis, immunoblotting, and N-terminal microsequencing have considerably facilitated the identification of immunogenic proteins in ovine brucellosis (15, 16). Among the proteins identified by these methods, one with an apparent molecular mass of 45 kDa was recognized by sera

from *Brucella*-infected sheep, and its N-terminal sequence showed homology to a dihydrolipoamide succinyltransferase (SucB) described in many bacteria (2, 5, 9, 13, 19). A monoclonal antibody (MAb) was raised against this protein (16) to allow easy screening of genomic libraries to clone the corresponding gene. The present report describes the cloning and the nucleotide sequence of the gene termed *sucB* encoding dihydrolipoamide succinyltransferase (E2o), an enzyme of the α -ketoglutarate dehydrogenase complex, and its expression in *Escherichia coli*.

Specificity of the anti-SucB MAb. The MAb raised against *Brucella* SucB did not cross-react with *E. coli* and other bacteria closely genetically related to *Brucella*, such as *Ochrobactrum anthropi*, *Phyllobacterium rubiacearum*, *Rhizobium leguminosarum*, and *Agrobacterium tumefaciens* (20; data not shown). Thus, the MAb appeared to be specific for *Brucella* and therefore particularly useful for screening genomic libraries constructed in *E. coli*.

Cloning of the *B. melitensis* *sucB* gene and its expression in *E. coli*. A *B. melitensis* 16M genomic library was constructed in lambdaGEM-12 *Xho*I half-site arms (Promega, Madison, Wis.) by following the instructions of the manufacturer. Briefly, *B. melitensis* 16M DNA, extracted and purified as described previously (17), was partially digested for 30 min at 37°C with *Sau*3 AI (Promega) at 0.014 U/ μ g of DNA, the enzyme concentration giving the highest percentage of fragments ranging from 15 to 23 kb. DNA fragments were partially filled in with dGTP and dATP, by using Klenow DNA polymerase (Promega), ligated with T4 DNA ligase (Promega) to lambda-GEM-12 digested with *Xho*I, and partially filled in with dTTP and dCTP. Recombinant phage DNA was packaged in vitro with the Packaging System (Promega), and the library was titrated by determination of the number of PFU that appeared after infection of *E. coli* KW251 cells (Promega). Recombinant phages were transferred to nitrocellulose filters, and phages expressing the *sucB* gene were identified by reactivity with the anti-SucB MAb. DNA of a positive phage was extracted from culture supernatants of *E. coli* KW251 cells infected with the phage and cultured until lysis was observed. Phage DNA was then cut with *Not*I, *Bam*HI, *Eco*RI, or *Sac*I, and restriction fragments

* Corresponding author. Mailing address: Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France. Phone: 33 2 47 42 78 72. Fax: 33 2 47 42 77 79. E-mail: zygmunt@tours.inra.fr.

† Present address: Louisiana State University, Baton Rouge, LA 70803.

‡ Present address: Station de Pathologie Aviaire et Parasitologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France.

FIG. 1. Immunoblotting after SDS-PAGE of *E. coli* (pMZ4503) cells expressing the *sucB* gene of *B. melitensis* 16M with anti-SucB MAb (lane 1), with sera from brucellosis-negative sheep (lanes 3 and 4), and with sera from naturally infected sheep (lanes 5 to 10), all adsorbed with *E. coli* JM109 cells carrying the control vector pGEM7Zf+. Lane 2, immunoblotting after SDS-PAGE of *E. coli* JM109 carrying the control vector pGEM7Zf+ with anti-SucB MAb.

were ligated into pGEM-5Zf+ (Promega) cut with *NotI* or into pGEM-7Zf+ (Promega) cut with *BamHI*, *EcoRI*, or *SacI*, respectively. Competent *E. coli* JM109 cells (Promega) were transformed with recombinant plasmid DNA as described previously, and bacteria were spread on Luria-Bertani (LB) broth-ampicillin (50 μ g/ml) plates containing isopropyl-1-thio- β -D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). *E. coli* JM109 colonies bearing recombinant plasmids were transferred to nitrocellulose, lysed with 10% sodium dodecyl sulfate (SDS), and screened with the anti-SucB MAb by a colony blotting technique. One positive colony was found bearing a plasmid with a large *BamHI* insert of about 15 kbp. This plasmid was named pMZ4501. A 6.5-kbp *NotI*-*BamHI* fragment from this insert was further subcloned into pCR2.1 (In Vitrogen, San Diego, Calif.), resulting in plasmid pMZ4503. Expression of *sucB* in *E. coli* bearing plasmid pMZ4503 was further confirmed by immunoblotting with the anti-SucB MAb (Fig 1, lane 1). The MAb detected one band with an apparent molecular mass of 45 kDa, which was overproduced in *E. coli* as demonstrated by Coomassie blue staining (data not shown). Control *E. coli* cells bearing nonrecombinant pGEM-7Zf showed no reaction at all with the anti-SucB MAb (Fig. 1, lane 2).

DNA sequence analysis of the *Brucella melitensis* 16M *sucB* gene. Recombinant plasmid pMZ4503 bearing the *B. melitensis* 16M *sucB* gene was sequenced for both strands by the chain termination method of Sanger et al. (11). Computer analysis of the sequence data was performed by BLAST analysis through the National Center for Biotechnology Information. Nucleotide sequencing of the 6.5-kbp *NotI*-*BamHI* fragment revealed the presence of two partial and two complete open reading frames (ORFs). Comparison of the ORFs to those in the GenBank database by using BLAST showed that three of the

ORFs encoded proteins homologous to SucA, SucB, and LpdA previously identified in *E. coli* (12). The first partial ORF contained 713 codons with 80.7% amino acid sequence identity to the *sucA* gene product of *B. abortus* S19 (GenBank accession no. AF07932) and 41.7% identity to the *sucA*-encoded E1o protein of *E. coli* (GenBank accession no. X00661). There was a complete ORF immediately downstream of the partial *sucA* gene that contains 409 codons with 88% amino acid sequence identity to the *sucB* gene product of *B. abortus* strain S19 and 51.2% identity to the *sucB*-encoded E2o protein of *E. coli* (GenBank accession no. X00664) (13). The N-terminal amino acid sequence of the protein deduced from the nucleotide sequence matched the first 14 amino acids of the protein identified by two-dimensional electrophoresis and N-terminal microsequencing (16). The differences between the *B. abortus* S19 and *B. melitensis* 16M *sucB* genes consisted of the following: single nucleotide substitutions; one, two, or three nucleotide deletions; one nucleotide addition; and, most importantly, a 42-bp deletion in *B. abortus* S19 *sucB* (Fig. 2). These nucleotide substitutions and additions in the *B. abortus* S19 *sucB* gene relative to the *B. melitensis* 16M *sucB* gene altered the predicted amino acid sequence for many amino acids. The 42-bp deletion (coding for 14 amino acids) could possibly have a greater importance and perhaps cause an antigenic shift, as previously described for other proteins (4).

The nucleotide sequence of the *sucB* downstream region revealed a third ORF, ORF1, which is 639 nucleotides long, starts 61 nucleotides downstream of *sucB*, and codes for a protein with 29% amino acid identity to a putative amino acid efflux-like protein of *E. coli* (GenBank accession no. P27846) (21). The last ORF is a sequence homologous to the *lpdA* gene coding for a dihydrolipoamide dehydrogenase and starts 2,762 nucleotides downstream of *sucB*.

The genetic organization of the 6.5-kbp *suc* region of the *B. melitensis* chromosome is summarized in Fig. 3. This organization appears to be similar to that of *Rhodobacter capsulatus* and *Rhizobium leguminosarum*, in which the *lpd* gene is also located downstream of the *sucB* gene (5, 19). This organization is different from that of *E. coli*, in which *sucC* and *sucD* are located between the *sucB* and *lpdA* genes (12, 14). The close phylogenetic relationship between *Brucella*, *Rhodobacter capsulatus*, and *Rhizobium leguminosarum* could explain this similar organization.

Serum activities. The antibody responses of naturally infected sheep against recombinant SucB protein were analyzed. pMZ4503-transformed *E. coli* cells were cultured in LB broth, and total cell protein extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting as described previously (22). All sera have been adsorbed with *E. coli* JM109 carrying plasmid pGEM7Zf+ to remove nonspecific antibodies reacting with proteins from *E. coli*. Figure 1 shows the positive reaction with infected sheep sera of the recombinant SucB protein showing an apparent molecular mass of 45 kDa (lanes 5 to 10) corresponding to the SucB protein migration identified by using a MAb against the recombinant protein (lane 1). Antibody responses against the recombinant SucB protein were detected in all naturally infected sheep and not in *Brucella*-free sheep (lanes 3 and 4).

In other intracellular pathogens, such as *Coxiella burnetii*, SucB has also been found as immunogenic protein reacting

```

sucB-B.mel1  ATGGCTACCGAAATTCGCGTTCCACGCTTGGGGAGTCCGTTACCGAGGCAACCATCGGAAAGTGGTTCA
sucB-B.ab    ATGGCTAC---GATTCGCGTTCCACGCTTGGGGAGTCCGTTAGC-----
*****
sucB-B.mel1  AGAAGGCTGGTGAAGCCATTGCTGTGATGAGCCGCTGGTGGAAGTGAACCCGACAAGGTGACGGTCGA
sucB-B.ab    -----ATTGCTGTGATGAGCCGCTGGTGGAAGTGAACCCGACAAGGTGACGGTCGA
*****
sucB-B.mel1  AGTGCCCGCACCGGCTGCGGGTGTCTGGCCGAAATCACGGCCAAAGAAGGCGATACGGTCGAAGTGGGC
sucB-B.ab    AGTGCCCGCACCGGCTGCGGGTGTCTGGCCGAAATCACGGCCAAAGAAGGCGATACGGTCGAAGTGGGC
*****
sucB-B.mel1  GAACTGCTCGGCCAGATTTCAGTGACGGGGCAGCGGTTGCCGCCGACCGGCCAGAAGAAGGAGGAAG
sucB-B.ab    GCACTGCTCGGCCAGATTTCAGTGACGGGGCAGCGGTTGCCGCCGACCGGCCAGAAGAAGGAGGAAG
* *****
sucB-B.mel1  CCAAGCCTGCAGCCGCGCTCCTGCTGCTGCACCGGTCGCATCGGCTTCGTCGGGCCCGGCCATGCAGCC
sucB-B.ab    CCAAGCCTGCAGCCGCGCTCCTGCTGCTGCACCGGTCGCATCGGCTTCGTCGGGCCCGGCCATGCAGCC
*****
sucB-B.mel1  GCGCGCGGCTGCTGCGAAGCTTCTGGCTGAAAGCGGTCTTTCGCCCGATCAGGTCGAAGGTTCCGGCAAG
sucB-B.ab    GCGCGCGGCTGCTGCGAAGCTTCTGGCTGAAAGCGGTCTTTCGCCCGATCAGGTCGAAGGTTCCGGCAAG
*****
sucB-B.mel1  CGCGGCCAGGTG-CTCAAAGGTGACGTTT-TCGAGGCCATCACGAAAGGC-GTTTCGGCGGCTCCTGCCG
sucB-B.ab    CGCGGCCAGGTGGCTCAAAGGGGACATTCTCGAGGCCATCACGAAAGGCCTTCGGCGGCTCCTGCCG
*****
sucB-B.mel1  CTCCGGTTGCCGCGCGTCCGGCCTCCTCGGCTGATGACGCATCGCGCGAAGAGCGTGTGAAGATGACCCG
sucB-B.ab    CTCCGGTTGCCGCGCGTCCGGCCTCCTCGGCTGATGACGCATCGCGCGAAGAGCGGTTGAAGATGACCCG
*****
sucB-B.mel1  TCTGCGCCAGACCATTGCGCGCGCCTGAAGGATGCCGAGAACACGGCTGCCATGCTCACCACCTTCAAC
sucB-B.ab    TCTGCGCCAGACCATTGCGCGCGCCTGAAGGATGCCGAGAACACGGCTGCCATGCTCACCACCTTCAAC
*****
sucB-B.mel1  GAGGTGGACATGTGCGCAGTCATGGAGCTGCGTAACAAGTATAAGGACGCTTTGAAAAGAAGCATGGCG
sucB-B.ab    GAGGTGGACATGTGCGCAGTCATGGAGCTGCGTAACAAGTATAAGGACCGTCTTGAAAAGAAGCATGGCG
*****
sucB-B.mel1  TGAAGCTTGGCTTCATGGGCTTCTTACCACAGGCAGTGACGCATGCGCTGAAGGAAATCCCGGCTGTCAA
sucB-B.ab    TGAAGCTTGGCTTCATGGGCTTCTTACCACAGGCAGTGACGCATGCGCTGAAGGAAATCCCGGCTGTCAA
*****
sucB-B.mel1  CGCCGAGATCGACGGCACGGACATCGTCTACAAGAACTTCTGCCATGTGCGCATGGCTGTGCGCACCGAC
sucB-B.ab    CGCCGAGATCGACGGCACGGACATCGTCTACAAGAACTTCTGCCATGTGCGCATGGCTGTGCGCACCGAC
*****
sucB-B.mel1  AAGGGCCTCGTTCGTTCCGGTATCCCGGATGCCGACAGATGTCGATTGCGGAAGTGAAAAGGAACTGG
sucB-B.ab    AAGGGCCTCGTTCGTTCCGGTATCCCGGATGCCGACAGATGTCGATTGCGGAAGTGAAAAGGAACTGG
*****
sucB-B.mel1  CACGTCTCGCCAGGGCTGCGCGTGACGGCTCGCTTTCATGGCCGATATGCAGGGCGGCACCTTTACCAT
sucB-B.ab    CACGTCTCGCCAGGGCTGCGCGTGACGGCTCGCTTTCATGGCCGATATGCAGGGCGGCACCTTTACCAT
*****
sucB-B.mel1  CACCAATGGCGGTGTTTACGGTTGCTCATGTCCACGCGATCCTCAATGCGCGCAGTCGGGCATTCTC
sucB-B.ab    CACCAATGGCGGTGTTTAC---TCGCTCATGTC-ACGC-GATCT---ATGCGCG--AGTCGGAC--TCTC
*****
sucB-B.mel1  GGTATGCACAAGATCCAGGAACGCCGATGGTCGTCGGTGGCCAGATCGTGGTCCGCCGATGATGTATC
sucB-B.ab    GGTATGTACAAGATCCAGGAATGCCGATGGTCGTCGGTGGCCAGATCGTGGTCCGCCGATGATGTATC
*****
sucB-B.mel1  TGGCGCTTTCCTACGATCACCGCATCGTGGACGGCAAGGAAGCCGTGACCTTCCTCGTGCCTGTGAAGGA
sucB-B.ab    TGGCGCTTTCCTACGATCACCGCATCGTGGACGGCAAGGAAGCCGTGACCTTCCTCGTGCCTGTGAAGGA
*****
sucB-B.mel1  AAGCCTGGAAGATCCGGAACGTCTGGTTCTCGATCTCTAG
sucB-B.ab    AAGCCTGGAAGATCCGGAACATCTGGTTCTCGATCTCTAG
*****

```

FIG. 2. Alignment of the *B. melitensis* (B. mel) 16M *sucB* and *B. abortus* (B. ab) S19 *sucB* nucleotide sequences. Identical nucleotides are indicated by stars, and gaps are indicated by hyphens.

FIG. 3. Organization of the *B. melitensis* 16M *suc* genes. The 6,246-bp *NotI*-*BamHI* fragment comprised, besides the *sucB* gene, part of the *sucA* gene, ORF1 coding for an amino acid efflux-like protein, and part of the *lpdA* gene. Arrows indicate the direction of transcription.

with sera from Q fever patients (9). Possibly, recombinant SucB could be used in association with other immunogenic proteins such as BP26 for the serodiagnosis of ovine brucellosis (3, 7, 10, 18).

Nucleotide sequence accession number. The nucleotide sequence of the *B. melitensis* 16M *sucB* gene and flanking regions has been deposited in GenBank under accession no. AF235020.

REFERENCES

- Alton, G. G. 1990. *Brucella melitensis*, p. 384–409. In K. Nielsen and J. R. Duncan (ed.), Animal brucellosis CRC Press, Boca Raton, Fla.
- Buck, D., M. E. Spencer, and J. R. Guest. 1986. Cloning and expression of the succinyl-CoA synthetase genes of *Escherichia coli* K12. J. Gen. Microbiol. **132**:1753–1762.
- Cloekaert, A., H. S. Debbbarh, N. Vizcaino, E. Saman, G. Dubray, and M. S. Zygmunt. 1996. Cloning, nucleotide sequence, and expression of the *Brucella melitensis* bp26 gene coding for a protein immunogenic in infected sheep. FEMS Microbiol. Lett. **140**:139–144.
- Cloekaert, A., J.-M. Verger, M. Grayon, M. S. Zygmunt, and O. Grépinet. 1996. Nucleotide sequence and expression of the gene encoding the major 25-kilodalton outer membrane protein of *Brucella ovis*: evidence for antigenic shift, compared with other *Brucella* species, due to a deletion in the gene. Infect. Immun. **64**:2047–2055.
- Dastoor, F. P., M. E. Forrest, and J. T. Beatty. 1997. Cloning, sequencing, and oxygen regulation of the *Rhodobacter capsulatus* α -ketoglutarate dehydrogenase operon. J. Bacteriol. **179**:4559–4566.
- Debbbarh, H. S., A. Cloekaert, M. S. Zygmunt, and G. Dubray. 1995. Identification of seroreactive *Brucella melitensis* cytosoluble proteins which discriminate between antibodies elicited by infection and Rev. 1 vaccination in sheep. Vet. Microbiol. **44**:37–48.
- Debbbarh, H. S., M. S. Zygmunt, A. Cloekaert, and G. Dubray. 1996. Competitive enzyme-linked immunosorbent assay using monoclonal antibodies to the *Brucella melitensis* BP26 protein to evaluate antibody responses in infected and *B. melitensis* Rev. 1 vaccinated sheep. Vet. Microbiol. **53**:325–337.
- Jimenez de Bagues, M. P., C. M. Marin, J. M. Blasco, I. Moriyon, and C. Gamazo. 1992. An ELISA with *Brucella* lipopolysaccharide antigen for the diagnosis of *B. melitensis* infection in sheep and for the evaluation of serological responses following subcutaneous or conjunctival *B. melitensis* strain Rev 1 vaccination. Vet. Microbiol. **30**:233–241.
- Nguyen, S. V., T. Yamaguchi, H. Fukushi, and K. Hirai. 1999. Characterization of the *Coxiella burnetii* *sucB* gene encoding an immunogenic dihydrolipoamide succinyltransferase. Microbiol. Immunol. **43**:743–749.
- Salih-Alj Debbbarh, H., A. Cloekaert, G. Bézard, G. Dubray, and M. S. Zygmunt. 1996. Enzyme-linked immunosorbent assay with partially purified cytosoluble 28-kilodalton protein for serological differentiation between *Brucella melitensis*-infected and *B. melitensis* Rev. 1-vaccinated sheep. Clin. Diagn. Lab. Immunol. **3**:305–308.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
- Spencer, M. E., and J. R. Guest. 1982. Molecular cloning of four tricarboxylic acid cycle genes of *Escherichia coli*. J. Bacteriol. **151**:542–552.
- Spencer, M. E., M. G. Darlison, P. E. Stephens, I. K. Duckenfield, and J. R. Guest. 1984. Nucleotide sequence of the *sucB* gene encoding the dihydrolipoamide succinyltransferase of *Escherichia coli* K12 and homology with the corresponding acetyltransferase. Eur. J. Biochem. **141**:361–374.
- Spencer, M. E., and J. R. Guest. 1985. Transcription analysis of the *sucAB*, *aceEF* and *lpd* genes of *Escherichia coli*. Mol. Gen. Genet. **200**:145–154.
- Teixeira-Gomes, A. P., A. Cloekaert, G. Bézard, G. Dubray, and M. S. Zygmunt. 1997. Mapping and identification of *Brucella melitensis* proteins by two-dimensional electrophoresis and microsequencing. Electrophoresis **18**:156–162.
- Teixeira-Gomez, A. P., A. Cloekaert, G. Bézard, R. A. Bowden, G. Dubray, and M. S. Zygmunt. 1997. Identification and characterization of *Brucella ovis* immunogenic proteins by two-dimensional electrophoresis and immunoblotting. Electrophoresis **18**:1491–1497.
- Verger, J.-M., F. Grimont, P. A. D. Grimont, and M. Grayon. 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. **35**:292–295.
- Vizcaino, N., A. Cloekaert, G. Dubray, and M. S. Zygmunt. 1996. Cloning, nucleotide sequence, and expression of the gene coding for a ribosome releasing factor-homologous protein of *Brucella melitensis*. Infect. Immun. **64**:4834–4837.
- Walshaw, D. L., A. Wilkinson, M. Mundy, M. Smith, and P. S. Poole. 1997. Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*. Microbiology **143**:2209–2221.
- Yanagi, M., and K. Yamasato. 1993. Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107**:115–120.
- Zakataeva, N. P., V. V. Aleshin, L. L. Tokmakova, P. V. Troshin, and V. A. Livshits. 1999. The novel transmembrane *Escherichia coli* proteins involved in the amino acid efflux. FEBS Lett. **452**:228–232.
- Zygmunt, M. S., H. S. Debbbarh, A. Cloekaert, and G. Dubray. 1994. Antibody response to *Brucella melitensis* outer membrane antigens in naturally infected and Rev1 vaccinated sheep. Vet. Microbiol. **39**:33–46.