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Nucleotide Sequence of the *ospAB* Operon of a *Borrelia burgdorferi* Strain Expressing OspA but Not OspB

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The nucleotide sequence of a 1.6-kb clone containing the gene for outer surface protein A (OspA) of a German strain (GÖ2) of *Borrelia burgdorferi* was determined. The deduced amino acid sequence showed a homology of 82% to the OspA molecules from three other *B. burgdorferi* strains. The best-conserved region was recognized at the 36 amino-terminal amino acids of OspA. OspB could not be identified in the strain investigated, probably because the nucleotide sequence of the *ospAB* operon prevented expression of the OspB gene.

Lyme borreliosis is a multisystem disorder caused by the tick-borne spirochete *Borrelia burgdorferi*. Antibiotic treatment in the early stage of the disease can prevent the disease, but such treatment often fails in later stages. Without treatment, the infection may develop into a chronic disease with various clinical manifestations (30, 31). The first immune response of the host detected after the infection is cell mediated; thereafter, the humoral response can be recognized by antibodies against antigens identified by immunoblots (34). Some of these immunodominant antigens are well characterized (5, 8, 19, 31). However, the question of whether the immune response is protective is still open. Reinfections with *B. burgdorferi* have been reported (33). Different animal models have been used in order to obtain more information about the protective role of anti-*B. burgdorferi* antibodies (12, 24–26, 28). Schmitz et al. (25) demonstrated that the sera of hamsters infected with *B. burgdorferi* could prevent Lyme arthritis in irradiated hamsters challenged with the spirochete. Schaible et al. (24) reported that monoclonal antibodies to outer surface protein A (OspA) are able to prevent the disease in mice with severe combined immunodeficiency if these antibodies are simultaneously injected. Those authors suggest that this is a promising approach to developing a vaccine against Lyme disease.

To gain ground with this idea, it is important to find out exactly how the OspA molecules of different *B. burgdorferi* strains vary. To our knowledge, three sequence determinations of OspA genes have been reported (2, 4a [GenBank accession no. M38375], 32). In this study, we present a further sequence of the gene for OspA and flanking regions from a further *B. burgdorferi* strain which did not express OspB.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. burgdorferi* GÖ2 was isolated from a tick found near Göttingen, Germany, by the method of Preac-Mursic et al. (21). The North American strain B31 (ATCC 35210) was also used.

The spirochetes were grown in the complex medium described by Barbour (1) at 37°C for 5 to 7 days. From day 3 on, the culture was diluted daily 1:2 with fresh medium. Thereafter, the culture was centrifuged at 4°C at 10,000 × *g*. The pellet was washed four times with phosphate-buffered saline (PBS), centrifuged, and lyophilized.

Escherichia coli Y1089 and Y1090 were provided by Stratagene Ltd. (Heidelberg, Germany).

Immunization of rabbits. For immunization, 5×10^6 viable spirochetes in 0.5 ml of 0.9% (wt/vol) NaCl were injected intravenously into a rabbit. This procedure was repeated twice at intervals of 6 weeks. The antibody response was investigated by immunoblots.

Electrophoresis and immunoblotting. The proteins were separated by standard sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (15). The gels were stained with Coomassie blue. Electroblotting was performed with a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Mass.) as described by Gültekin and Heermann (7). The bound antibodies were detected by peroxidase-labeled anti-rabbit or anti-human antibodies (Dako, Copenhagen, Denmark).

Elution of antibodies from blot membranes. For the elution of specific antibodies from immunoblots, the method of Olmsted (18) was used. Serum specimens from patients with Lyme disease were obtained from the *B. burgdorferi* routine diagnostic laboratory, Department of Medical Microbiology, University of Göttingen, Göttingen, Germany.

DNA preparation. The cells of a 500-ml *B. burgdorferi* culture were prepared and the DNA was extracted as described by Nakamura et al. (17), with some modifications (10). The cells were suspended after centrifugation in 1 ml of 50 mM Tris HCl (pH 7.6) containing 25% (wt/wt) sucrose and 2 mg of lysozyme (Boehringer, Mannheim, Germany) and incubated for 30 min at 37°C. EDTA (0.4 ml, 0.25 M, pH 8.0) and 0.16 ml of 10% (wt/wt) SDS were added and mixed. This lysate was digested with 0.5 mg of proteinase K (Boehringer) for 60 min at 37°C and extracted with phenol-chloroform. The DNA was precipitated with ethanol (22).

Preparation and screening of the expression library. Genomic DNA from *B. burgdorferi* was completely digested with *Eco*RI (Pharmacia, Uppsala, Sweden) and ligated to DNA from phage lambda gt11 (Bethesda Research Laboratories, Gaithersburg, Md.) which had previously been digested with *Eco*RI and packaged with the DNA Packaging Kit (Boehringer) as described by the manufacturer. Competent *Escherichia coli* Y1090 (100 μl) was transfected with 1 μl of the suspension of recombinant bacteriophage at 37°C

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for 60 min and added to 3 ml of soft agar containing $MgSO_4$ (10 mM), ampicillin (0.1%), and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.3 mM). The soft agar was then plated onto Luria-Bertani plates containing 0.01% ampicillin and incubated overnight at 37°C. Replicas of the plates were obtained by carefully placing dry nitrocellulose filter disks (Schleicher & Schüll, Dassel, Germany) on the soft agar for 30 min at 37°C. The disks were then removed, and their positions on the plate were marked with a needle. The filters were rinsed with PBS and incubated with 5% (wt/wt) milk powder (Uelzena, Uelzen, Germany) in 5 ml of PBS for 30 min at room temperature. Sera of the rabbit taken prior to and after immunization were then added at a final dilution of 1:1,000 and incubated for 60 min at room temperature. Positive clones were detected by standard methods after four washes (5 min each) with 0.05% (wt/wt) Tween in PBS with peroxidase-labeled anti-rabbit antibodies of mice (Dako). Positive clones were identified, and the phage were isolated and stored at 4°C in 0.5 ml of SM buffer (22) containing 5 μl of chloroform. Eight of about 5,000 plaques showed a positive reaction.

Expression of the protein in *E. coli* Y1089. *E. coli* Y1089 was infected with the recombinant bacteriophage according to the method of Huynh et al. (11). The lysogenic *E. coli* strain Y1089 was incubated in 3 ml of Luria-Bertani medium containing 10 μl of 1 M $MgSO_4$ and 3 μl of ampicillin overnight at 32°C. Luria-Bertani medium (2 ml) was then added, and the mixture was incubated at 45°C for 20 min. IPTG was added to a final concentration of 10 mM. This mixture was incubated at 37°C for 2 h and centrifuged at 1,500 × *g* for 15 min. The resulting pellet was suspended in 50 μl of PBS at −20°C. After being thawed, the cells were lysed, and the proteins were investigated.

Nucleotide sequencing. The *Eco*RI insert from the lambda gt11 clone was isolated and subcloned into the plasmid pUC18. The plasmid DNA was prepared by a plasmid preparation kit (DIAGEN, Düsseldorf, Germany) as described by the manufacturer. Double-stranded DNA was sequenced by the dideoxy chain termination procedure (23) with the Sequenase sequencing kit (U.S. Biochemicals, Cleveland, Ohio) and ^{35}S-dATP (Amersham, Braunschweig, Germany).

Determination of amino-terminal amino acids. The proteins were separated by SDS-PAGE, blotted onto a PVDF membrane, and stained with Coomassie blue. The sequence was investigated with an automatic gas-phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, Calif.) (13).

Nucleotide sequence accession number. The sequence of the nucleotide fragment has been submitted to the EMBL Data Library under accession number X60300.

RESULTS

Cloning and expression. A *B. burgdorferi* strain (GÖ2) was isolated from a tick from the Göttingen, Germany, area. Investigations by electroblotting and Coomassie blue or immunostaining showed predominating bands near 23 and 32 kDa, which differs from the pattern for North American strain B31 (Fig. 1). These two proteins and the OspA of strain B31 were recognized by the serum of a rabbit previously immunized by viable borrelia of strain GÖ2 (Fig. 1, lanes 5 and 6). The 32-kDa protein was investigated by an automatic amino acid sequencer using a Coomassie-stained PVDF membrane. Only about 10% (15 pmol) of the 150 pmol of protein bound to the membrane could be analyzed. Eleven amino-terminal amino acids in the order MKKYLLGIGLI were detected. An identical sequence was also reported by other authors characterizing OspA.

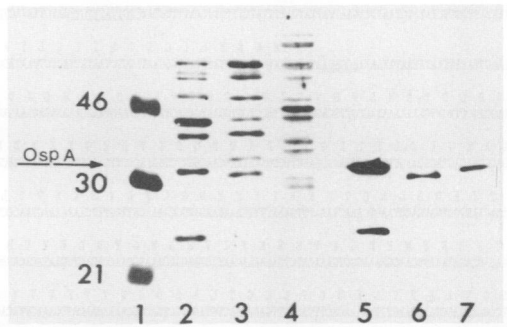

FIG. 1. Electroblot after SDS-PAGE of *B. burgdorferi* strains GÖ2 (lanes 2 and 5) and B31 (lanes 3 and 6) and of the *E. coli* lysate of the OspA-expressing clone (lanes 4 and 7). Lanes 1 through 4 were stained with Coomassie blue, and lanes 5 through 7 were stained with the serum of a rabbit immunized with GÖ2. Numbers on the left indicate the molecular masses (in kilodaltons) of the standards used (lane 1). Strain GÖ2 shows only one band (32 kDa) (lane 2) in the vicinities of OspA (31 kDa) and OspB (34 kDa) of strain B31 (lane 3). A strong additional band is demonstrated at 23 kDa of strain GÖ2 (lanes 2 and 5).

OspB could not be identified by Coomassie staining in the strain we investigated (Fig. 1). Lyme disease patient antibodies eluted from OspB of strain B31 by the method of Olmsted (18) showed no reaction in the immunoblot of GÖ2 but a reaction with the OspB of B31 (data not shown).

To find out the differences between the strains with regard to OspA and OspB, we isolated the DNA of strain GÖ2. The DNA was completely digested by *Eco*RI, integrated in phage lambda gt11, and transfected into *E. coli*. The resulting expression products were screened with serum from the rabbit mentioned above. We expected a protein with a molecular mass of about 32 kDa or less if an *Eco*RI cleavage site was situated in the gene. In any case, the expression product should not be fused to β-galactosidase, because OspA should be expressed by its own promoter in *E. coli*, as described by Howe et al. (10) and Bergström et al. (2). A positive *E. coli* clone producing a 32-kDa protein was identified (Fig. 1, lane 7), and the DNAs of the phages were isolated (22) and digested again with *Eco*RI. A fragment about 1.6 kbp long was isolated.

Sequence analysis. The resulting *B. burgdorferi* DNA was subcloned in pUC18 and sequenced by the standard methods of Sanger et al. (23). The nucleotide sequence of OspA and flanking regions and the deduced amino acid sequence of OspA are shown in Fig. 2.

The sequenced DNA fragment had a total length of 1,361 bp. Promoter regions (2) were recognized in positions 68 to 79 and 90 to 101. These sequences also have their functions in *E. coli*, because the OspA was expressed from its own promoter, as mentioned above.

The open reading frame of the OspA gene starts with ATG in positions 145 to 147, has a length of 822 bp, and codes for a protein with 274 amino acids. Two ribosome-binding sites with the consensus sequence AGGAGA were identified. One is in positions 133 to 138 upstream from the OspA gene, and the other is downstream in positions 969 to 974. The last one was necessary for the expression of OspB. Its gene is tandemly arrayed following the OspA gene and the ribosome-binding site (2). In the strain we analyzed, a potential start codon for OspB in positions 979 to 981 followed the

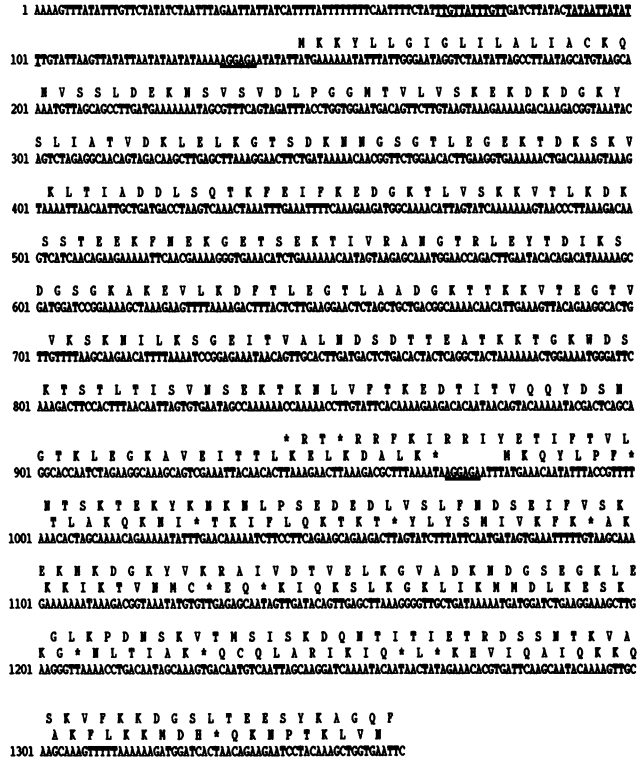

FIG. 2. Nucleotide sequence of the OspA gene and flanking regions of *B. burgdorferi* GÖ2. The deduced amino acid sequence of OspA had a length of 274 amino acids (positions 145 to 967). The ribosome-binding sites are underlined with a double line, putative promoter sequences are underlined once, and stop codons are marked with asterisks. The tandemly arrayed OspB gene should follow in the same reading frame, starting with ATG in position 979. Mutations prevent the expression of OspB. Another reading frame (shown from position 942) demonstrates impressive homologies to OspB from strain B31 (Fig. 3).

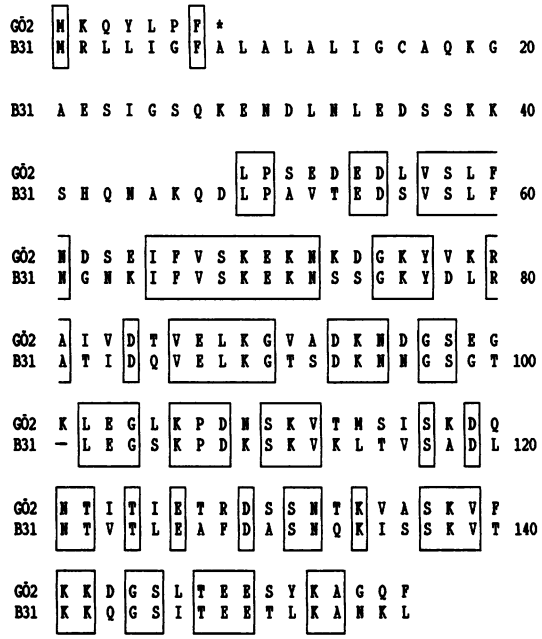

FIG. 3. Comparison of the amino-terminal amino acid sequence of OspB from strain B31 and the deduced amino acid sequence composed of two different reading frames (see Fig. 2, positions 979 to 1002 and 1038 to 1361) of a nucleotide fragment of GÖ2. The boxes indicate homologies. It was demonstrated that the gene or a gene fragment for OspB also exists in strain GÖ2, although the gene cannot be expressed.

second ribosome-binding site, but in this frame, several stop codons exist. Another open reading frame (Fig. 2, from position 951) in this region showed impressive homologies to the OspB from B31 (Fig. 3), but no start codon exists. Thus, the expression of OspB is not possible. This result is in agreement with the findings of the PAGE and blotting experiments in which OspB was not detectable (Fig. 1).

Characterization of the 23-kDa protein. The amino-terminal amino acids of the dominant protein in the vicinity of 23 kDa (p23) (Fig. 1, lanes 2 and 5) were determined after electroblotting to be ME?YLSY?K. This protein was also preferentially recognized by antibodies of the immunized rabbit. To investigate whether there were cross immunoreactions between p23 and OspA, antibodies were eluted from the bands by the method of Olmsted (18). The isolated immunoglobulins showed no cross-reactions (data not shown); thus, the two proteins demonstrated distinct antigenic properties.

DISCUSSION

OspA and OspB, with molecular masses of approximately 31 and 34 kDa, were dominant structures in many *B. burgdorferi* strains (26, 34, 35). Their genes are arranged tandemly on a linear plasmid and cotranscribed (2, 9, 21). The amino acid sequences of OspA and OspB of strain B31 showed a high degree of homology (53%). Thus, it was suggested that the two genes had a common phylogenetic precursor. Different authors (4, 28) report antigenic variants demonstrated by immunoblots or monoclonal antibodies. OspB seems to be more heterogeneous than OspA.

The nucleotide sequence of the tandem gene of strain B31 for OspA and OspB was determined by Bergström et al., and in strains ZS7 (32) and N40 (4a [GenBank accession no. M38375]), the OspA gene was sequenced.

In this study, we have analyzed the sequence of the gene for OspA in another *B. burgdorferi* strain (GÖ2) to obtain exact molecular data on the heterogeneity.

In Fig. 4, a comparison of the amino acid sequences of four different OspA molecules is presented. The sequence data on OspA already published differ in only 3 of 273 amino acids (positions 39, 149, and 164). However, our results deviate in 46 further amino acids and 1 additional aspartic acid (position 174), demonstrating a homology of 82% to the others. Thirty-six amino-terminal amino acids were identical in all isolates. The variations were distributed almost regularly along the remaining protein. The conservation in the region of the amino terminus possibly indicates that here is an essential functional part of the molecule. This sequence shows homologies to the recognition site of a signal peptidase II enzyme (2). The processed protein should have cysteine (position 17) at its amino terminus. It was demonstrated that the cysteine was covalently bound to a fatty acid, a potential anchor to the membrane (3).

The sequence determination for the 32-kDa protein of our strain showed only a small amount of unprocessed OspA. We speculate that the protein band consists mainly of OspA blocked to Edman degradation. It remains unclear whether this blocked molecule was processed or not. The blot

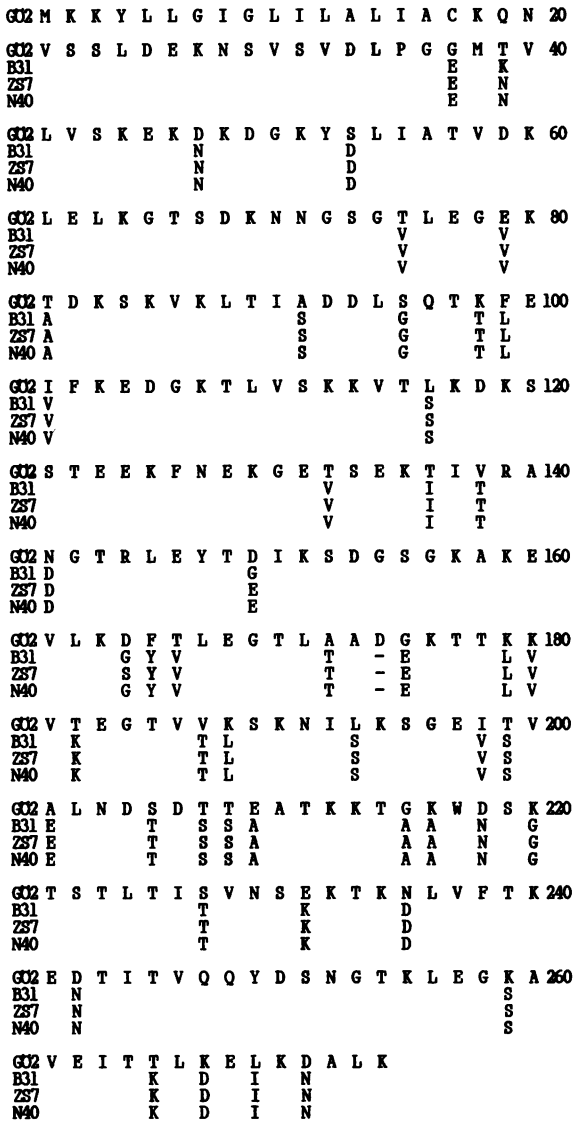

FIG. 4. Comparison of the deduced amino acid sequences of OspA from strain GÖ2 with those of OspAs from strains B31, ZS7, and N40. Whereas the proteins from B31, ZS7, and N40 differ only in positions 39, 149, and 164, GÖ2 shows differences in an additional 47 positions.

experiments (Fig. 1) showed only one single band in the vicinity of 32 kDa.

Schubach et al. (27) recently reported on the mapping of antibody-binding domains of OspA. They found that no antibodies bound to the first 61 amino-terminal amino acids, which suggests that this domain is not exposed to the bacterial surface.

The heterogeneity of the amino acid sequence and the distribution of the exchanges made it questionable whether any epitopes exist that react with neutralizing antibodies common to all or nearly all OspA molecules of various *B. burgdorferi* strains.

Although the ribosome-binding site and the start codon exist, our sequence data demonstrate that OspB could not be expressed. Possibly, a deletion event led to a frameshift (Fig. 2). In another frame, impressive sequence homologies to OspB of strain B31 were found, as shown in Fig. 3. Thus, it was demonstrated that the gene of OspB was not eliminated in the strain we investigated but rather that the sequence of the gene did not allow expression.

Bundoc and Barbour (4) reported on a clonal polymorphism of OspB and described also a strain which did not produce OspB but did produce an additional 18.5-kDa protein. We also found a further dominant protein with a molecular mass of about 23 kDa (p23) (Fig. 1). The amino-terminal amino acids were determined to be ME?YLSY?K and are possibly identical with those of a 22-kDa protein with the sequence MEKYLSYIK that was characterized by Luft et al. (16). It is still unclear whether the 23-kDa protein shows further similarities to the immunoreactive protein pC (34), other proteins in the range of 20 to 24 kDa (14), or P22-A, recently reported by Simpson et al. (29). The cloning and sequencing of the p23 gene is under investigation.

In our study, we characterized the heterogeneity of OspA and OspB of *B. burgdorferi* at the molecular level. The variations of OspA and the nucleotide sequence leading to the absence of OspB might reflect antigenic drift. However, the mechanism for this antigenic change is different from that of the variations of *Borrelia hermsii* (6, 20), the agent for relapsing fever.

Further investigations have to be performed to obtain more information about the variations of antigenic structures in the context of the virulence of *B. burgdorferi* or a future vaccine.

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