Oligopeptide permease in *Borrelia burgdorferi*: putative peptide-binding components encoded by both chromosomal and plasmid loci

James L. Bono, Kit Tilly, Brian Stevenson, Dan Hogan and Patricia Rosa

Author for correspondence: Patricia Rosa. Tel: +1 406 363 9209. Fax: +1 406 363 9204. 

e-mail: patricia_rosa@nih.gov

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, 903 South Fourth Street, Hamilton, MT 59840, USA

To elucidate the importance of oligopeptide permease for *Borrelia burgdorferi*, the agent of Lyme disease, a chromosomal locus in *B. burgdorferi* that encodes homologues of all five subunits of oligopeptide permease has been identified and characterized. *B. burgdorferi* has multiple copies of the gene encoding the peptide-binding component, OppA; three reside at the chromosomal locus and two are on plasmids. Northern analyses indicate that each oppA gene is independently transcribed, although the three chromosomal oppA genes are also expressed as bi- and tri-cistronic messages. Induction of one of the plasmid-encoded oppA genes was observed following an increase in temperature, which appears to be an important cue for adaptive responses *in vivo*. The deduced amino acid sequences suggest that all five borrelial OppA homologues are lipoproteins, but the protease-resistance of at least one of them in intact bacteria is inconsistent with outer-surface localization. Insertional inactivation of a plasmid-encoded oppA gene demonstrates that it is not essential for growth in culture.

Keywords: oligopeptide permease, oppA, plasmids, Lyme disease, spirochaetes

INTRODUCTION

*Borrelia burgdorferi*, the spirochaetal agent of Lyme disease (Burgdorfer et al., 1982), is maintained in nature by an infectious cycle between tick vectors and small rodent hosts (Lane et al., 1991). Like many bacterial pathogens, *B. burgdorferi* must cope with an array of changing environmental conditions to successfully persist and proliferate in each location and to ensure subsequent transmission between hosts. To accomplish this, *B. burgdorferi* presumably must regulate gene expression in response to environmental cues. It is unknown how *B. burgdorferi* senses environmental changes and orchestrates the appropriate adaptive responses. In other bacteria, oligopeptide permeases can be involved in sensing pertinent environmental signals in the form of peptides, culminating in diverse responses such as expression of virulence determinants, sporulation, conjugation or competence (Clewell, 1993; Leonard et al., 1996; Perego et al., 1991; Rudner et al., 1991). In addition to these signalling roles, the major role for most bacterial oligopeptide permeases is in nutrient uptake, by recycling cell wall and external peptides and transporting them into the cell, where they can be degraded into constituent amino acids for protein synthesis (Goodell & Higgins, 1987; Payne & Gilvarg, 1968).

We are interested in the potential role of oligopeptide permease in the transmission of *B. burgdorferi* between ticks and mammals. To initiate this investigation, we have identified genes that probably encode the *B. burgdorferi* oligopeptide permease, including homologues of the peptide-binding component OppA, the transmembrane components OppB and OppC, and the associated ATPases OppD and OppF. A locus on the *B. burgdorferi* linear chromosome contains three oppA gene homologues, located adjacent to single copies of oppB, oppC, oppD and oppF gene homologues. Two plasmid loci, one on a 26 kb circular plasmid (cp26) and the other on a 54 kb linear plasmid (lp54), carry individual oppA gene homologues. We have characterized the transcripts from all five oppA genes and have demonstrated that a protein product from the oppA gene on cp26 can be detected in *B. burgdorferi* lysates,

The GenBank accession numbers for the opp nucleotide sequences reported in this paper are AF000365 (oppAIV), AF000366 (chromosomal opp locus) and AF000948 (oppAIV).
but is not surface-exposed on intact bacteria. We have begun a genetic investigation of the biological roles of the individual \( \textit{oppA} \) genes and oligopeptide permease in \( \textit{B. burgdorferi} \) by inactivating the \( \textit{oppA} \) gene on cp26.

**METHODS**

\( \textit{B. burgdorferi} \) strains and growth conditions. \( \textit{B. burgdorferi} \) B31 (ATCC 35210), the prototype strain for \( \textit{B. burgdorferi sensu stricto} \), was originally isolated from a tick collected on Shelter Island, NY (Burgdorfer et al., 1982). Wild-type B31 used in RNA and protein analyses was low passage and infected. \( \textit{oppA} \)V: \( \textit{gyrB} \) mutant B31-82 was derived from high passage, non-infectious B31 by allelic exchange with recombinant plasmid pKK74, as described below. Cultures were grown in BSK-H medium (Sigma) supplemented with 6% (v/v) rabbit serum at 34 °C (Barbour, 1984), unless indicated otherwise. Single colonies in solid medium were obtained as described previously (Rosa et al., 1996).

Cloning and sequencing of the \( \textit{opp} \) loci. While characterizing positive clones from a library of \( \textit{B. burgdorferi} \) B31 DNA that had been screened with a \( \textit{guaB} \) probe (Margolis et al., 1994), we identified an open reading frame homologous to \( \textit{OppA} \) (probability = \( 5.6 \times 10^{-14} \) that the observed similarity to \( \textit{S. typhimurium} \) \( \textit{OppA} \) arose by chance), adjacent to \( \textit{guaB} \) (Rosa et al., 1996). Sequence comparisons to protein sequence databases with this sequence (subsequently designated \( \textit{OppA} \)V) were performed using the BLAST network service (National Center for Biotechnology Information) and a BLASTP search (Altschul et al., 1990). A closely related sequence from \( \textit{B. coriaceae} \) was also found; this sequence was a chromosomally encoded gene that was used as a PCR target sequence for the detection of \( \textit{B. coriaceae} \) (Zingg & LeFebvre, 1994).

An alignment of the \( \textit{B. burgdorferi} \) \( \textit{oppA} \)V and \( \textit{B. coriaceae} \) sequences permitted the identification of conserved amino acid residues, and a pair of degenerate oligonucleotides designed from these regions (Table 1, primers \( \textit{oppA} \).7 and \( \textit{oppA} \).4) was used in PCR amplification with total genomic DNA. Although the amplification product migrated as a single band on an agarose gel, restriction digests indicated that it contained a mixture of DNA sequences. The undigested PCR fragments were cloned into the pCRII vector (Invitrogen) and sequenced to confirm that the cloned segments of \( \textit{B. burgdorferi} \) DNA were derived from \( \textit{oppD} \) and \( \textit{oppF} \) homologues. PCR fragments (Table 1, primers oppD/F5′–oppD3′ and oppF/F5′–oppF3′) were used as probes in Southern blots to map the borrelial \( \textit{oppD} \) and \( \textit{oppF} \) genes to a chromosomal locus that is linked to the \( \textit{oppA} \) homologues (Fig. 1b and data not shown), and to screen the genomic library of B31 DNA. Sequence analysis of the genomic clones identified borrelial \( \textit{oppB} \) and \( \textit{oppC} \) homologues immediately upstream of \( \textit{oppD} \) and \( \textit{oppF} \) genes (see Fig. 2).

The nucleotide sequences of all \( \textit{opp} \) genes were obtained from both strands using either Sequenase 2.0 (US Biochemicals) or a 373A automated DNA sequencer (Applied Biosystems). The nucleotide sequences of \( \textit{B. burgdorferi} \) \( \textit{opp} \) genes and flanking DNA on cp26 (3–5 kb), \( \textit{lp54} \) (2–5 kb) and the chromosome (12–2 kb) have been deposited in GenBank (accession numbers AF000948, AF000365 and AF000366, respectively). Our sequence for the \( \textit{opp} \) loci differs from unedited genomic sequence determined by The Institute for Genomic Research and available at http://www.tigr.org as follows: four nucleotide substitutions in \( \textit{oppA} \) resulting in four amino acid changes; four nucleotide substitutions in \( \textit{oppA} \) resulting in two amino acid changes; two nucleotide substitutions in \( \textit{oppA} \) resulting in one amino acid change; one nucleotide substitution in \( \textit{oppC} \) resulting in an amino acid change and one nucleotide insertion in \( \textit{oppC} \) resulting in an altered reading frame for 76 amino acids and truncation of 87 amino acids; no nucleotide differences in \( \textit{oppD} \) and \( \textit{oppF} \); one nucleotide difference in \( \textit{oppA} \) that did not alter the amino acid sequence; and two nucleotide differences in \( \textit{oppA} \) that resulted in two amino acid changes. There were also four nucleotide differences (one substitution, three insertions/deletions) in the \( \textit{oppA} \)-\( \textit{oppA} \)III intergenic region.

Southern blot analysis of the \( \textit{opp} \) loci. Total genomic DNA was isolated from \( \textit{B. burgdorferi} \) as previously described (Rosa & Schwan, 1989). Total plasmid DNA, including both linear and circular molecules, was isolated from \( \textit{B. burgdorferi} \) with Qiagen columns following the manufacturer’s instructions. Southern blot analyses (Southern, 1975) were performed after DNA was separated on a 0.8% (w/v) agarose gel by field inversion electrophoresis for 24 h at 7 V cm–1 with program 3 of a PIII-200 programmable power inverter (MJ Research). DNA was transferred to Biotrans nylon membranes (ICN), prehybridized and hybridized with radioactive probes in 6 × SSC (where 20 × SSC is 3 M sodium chloride, 0.3 M sodium citrate), 0.1% (w/v) SDS, 0.5% (w/v) non-fat dry milk, 1 mM sodium pyrophosphate at 55 °C (high stringency) or 45 °C (low stringency) in rotating bottles in a hybridization oven (Bellco). Probe fragments were generated by PCR amplification and radiolabelled with [α-32P]dATP (Du Pont) by random priming (Life Technologies). Blots were washed in 0.1 × SSC, 0.1% (w/v) SDS at 55 °C (high stringency) or 2 × SSC, 0.1% (w/v) SDS at room temperature (low stringency) and visualized by autoradiography.

Northern blot analysis of the \( \textit{opp} \) genes. Total borrelial RNA was extracted from exponential-phase cultures using the
RNAgents Total RNA isolation system (Promega) or the Ultraspec RNA isolation system (Biotecx) according to the manufacturer's instructions. For temperature shift experiments, borreliae were grown at 23°C to mid-exponential phase, diluted 1:100 in fresh medium, and grown to mid-exponential phase at 35°C. RNA was denatured with glyoxal in 0.5 M sodium phosphate, pH 7.0, and 1 mM EDTA in rotating bottles in a hybridization oven (Bellco). Probe fragments were generated by PCR amplification and radiolabelled with [γ-32P]ATP (Du Pont) by random priming (Life Technologies). Membranes were washed at 55°C in 0.2× SSC, 0.1% (w/v) SDS.

**Table 1. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Our designation</th>
<th>Sequence (5'→3')</th>
<th>Gene</th>
<th>Use for oligonucleotides</th>
</tr>
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<tr>
<td>U157F + HpaI</td>
<td>ACCGTTACTTAACTCCTATTATATGAT</td>
<td>gyrB</td>
<td>Mutant construction</td>
</tr>
<tr>
<td>1905R + BclI</td>
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<td>Probe</td>
</tr>
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<td>oppBC</td>
<td>Probe</td>
</tr>
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<td>Specific probe</td>
</tr>
<tr>
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<td>oppBI</td>
<td>Specific probe</td>
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<td>oppF 3'</td>
<td>WAYWGAWAVRTCWARWGC</td>
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This fragment was ligated with HpaI/BglIII-digested pHDH3 (Rosa et al., 1996), a genomic clone isolated from the *B. burgdorferi* B31 DNA library whose 3.5 kb insert is derived from cp26 and spans the oppAIV and guaB genes. The resultant plasmid, pPKK73, contains a gyrB' insertion and corresponding deletion of oppAIV, between the HpaI site located approximately 400 bp within the gene to the BglIII site 30 bp beyond the 3' end of the gene (Fig. 2). pKK73 contains only a small segment of oppAIV upstream of the gyrB' insertion, so those sequences were replaced with the 2 kb PstI–HpaI fragment from pHDH62, another cp26-derived genomic clone whose insert extends approximately 1.5 kb 5' of oppAIV, to create pKK74. This construct has approximately 2 kb and 1.4 kb of cp26 sequence flanking gyrB' on the 5' and 3' ends, respectively. pKK74 was digested with PvuII to inactivate the bla gene (which removes the ability of this plasmid to confer ampicillin resistance) and the large fragment was purified from an agarose gel using a Unidirectional Electroelutor (model UEA, IBI). This DNA was used to transform *B. burgdorferi* B31 by electroporation as previously described (Rosa et al., 1996; Samuels et al., 1994).

Coumermycin-resistant colonies were screened by PCR to identify transformants with targeted insertion of gyrB' inoppAIV. The structure of mutant oppAIV on cp26 was confirmed by PCR with five different primer pairs between gyrB and cp26 flanking sequences and by Southern blot analysis of plasmid DNA from wild-type and oppAIV mutant borreliae with both oppAIV and gyrB probes. The results were consistent only with the deletion-insertion structure of the anticipated oppAIV mutant (ΔoppAIV::gyrB' cp26). Although...
plasmids can be present at multiple copies per spirochaete (Kitten & Barbour, 1992), we have found no examples of transformants in which the selectable marker was present in only some copies of the plasmid (Rosa et al., 1996; Tilly et al., 1997).

PCR conditions for cloning, generating probes and analysing recombinants. Oligonucleotides used as PCR primers for cloning and generating probes are described in Table 1. Coumermycin-resistant colonies were screened for the presence of the gyrB gene within oppAlV by PCR in a 9600 DNA Thermal Cycler (Perkin Elmer/Applied Biosystems) as previously described (Rosa et al., 1996). PCR conditions for cloning, generating probes and analysing recombinants were 25 cycles of 94°C for 1 min, 50°C for 0.5 min and 70°C for 3 min. Probe fragments were amplified through two rounds of PCR with a 1:100 dilution of the first reaction to minimize carryover of the template genomic DNA in the final PCR product. PCR conditions with degenerate primers were 30 cycles of 94°C for 1 min, 50°C for 2.0 min and 65°C for 2 min, using 1 μg (150 pM) primer per 100 μl reaction mixture.

SDS-PAGE and Western blotting of proteins. B. burgdorferi extracts were prepared from equal culture volumes by pelleting the bacteria, washing twice in PBS, and resuspending in sample buffer (Sambrook et al., 1989). After boiling for 5 min, lysates were separated by electrophoresis through 12.5% (w/v) or 10% (w/v) polyacrylamide gels (King & Swanson, 1978) in a Mini-Protean II cell (Bio-Rad). Proteins were blotted to nitrocellulose (Life Technologies), probed with polyclonal serum (anti-OppAIV, see below, or anti-OspC, Schwan et al., 1993) or hybridoma supernatant (anti-OspB monoclonal H4610, Rosa et al., 1992), and antibody-bound proteins were detected as previously described (Tilly et al., 1997) using enhanced chemiluminescence reagents as recommended by the manufacturer (Amersham). OppAlV was cloned, expressed and purified as a fusion protein with maltose-binding protein (MBP), according to the manufacturer's instructions (New England Biolabs). Polyclonal rabbit antiserum was raised against the purified recombinant OppAlV-MBP fusion protein. OppAlV were individually cloned and expressed in the pHClII vector (Invitrogen). The proteinase K sensitivity of borrelial proteins on viable spirochaetes was assayed by a modification of the protocol described by Barbour et al. (1984). Briefly, spirochaetes were pelleted and washed once in PBS-Mg²⁺, resuspended at a concentration of 2 x 10⁹ cells ml⁻¹ and incubated at room temperature for 40 min with varying amounts of proteinase K (0, 0.04, 0.2 and 1 mg ml⁻¹). After inhibiting proteinase K with PMSF, bacteria were pelleted, washed twice in PBS-Mg²⁺, resuspended in sample buffer and boiled for 5 min before analysis by SDS-PAGE and immunoblotting as described above.

RESULTS

Plasmid and chromosomal copies of oppA

We initially identified a homologue of oppA on cp26 of B. burgdorferi, located approximately 300 bp downstream of the guaAB locus and in the opposite orientation (Rosa et al., 1996). No open reading frames homologous to other components of oligopeptide permease were located in either the upstream (1.6 kb) or downstream (3.1 kb) flanking regions. Additional copies of oppA were subsequently identified elsewhere in the genome by a Southern blot of total undigested borrelial DNA hybridized at low stringency with a probe derived from the cp26 oppA gene (Fig. 1a). In addition to the supercoiled and nicked forms of cp26 (indicated by hybridization with a guaA probe), this probe also hybridized to the sheared chromosomal DNA and to a 54 kb linear plasmid (lp54, Fig. 1a). Southern blots of total genomic DNA digested with restriction enzymes that did not cut within the cp26 oppA gene contained multiple fragments that hybridized to the oppA probe at low stringency. For example, the oppA probe hybridized with four EcoRI fragments (Fig. 1b); washing this blot at increased stringency led to greatly reduced hybridization with the three larger fragments (not shown). These results suggested the presence of additional oppA genes on the chromosome and lp54.

Chromosomal locus encoding all five components of oligopeptide permease

Since many bacteria have the genes encoding all five components of oligopeptide permease at a chromosomal locus, we looked for a similar organization in B. burgdorferi. Alignments of the amino acid sequences of the ATPase subunits, OppD and OppF, from a diverse group of bacteria have identified several highly conserved regions (Higgins et al., 1986; Hyde et al., 1990; Mimura et al., 1991). Degenerate oligonucleotides from these regions (Table 1) were used in PCR with B.
The Southern blot of EcoRI-digested DNA. Sequence analyses of PCR products of the anticipated sizes confirmed their identities as portions of oppD and oppF. An amplification product spanning oppD and oppF was also obtained, indicating that these genes are adjacent in B. burgdorferi, as is true in other bacteria. The Southern blot of EcoRI-digested DNA hybridized with the oppA probe was rehybridized with an oppD probe, after the oppA signal had decayed (Fig. 1b); 6·5 kb and 9 kb EcoRI fragments were recognized by both probes. Similar co-hybridization of oppA and oppD probes was seen with borrelial DNA digested with several other enzymes (not shown). The oppD probe only hybridized to chromosomal DNA in undigested DNA (not shown), suggesting the presence of a chromosomal locus in B. burgdorferi with homologues of the oppA, oppD and oppF genes.

To more fully characterize this chromosomal locus, oppA and oppD probes were used to screen a B. burgdorferi genomic library (described in Methods). Sequence analysis of a number of positive clones defined a 10 kb chromosomal locus that contained seven open reading frames (Fig. 2), whose deduced amino acid sequences included homologues of three substrate-binding OppA proteins, the integral membrane proteins OppB and OppC, and the ATPases OppD and OppF. In addition to the previously characterized oppA gene on cp26, an oppA homologue unlinked to other permissive components was identified on lp54 (Fig. 2). We designated the chromosomal oppA homologues as oppAI, II and III, the cp26 homologue as oppAIV and the lp54 homologue as oppAV (Fig. 2). An open reading frame encoding a homologue of enolase resides approximately 1 kb downstream of oppA; there are no other readily identifiable sequences within approximately 1 kb upstream of the chromosomal locus or 0·6 kb on either side of oppAV on lp54.

The three loci shown diagrammatically in Fig. 2 account for all the EcoRI fragments that hybridized to the oppA probe (Fig. 1b); the smallest fragment is from cp26, the 6·5 kb fragment is part of the chromosomal locus (oppAI–III), and the remaining two fragments of approximately 5 and 9 kb are from lp54 or the remainder of the chromosomal locus (oppAI–II). Although the oppD probe also weakly hybridized to a 9 kb fragment (Fig. 1b), the location of EcoRI sites in the chromosomal locus indicates that this fragment is not derived from the oppD gene at this site; a related gene may be further upstream of oppAI. Alternatively, the 9 kb oppD and oppA fragments could be unrelated, co-migrating fragments. From this analysis (and digests with other restriction enzymes) there is no indication of additional oppA genes in the B. burgdorferi genome.

Characteristic sequence motifs and predicted structures

The linkage of three oppA homologues to homologues of oppBCDF at the chromosomal locus strongly suggests that they collectively encode a borrelial oligopeptide permease. In addition to overall sequence similarity, the deduced borrelial Opp proteins have predicted structural features and sequence motifs that are consistent with their proposed transport functions and inclusion in the ABC (ATP-binding cassette) transporter family (Higgins, 1992; Higgins et al., 1986).

Like many nucleotide-binding proteins, the B. burgdorferi OppD and OppF homologues contain two short sequence motifs known as Walker boxes that are proposed to contribute to the nucleotide-binding fold in the predicted structure (Walker et al., 1982). ATP-binding components of ABC transporters (such as OppD and OppF) also exhibit a highly conserved sequence known as the loop 3 region, which is thought to interact directly with the transmembrane components (OppB and OppC), and couple ATP hydrolysis to the transport process (Higgins, 1992; Hyde et al., 1990; Mimura et al., 1991); this region is also found in the B. burgdorferi OppD and OppF homologues.

Hydropathy analyses of the deduced borrelial OppB and OppC homologues indicate five to six potential membrane-spanning domains (not shown), consistent with the proposed structures of these integral membrane components in other bacteria (Dassa & Hofnung, 1985; Higgins, 1992; Pearce et al., 1992). A unique feature of the B. burgdorferi OppC sequence relative to OppC of other bacteria is an additional 53 residues within the predicted periplasmic loop between the first and second membrane-spanning regions.

The solute-binding components confer high-affinity substrate specificity upon the ABC transport systems with which they interact. Tam & Saier (1993) have defined eight families or clusters of bacterial solute-binding proteins based on amino acid sequence similarity. The deduced borrelial OppA sequences are most similar to members of cluster 5, which includes the peptide- and nickel-binding proteins, and each OppA homologue contains a good match to the consensus...
signature sequence for cluster 5 solute-binding proteins (underlined in Fig. 3).

OppA sequence comparisons

B. burgdorferi OppA homologues share approximately 25% identity with OppA proteins from Bacillus subtilis (Perego et al., 1991; Rudner et al., 1991) and Salmonella typhimurium (Hiles et al., 1987a), similar to the degree of sequence identity shared between the OppA proteins of the latter two, and with TraC from Enterococcus faecalis, a plasmid-encoded OppA homologue that is a receptor for a peptide pheromone (Tanimoto et al., 1993) (Table 2). Relatedness among the five B. burgdorferi OppA homologues ranges from 44% to 64% identity and does not correlate with genomic location.
Expression of oppA genes

The organization of the chromosomal opp genes suggests that oppAI, II and III could be individually transcribed, with oppBCDF forming an operon. Sizeable intergenic regions separate the B. burgdorferi oppA genes (oppAI-II, 119 bp; oppAI-III, 139 bp; oppAIIV-oppB, 335 bp), whereas only a few bases separate the oppB, oppC, oppD and oppF genes (oppB-C, 12 bp; oppC-D, 11 bp; oppD-F, 3 bp; see gene diagram in Fig. 2). An alignment of approximately 100 nucleotides upstream of the initiation codon for each oppA gene indicated very little conservation of sequence in the potential promoter regions (not shown). Computer analysis of these regions using the program MAFFT search did not identify any sequence that had greater than 60% identity with that of a consensus sigma 70 promoter from E. coli, in contrast to several other borrelial genes, which have sigma 70-like promoters (Bergstrom et al., 1989; Reindl et al., 1993; Simpson et al., 1994; Stevenson et al., 1996; Wallich et al., 1993; Zhang et al., 1997). The regions between the chromosomal oppA genes contain short, imperfect inverted repeats, but no sequences resembling rho-independent terminators.

Since the B. burgdorferi oppA genes share significant sequence identity, probes encompassing them cross-hybridize (Fig. 1 and data not shown), so developing gene-specific probes was a necessary prelude to undertaking studies of oppA gene expression. Within the most divergent portion of each gene (encoding amino acid residues 230-300, Fig. 3), the oppA genes share less than 60% nucleotide identity with each other. Probes from these regions were shown to be specific for the oppA gene from which they were derived by hybridization to plasmid DNA containing cloned segments from individual oppA genes (Fig. 4a). The probes did not cross-react with other oppA genes, even when the blots were overexposed.

Expression of B. burgdorferi opp genes in mid-exponential-phase cultures was monitored by Northern blot analysis of total RNA with probes for oppAI-V, oppBC and oppD. Three transcripts were detected when a probe specific for oppAI was used (Fig. 4b). The sizes of these transcripts correspond to those predicted for messages that encompass one (18 kb), two (4-1 kb) or three (6-2 kb) oppA genes (Fig. 4b, oppAI, arrows 1, 2 and 3, respectively). Similar results were obtained using the oppAI and oppAIIV probes (not shown); the large 6-2 kb transcript was the least abundant, but detectable with all three probes. These results suggest that transcription initiates at each oppA gene, but can also proceed through the intergenic regions and downstream oppA genes at the chromosomal locus. Alternatively, processing of a single large transcript initiating upstream of oppAI could result in a similar banding pattern. Only a 1-8 kb transcript was detected for each of the plasmid-located oppAIV and oppAV genes (Fig. 4b). As a preliminary step toward assessing the functional significance of the five B. burgdorferi OppA proteins, we inactivated the oppAIV gene by allelic exchange (described in Methods). No oppAIV transcript was detected in the oppAIV mutant, although it did contain transcripts of the three chromosomal oppA genes (Fig. 4b).

Different preparations of RNA yielded varying proportions of mono-, bi- and tri-cistronic transcripts from the chromosomal oppA genes, but their relative abundances did not correlate with known culture variables. Transcripts of the three chromosomal oppA genes and of oppAIV (on cp26) were not more abundant in B. burgdorferi cultures that were grown at 23 °C and then shifted to 35 °C (data not shown), but the transcript from oppAIV (on lp54) was consistently increased in cultures shifted to 35 °C (Fig. 4b, oppAIV). The oppAIV transcript was also present at a much lower level than those of the chromosomal oppA genes or oppAIV, even after induction at 35 °C; the oppAIV blot was exposed for approximately 6 d, compared to 6 h exposures for the oppAI and oppAIV blots (Fig. 4b). Hybridization of the same RNA preparations with a probe to the constitutively expressed flagellin gene indicated comparable amounts of RNA in all samples (Fig. 4c) and the quality of each RNA was demonstrated by the integrity of the rRNA bands in ethidium bromide-stained gels (Fig. 4b and data not shown).

No distinct transcripts were detected with probes from the oppB, oppC and oppD genes; instead, a broad band was visible after relatively long exposures with the oppD probe (45 h, Fig. 4b) or a larger probe spanning oppB and oppC (not shown). No oppA or oppD probe detected a transcript that was long enough to extend from oppAI through oppD.

Synthesis of OppAIV

To begin studies of OppA protein levels and location, OppAIV was synthesized as a recombinant fusion with maltose-binding protein in E. coli and a rabbit polyclonal serum was raised against the purified recombinant protein. Only faint reactivity was evident when an immunoblot containing a protein lysate from the
Fig. 4. (a) High-stringency Southern blot analysis of cloned oppA genes with specific probes. Recombinant plasmids containing individual *B. burgdorferi* oppA genes were digested with EcoRI (except oppAIV), separated by agarose gel electrophoresis, blotted and hybridized at 55 °C with oppA gene-specific probes, as indicated at the top of each panel. Plasmid DNA was as follows: lane 1, oppAI; lane 2, oppAII; lane 3, oppAlll; lane 4, oppAIV; lane 5, oppAV.

(b) Analysis of oppA transcripts. Left panel, ethidium-bromide-stained gel of RNA from *B. burgdorferi* indicating the mobilities of 55, 165 and 235 rRNAs relative to the hybridizing bands on the blots. Remaining panels, Northern blots were hybridized with probes as indicated above each panel. The oppAII and oppAIV probes were hybridized with RNA from *B. burgdorferi* oppAIV mutant (mutant) and wild-type (WT) bacteria. Arrows indicate the mobilities of mono- (1), bi- (2) and tri-cistronic (3) oppA transcripts hybridizing with the oppAII probe. We assume that the hybridizing band (*) below the 235 RNA band results from smaller RNA species being pushed in front of the rRNA and is not a discrete oppA transcript. The oppAV probe was hybridized with RNA from wild-type *B. burgdorferi* grown at 23 °C or shifted to 35 °C. The oppD probe was hybridized with RNA from wild-type *B. burgdorferi* grown at 35 °C. Mobilities of size standards (0.24–9.5 kb RNA ladder, Life Technologies) are indicated on the left of the ethidium-stained gel and first Northern blot panel. (c) The blots described above were allowed to decay and hybridized with a flagellin probe to assure equivalent loading of RNA in each lane.
**Fig. 5.** Immunoblot analysis of wild-type and oppAIV mutant *B. burgdorferi*. Lysates of oppAIV mutant B31-82 (lane 1) and wild-type *B. burgdorferi* grown at 23 °C (lane 2) or shifted to 35 °C (lane 3) were separated by SDS-PAGE and identical blots were incubated with (a) OppA or (b) OspC-specific sera. Mobilities of protein size standards (kDa) are indicated. The 35 kDa protein recognized by the anti-OspC antiserum in all samples has been noticed previously and is unrelated to OspC (Norris et al., 1992; Schwan et al., 1993; Tilly et al., 1997).

**Fig. 6.** Proteinase K sensitivity of OppAIV on intact *B. burgdorferi*. Live, unfixed bacteria were treated with different concentrations of Proteinase K: 1 mg ml⁻¹ (lane 1), 0.2 mg ml⁻¹ (lane 2), 0.04 mg ml⁻¹ (lane 3), and 0.0 mg ml⁻¹ (lane 4). Lysates of treated bacteria were separated by SDS-PAGE and either stained with Coomassie brilliant blue (a), or analysed by immunoblot with OspB (b) and OppAIV (c) antiserum. The mobility of proteinase K (pk) is indicated on the Coomassie-stained gel. The mobilities of protein size standards (kDa) are indicated.

oppAIV mutant was probed (Fig. 5a, lane 1), indicating that the majority of the protein recognized by this antiserum in wild-type *B. burgdorferi* lysates (Fig. 5a, lanes 2 and 3) is the oppAIV gene product. The other four *B. burgdorferi* OppA proteins were also synthesized as recombinant proteins in *E. coli* and bacterial lysates were tested by immunoblotting; the OppAIV antiserum cross-reacted only very weakly with OppAI, II, III and V (data not shown).

Lysates of wild-type *B. burgdorferi* grown at 23 °C and then shifted to 35 °C were probed by immunoblotting to determine if there were an increase in OppAIV, as had been found for OspC (Schwan et al., 1995). Bacteria grown at both temperatures contained similar amounts of OppAIV (Fig. 5a, compare lanes 2 and 3), consistent with oppAIV transcript levels, whereas OspC was only detected in the culture shifted to 35 °C (Fig. 5b, compare lanes 2 and 3).

**Surface inaccessibility of OppA**

To determine if the borrelial OppA proteins were surface-exposed, intact borreliae were treated with varying amounts of proteinase K and bacterial lysates were immunoblotted with OppAIV and OspB polyclonal sera (Fig. 6). Whereas only a slight diminution in OppAIV was seen at even the highest concentration of proteinase K (1 mg ml⁻¹; lane 1, Fig. 6), OspB, a surface-exposed lipoprotein, was completely degraded at the lowest concentration of proteinase K (0.04 mg ml⁻¹; lane 3, Fig. 6), relative to the control lysate without
proteinase K treatment (lane 4, Fig. 6). Analysis of the same bacterial lysates by Coomassie blue stain indicated that two other surface-exposed lipoproteins, OspA and OspC, were also degraded by proteinase K treatment, although OspA was somewhat resistant at the lowest concentration (0.04 mg ml⁻¹; lane 3, Fig. 6). We conclude that, in contrast with several borrelial outer-surface lipoproteins, OppAIV of intact organisms resists digestion by proteinase K and may have a periplasmic location.

DISCUSSION

Multiple substrate-binding components in *B. burgdorferi*

Typically, the genes encoding all the components of oligopeptide permease are present at a single chromosomal locus. An interesting feature of the putative *B. burgdorferi* transporter is the presence of multiple substrate-binding components (OppA homologues), encoded by both plasmid and chromosomal loci. Some other bacteria have additional substrate-binding components encoded on plasmids or elsewhere on the chromosome (Alloing et al., 1994; Jenkinson et al., 1996; Leonard et al., 1996; Ruhfel et al., 1993; Tanimoto et al., 1993). It is intriguing to consider the possibility that the *B. burgdorferi* plasmid-encoded OppA homologues recognize peptide signals that induce an adaptive response, since these plasmids also carry outer-surface protein genes that are differentially expressed during transmission from ticks to mammals (de Silva et al., 1996; Schwan et al., 1995).

In addition to multiple peptide-binding components for a single oligopeptide permease, separate transport systems can exist for peptides of different sizes or characteristics (Hiles et al., 1987b; Koide & Hoch, 1994). Components of the putative *B. burgdorferi* peptide permease are most similar to oligopeptide (Opp) transport components. However, substrate specificities cannot be assigned on the basis of sequence similarity alone, and biochemical and genetic studies are necessary before the physiological role and peptide specificity of this system in *B. burgdorferi* can be determined. Borreliae grow in the laboratory only in a complex, undefined medium, hence complicating *in vitro* studies to directly characterize peptide transport in these bacteria. Expression of oppAIV in a *Bacillus subtilis* oppA mutant did not restore general peptide transport, as assayed by resistance to the toxic peptide bialophos and failure of a methionine auxotroph to grow in medium in which peptides were the sole source of amino acids (unpublished data). These data suggest that OppAIV does not bind peptides non-specifically, but are inconclusive due to the nature of negative complementation results in a heterologous system.

**OppAIV not essential for in vitro growth**

As an initial step toward understanding the role of oligopeptide permease in the transmission and adaptation of *B. burgdorferi* between the arthropod and mammalian environments, we have inactivated oppAIV, the cp26-encoded gene. We suspect that the oppAIV product could play an important role in sensing environmental signals in the form of peptides. oppAIV was inactivated in a non-infectious, laboratory-adapted *B. burgdorferi*, with the intention of subsequently moving this mutation into an infectious isolate. However, we have not succeeded in transforming low-passage infectious *B. burgdorferi* and thus have been unable to directly test the effect of this mutation in the infectious cycle. Clearly, *B. burgdorferi* does not require OppAIV for growth in culture medium.

**Homologue of OppAII not essential for in vivo infectivity**

Das et al. (1996) previously described a 30 kDa protein (P30) from *B. burgdorferi* isolate N40 that is homologous to periplasmic substrate-binding proteins of Gram-negative bacteria. Extensive sequence similarity strongly suggests that p30 and oppAII are alleles of the same gene from two different *B. burgdorferi* isolates. The disparity in size between P30 and OppAII (30 kDa versus 58 kDa, respectively) derives from a base deletion in the p30 gene, which results in a frame shift and premature stop codon. P30 probably is not a functional substrate-binding protein because much of the peptide-binding pocket (Tame et al., 1994) is missing. Despite this mutation, *B. burgdorferi* N40 maintains a mammal–tick infectivity cycle (Fikrig et al., 1992). These data suggest that p30 represents a naturally occurring oppAII mutant and that there may be functional redundancy among the five *B. burgdorferi* OppA proteins such that they are not all essential in vivo.

**Cellular location of substrate-binding components**

The substrate-binding components of high-affinity transport systems are located in the periplasm of Gram-negative bacteria. In Gram-positive bacteria, which lack an outer membrane, these "periplasmic binding proteins" such as OppA, are surface-exposed lipoproteins that are anchored to the cytoplasmic membrane by an amino-terminal lipid moiety (Gilson et al., 1988; Perego et al., 1991). The deduced amino acid sequences of the *B. burgdorferi* OppA homologues suggest that they are membrane-bound lipoproteins, yet the surface-inaccessibility of one of these proteins (OppAIV) is consistent with a periplasmic location. The borrelial substrate-binding proteins may be periplasmic, but associated with the cytoplasmic membrane by a lipid moiety, thus combining features of both Gram-positive and Gram-negative bacteria. In another spirochaete, *Treponema pallidum*, a protein with homology to the substrate-binding components of ABC transporters is primarily associated with the cytoplasmic membrane (Akins et al., 1997).
Concluding remarks

In conclusion, we have characterized a chromosomal locus and two plasmid loci in *B. burgdorferi* that encode homologues of oligopeptide permease components. To determine the function of this transporter we are pursuing biochemical and genetic studies to investigate the peptide-binding characteristics and biological roles of the five different substrate-binding components. We speculate that *B. burgdorferi* might utilize peptide pheromones to elicit a concerted adaptive response to changing environmental conditions, such as those that the bacteria encounter as they cycle between ticks and mammals.

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