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Expression of Antigens from Chromosomal and Linear Plasmid DNA of *Borrelia coriaceae*

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Three recombinant plasmids containing DNA from *Borrelia coriaceae*, the putative agent of epizootic bovine abortion, expressed antigens in *Escherichia coli* that reacted with antibodies specific for *B. coriaceae*. Two of the recombinants each expressed a single high-molecular-weight antigen. The third recombinant expressed three smaller antigens. The DNA inserts were sized and mapped. Hybridization of the cloned inserts to pulsed-field electrophoresis samples of *B. coriaceae* whole-cell DNA revealed the origin of two of the inserts to be located in linear plasmids. One of these, expressing three antigens, was located in a 210-kilobase linear plasmid. A second recombinant expressed a single antigen but hybridized to at least three distinct linear plasmids. The third clone also expressed a single antigen but was demonstrated to be chromosomal in origin.

Borrelia coriaceae has been reported to be the putative agent of epizootic bovine abortion (14, 18). The organism is carried and transmitted by the soft-bodied *Ornithodoros coriaceus* tick. Because this tick feeds on animals and humans, *B. coriaceae* may pose an unappreciated health threat to vertebrates other than cattle. A member of the spirochete family, *B. coriaceae* typically has proven difficult to identify in the host and to isolate and grow in culture. Hence, conclusive evidence of the role of this organism as a pathogen is still lacking. Important information has been gained concerning other spirochetes when recombinant DNA methods were used to study their respective genomes and antigen expression. Notable examples are the syphilis agent *Treponema pallidum* (7, 8, 31, 32), the relapsing fever agent *Borrelia hermsii* (21, 25), and the Lyme disease agent *Borrelia burgdorferi* (3, 12, 13). Of particular importance in these studies was the discovery that major antigens from both *B. hermsii* and *B. burgdorferi* are encoded and expressed in linear plasmids (3, 12, 13, 25). In the *B. hermsii* report it was demonstrated that antigenic variation is due to translocation of genes to expression sites contained in linear plasmids.

It is not known whether *B. coriaceae* undergoes antigenic variation or whether it controls expression of genes by translocation. However, in this report we describe the cloning of genes into plasmid vectors expressing *B. coriaceae* antigens in *Escherichia coli*. One of the recombinants apparently contained an operon of three genes expressing three distinct antigens. This insert hybridized to a 210-kilobase (kb) linear plasmid. A second clone expressed a single antigen but hybridized to at least three distinct linear plasmids, thus indicating the possible presence of silent and expression-linked copies of this gene as described previously for *B. hermsii*. The third clone discussed in this report expressed a single antigen derived from the chromosome of *B. coriaceae*.

MATERIALS AND METHODS

Strains used in this study. *B. coriaceae* reference strain ATCC 43381 was maintained in modified BSKII medium (1) and grown at 33°C. Cells were harvested at the mid-log phase (approximately 2×10^8 to 4×10^8 cells per ml).

Purification of linear plasmids for cloning. Samples of 20 ml of cell culture were washed three times in phosphate-buffered saline containing 5 mM $MgCl_2$. The pellet was suspended in 400 μl of 15% sucrose in 50 mM Tris hydrochloride (pH 8.0)–50 mM EDTA. Then 100 μl of 10% sodium dodecyl sulfate and 25 μl of a 20 mM stock solution of proteinase K were added, and the mixture was incubated at 37°C for 1 h. The mixture of lysed cells was carefully loaded on to a linear sucrose gradient (10 to 40%) prepared in 50 mM Tris hydrochloride–50 mM EDTA. The sample was centrifuged in an SW41 rotor at 28,000 rpm for 20 h. Three-drop fractions were collected from the bottom of the tube through a no. 21 needle. Individual fractions were electrophoresed in a 0.3% agarose gel, stained with ethidium bromide, and illuminated with UV irradiation to determine which contained the linear plasmid DNA. The linear plasmid fractions were pooled, and the DNA was precipitated with 2.5 volumes of cold 95% ethanol, dried, and suspended in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (TE) buffer. The DNA concentration was determined spectrophotometrically in preparation for cloning into pUC plasmid expression vectors.

Cloning procedures. Enzymes used in these procedures were purchased from Bethesda Research Laboratories, Inc., Gaithersberg, Md., and used according to the manufacturer's specifications. Approximately 100 ng of the pooled linear plasmid fraction from *B. coriaceae* was partially digested with *Hin*dIII (1 U of enzyme, 15 min). The DNA was phenol extracted and ethanol precipitated by standard procedures as described above. The DNA pellet was dissolved in TE buffer. The pUC19 plasmid vector was prepared for ligation to the *B. coriaceae* plasmid DNA by digestion with *Hin*dIII, phenol extraction, ethanol precipitation, and suspension in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. The pUC plasmid and the digested linear plasmids were mixed and ligated with T4 DNA ligase at 13°C overnight.

Preparation of competent *E. coli* cells (JM83) and the subsequent transformation were performed by standard procedures (20). The transformed cells were plated on LB plates (22) containing 100 μg of ampicillin per ml and 50 μg of

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5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. White colonies representing *E. coli* cells containing recombinant plasmids were subcloned and screened for expression of *B. coriaceae* antigens.

Immunoassay of recombinant clones. After the recombinant clones were grown, they were transferred to nitrocellulose membranes by standard colony hybridization methods (10). Approximately 300 clones were assayed. The membranes were previously saturated in 24 mg of isopropyl-β-D-thiogalactopyranoside per ml to induce expression of the insert sequences. The cells were then lysed on the membranes by exposure to chloroform vapor, followed by gentle washing in 1% sodium dodecyl sulfate. The membranes were blocked with bovine serum albumin and Tween 20 by standard procedures (10). The lysed cells were then reacted with antibodies raised in rabbits inoculated with whole cells of *B. coriaceae*. A second (goat anti-rabbit) antibody conjugated to alkaline phosphatase was added, followed by the enzyme substrate, 5-bromo-4-chloro-3-indolylphosphate, and Nitro Blue Tetrazolium (Zymed Laboratories Inc., South San Francisco, Calif.). Sixteen clones appeared to react positively with the antiserum. These clones were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide gels) and immunoblot analysis as previously described (6, 17).

Restriction maps of the cloned genes. The *B. coriaceae* antigen genes were sized and mapped with restriction enzymes by standard procedures (20).

Location of the antigen genes in *B. coriaceae*. Positive clones expressing *B. coriaceae* antigens were grown in 20 ml of LB broth containing 100 mg of ampicillin per liter. The plasmids were isolated by an alkaline lysis procedure (5). The plasmids were digested with *Hin*dIII and radiolabeled with [α-^{32}P]dCTP (26). The labeled plasmids were used as probes and hybridized to Southern blots (30) of pulsed-field gels containing whole-cell *B. coriaceae* DNA encapsulated in agarose beads and electrophoresed as previously described (19, 24, 29).

RESULTS

Immunoassay of recombinant clones. The screening of the recombinant cells by colony hybridization produced 16 clones that appeared to react with the antiserum. These colonies were run in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis system. Figure 1 shows an immunoblot of these clones reacted with the antiserum directed against *B. coriaceae*. Of the 16 clones, 7 were strongly positive to the antiserum. Clones in lanes 4, 7, and 13 all synthesized three antigens of similar molecular masses ranging from approximately 27 to 30 kilodaltons (kDa). Lanes 9 and 11 represented clones expressing a single protein of approximately 35 kDa. Clones in lanes 15 and 16 expressed a *B. coriaceae* antigen of approximately 55 to 60 kDa. The expressed antigens appeared to represent full-length copies of proteins based on similar-sized reacting antigens in *B. coriaceae*. However, the smallest of the triplet proteins did not appear to be represented in *B. coriaceae*. It is possible that this protein is expressed in *B. coriaceae* but at much lower levels than in *E. coli*.

Sizing and mapping the DNA inserts. Representative recombinant plasmids for each of the three antigen expression types in Fig. 1 were further characterized. The recombinant plasmid expressing three antigens was designated pPL1. Several restriction enzymes were used in mapping the cloned gene(s): *Eco*RI, *Bam*HI, *Pst*I, *Kpn*I, *Bcl*I, *Hin*dIII, *Cla*I, *Nhe*I, *Nru*I, *Xba*I, *Bss*HII, *Sal*I, *Mlu*I, *Sst*I, *Sst*II, *Bal*I, *Bgl*II, *Eco*RV, and *Hin*cII. Only *Hin*cII cut the DNA (Fig. 2A). The size of the insert was determined to be 2 kb by *Hin*dIII digestion and agarose gel electrophoresis.

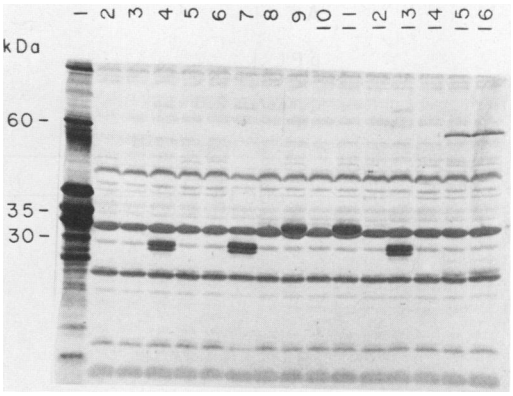

FIG. 1. Immunoblot screening for the expression of *B. coriaceae* antigens in *E. coli* cells containing recombinant plasmids. The blot was screened with antiserum raised in rabbits against whole-cell *B. coriaceae*. Lanes: 1, *B. coriaceae* lysed whole cells; 4, 7, and 13, three antigens between 27 and 30 kDa; 9 and 11, 35-kDa antigen; 15 and 16, 55- to 60-kDa antigen.

The recombinant plasmid expressing the 35-kDa protein was designated pPL2. The same restriction enzymes were used to map this insert. Figure 2B illustrates the enzymes that cut the DNA and their approximate locations. The size of the pPL2 gene was determined to be 1.5 kb.

The third recombinant, expressing the 55- to 60-kDa antigen, was designated pPL3. The size of the gene (1.9 kb) and a restriction map of the enzymes the cut within it are shown in Fig. 2C.

Location of the antigen genes. The recombinant plasmids were radiolabeled and used as probes to determine their location within the *B. coriaceae* cell. Figure 3 represents a pulsed-field gel containing several lanes of *B. coriaceae* whole-cell DNA. The bands at the bottom of Fig. 3 between 10 and 60 kb represent several linear plasmids as described previously (19). The band at approximately 210 kb also represents a linear plasmid. The intense staining band slightly above the 210-kb plasmid (approximately 225 to 250 kb) represents linearized or broken chromosomal DNA. The DNA remaining in the well is predominantly intact chromosomal DNA. The DNA in the gel was transferred to a nylon membrane, and the lanes were cut into strips for hybridization with the cloned probes.

Figure 4 is an autoradiograph of the gel in Fig. 3, which was hybridized with different probes. The first lane (labeled B. c. whole cell) was probed with labeled whole-cell *B. coriaceae* DNA. All of the genetic components of the *B. coriaceae* are apparent. The second lane was probed with pPL1, the recombinant expressing three antigens. The probe hybridized only to the 210-kb linear plasmid. The third lane was probed with pPL2, the gene that expressed the 35-kDa antigen. The DNA hybridized to what appeared to be three linear plasmids (60, 35, and 30 kb). The 35-kb band may in fact be a cluster of linear plasmids that were poorly resolved under these conditions (19).

The third probe, pPL3, which synthesized the 55- to

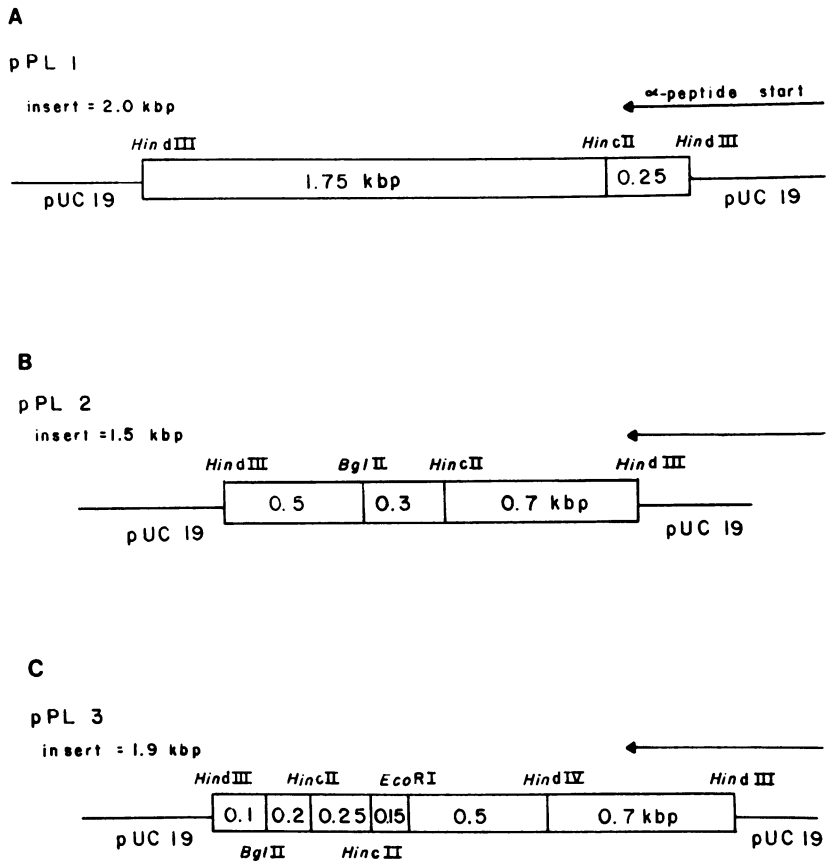

FIG. 2. (A) Restriction map of pPL1 expressing three *B. coriaceae* antigens. (B) Restriction map of pPL2 expressing the 35-kDa antigen. (C) Restriction map of pPL3 expressing the 55- to 60-kDa antigen.

60-kDa antigen, hybridized to the chromosome (Fig. 4, fourth lane). This plasmid hybridized to the linearized chromosomal DNA as well as to the intact chromosomal DNA remaining in the loading well. It was not surprising that a chromosomal gene was cloned from a linear plasmid-enriched fractionation. This is due to the fact that chromosomal DNA is easily broken and linearized during cell lysis and other physical manipulations. This high-molecular-weight linearized DNA is difficult to separate from the 210-kb linear plasmid in the sucrose fractionation and thus was pooled with the linear plasmids.

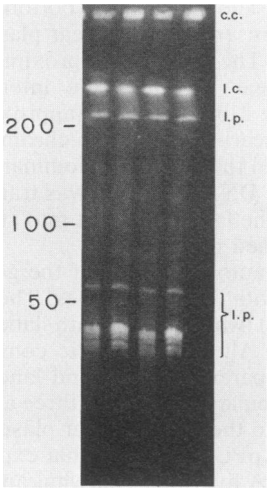

FIG. 3. Pulsed-field gel electrophoresis of four identical lanes of whole-cell *B. coriaceae* DNA encapsulated in agarose beads. l.p., Linear plasmid; l.c., linearized chromosome; c.c., circular chromosome. Sizes in kilobase pairs are indicated on the left side.

DISCUSSION

B. coriaceae has been described as the presumptive agent of epizootic bovine abortion, a disease endemic to the foothill ranges of California (16, 18). The spirochete was first isolated in 1985 (18) and was characterized as a new species in 1987 (14). It is known to by carried and transmitted by the soft-bodied *O. coriaceus* tick (14, 18). Because this tick frequently takes blood meals from humans, it is of critical importance to determine the role of *B. coriaceae* as a pathogen. This is particularly true in light of the serious diseases caused by other members of the *Borrelia* genus, among which are relapsing fever and Lyme disease.

A previous report described a genetic and antigenic characterization of *B. coriaceae* and its relationship to other spirochetes (19). The genetic complement present within *B. coriaceae* was described at that time. As seems to be typical among all *Borrelia* species, several linear plasmids are present in *B. coriaceae* as described by pulsed-field gel electrophoresis (2, 3, 19, 25). It has been reported that linear plasmids in *B. hermsii* and *B. burgdorferi* encode for and express outer surface proteins in these organisms (3, 21). It has also been reported that linear and circular plasmids in a

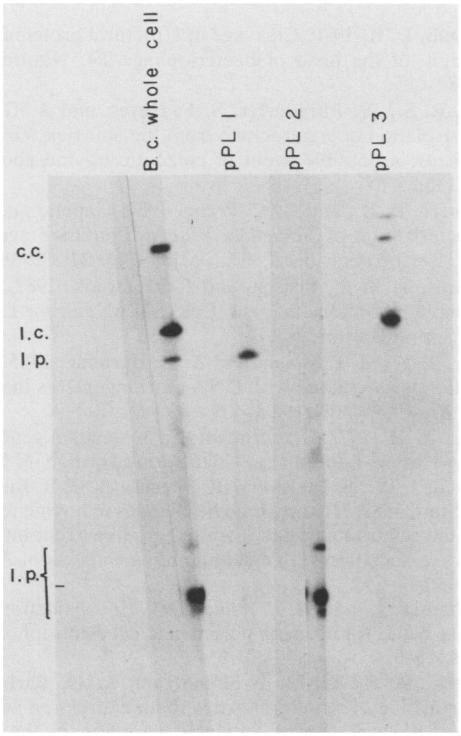

FIG. 4. Autoradiograph of the four lanes represented in Fig. 3 blotted to nylon membranes and probed with the recombinant genes. Lanes were probed with whole-cell *B. coriaceae* (B.c.) DNA, pPL1, pPL2, and pPL3. l.p., Linear plasmid; l.c., linearized chromosome; c.c., intact circular chromosome.

strain of *B. burgdorferi* are responsible for encoding virulence factors in the mammalian host (28). Based on this information, we investigated the genetic elements resident in *B. coriaceae*. Several clones were identified that contained recombinant plasmids expressing antigens that reacted with antiserum raised against *B. coriaceae*. Three of these clones, expressing five different antigens, were studied in detail.

One of these clones (pPL1) expressed three antigens and was derived from a 210-kb linear plasmid. The second clone (pPL2) expressed a 35-kDa antigen and hybridized to at least three of the smaller linear plasmids of *B. coriaceae*. The third clone (pPL3) expressed a 55- to 60-kDa antigen and was derived from the chromosome of *B. coriaceae*. These results are novel compared with what has been reported regarding antigen expression in other *Borrelia* species. In *B. burgdorferi* the two outer surface antigen genes that have been described are located in a juxtaposed operon position in a single linear plasmid (3, 12). The genes described for *B. hermsii* that are responsible for antigenic variation are located on different linear plasmids, but only one linear plasmid appears to contain the site for expression of these genes (21).

Recombinant pPL1 expressed three antigens of approximately 27, 28, and 30 kDa. It is not clear whether the two smaller antigens are degradation products of the largest one or whether unique proteins synthesized from distinct genes. Based on the fact that a 30-kDa protein is encoded in approximately 810 DNA base pairs, it is possible that up to three genes may be coded within the cloned 2-kb fragment in pPL1. Thus, it may be that these genes are linked in an operon configuration similar to outer surface proteins A and B described for *B. burgdorferi* (3). In support of this theory, the antigens were expressed in *E. coli* at the same levels with or without induction of expression by isopropyl-β-D-thiogalactopyranoside. This indicates that a promoter(s) for these genes was also cloned which functioned in *E. coli* independently of the repressed lactose promoter. Future work involving transposon mutagenesis and subcloning will determine the configuration of these genes in relation to a possible transcriptional unit. The function(s) of the 210-kb linear plasmid, other than encoding the antigen genes described here, is not known. However, it is of interest to note that only 1 of 19 restriction enzymes used for mapping this gene digested the DNA (*Hin*cII). The lack of restriction sites within this 2-kb clone is not readily explainable, since the battery of enzymes used to digest the DNA recognizes a wide range of purine and pyrimidine combinations.

The recombinant pPL2 expressed a 35-kDa antigen. The 1.5-kb cloned gene hybridized to at least three of the smaller linear plasmids in *B. coriaceae*. It is not known which of these plasmids were actually transcribing the gene. Conceivably, all of them may be actively transcribed in expressing this antigen. Another interpretation may be similar to what has been reported in *B. hermsii*; that is, there may be an expression site in one of the linear plasmids where antigen genes are transposed in and out for activation (21). Should this be the case, it may also indicate that *B. coriaceae* undergoes a similar method of antigenic variation as described in *B. hermsii*. Such a phenomenon could explain, at least in part, how *B. coriaceae* might invade and kill or injure the bovine fetus in spite of an active immune response by the unborn calf (23, 27; B. I. Osburn, unpublished data).

The fact that genes cloned from the linear plasmids expressed major antigens of approximately 34 kDa and 27 to 30 kDa is of interest. These appear to be size ranges of major antigens in *B. coriaceae*. These are essentially the same sizes as the two major outer surface proteins, A and B, of the lyme disease agent *B. burgdorferi* (31 and 34 kDa, respectively). Outer surface antigens identified in *B. hermsii* and other relapsing fever *Borrelia* spp. range from 30 to 43 kDa (4, 15). All of these antigens are encoded in linear plasmids in these organisms. It is conceivable, therefore, that the 34-kDa and the 27- to 30-kDa antigens are surface proteins in *B. coriaceae* as well.

The third recombinant, pPL3, expressed a 55- to 60-kDa antigen in *E. coli*. The 1.9-kb clone hybridized specifically to chromosomal DNA in the pulsed-field gel separation of whole-cell DNA from *B. coriaceae*. This is the first report of any *Borrelia* species housing and expressing an antigen gene located in the chromosome, although other interesting findings of *Borrelia* chromosomes have been reported (9, 11). The role of the expressed protein in *B. coriaceae* is not known.

B. coriaceae, like other species in this genus, is unique among procaryotic organisms. The maintenance of several linear plasmids and their role in the expression of antigens and possibly other proteins is of interest to the clinical investigator as well as to the basic research scientist. In this report several new findings are discussed for *B. coriaceae* which have not been described in other *Borrelia* organisms. The synthesis of antigens encoded in genes located in several discrete genetic components resident in *B. coriaceae* is intriguing. Future work should delineate other functional genes in the linear plasmids and the possible role of gene switching and antigenic variation. The role of the chromosome in antigen expression and possible virulence factors and gene switching is also in need of further study.

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