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Use of the "Blue Halo" Assay in the Identification of Genes Encoding Exported Proteins with Cleavable Signal Peptides: Cloning of a *Borrelia burgdorferi* Plasmid Gene with a Signal Peptide

MICHAEL GILADI,[1] CHERYL I. CHAMPION,[2] DAVID A. HAAKE,[1] DAVID R. BLANCO,[2] JEFF F. MILLER,[2] JAMES N. MILLER,[2] AND MICHAEL A. LOVETT[1,2]*

Departments of Medicine[1] and Microbiology and Immunology,[2] UCLA School of Medicine, Los Angeles, California 90024*

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We have recently reported a *phoA* expression vector, termed pMG, which, like Tn*phoA*, is useful in identifying genes encoding membrane-spanning sequences or signal peptides. This cloning system has been modified to facilitate the distinction of outer membrane and periplasmic alkaline phosphatase (AP) fusion proteins from inner membrane AP fusion proteins by transforming pMG recombinants into *Escherichia coli* KS330, the strain utilized in the "blue halo" assay first described by Strauch and Beckwith (Proc. Natl. Acad. Sci. USA 85:1576–1580, 1988). The lipoprotein mutation *lpp-5508* of KS330 results in an outer membrane that is leaky to macromolecules, and its *degP4* mutation greatly reduces periplasmic proteolytic degradation of AP fusion proteins. pMG AP fusions containing cleavable signal peptides, including the *E. coli* periplasmic protein β-lactamase, the *E. coli* and *Chlamydia trachomatis* outer membrane proteins OmpA and MOMP, respectively, and Tp 9, a *Treponema pallidum* AP recombinant, diffused through the leaky outer membrane of KS330 and resulted in blue colonies with blue halos. In contrast, inner membrane AP fusions derived from *E. coli* proteins, including leader peptidase, SecY, and the tetracycline resistance gene product, as well as Tp 70, a *T. pallidum* AP recombinant which does not contain a signal peptide, resulted in blue colonies without blue halos. Lipoprotein-AP fusions, including the *Borrelia burgdorferi* OspA and *T. pallidum* Tp 75 and TmpA showed halo formation, although there was significantly less halo formation than that produced by either periplasmic or outer membrane AP fusions. In addition, we applied this approach to screen recombinants constructed from a 9.0-kb plasmid isolated from the B31 virulent strain of *B. burgdorferi*. One of the blue halo colonies identified produced an AP fusion protein which contained a signal peptide with a leader peptidase I cleavage recognition site. The pMG/KS330r$^-$ cloning and screening approach can identify genes encoding proteins with cleavable signal peptides and therefore can serve as a first step in the identification of genes encoding potential virulence factors.

Enzymatic function of *Escherichia coli* alkaline phosphatase (AP) fusion proteins is dependent on the presence of sequences which mediate their export to the periplasmic space (30), such as alpha-helical membrane-spanning segments and signal peptides. On the basis of this principle, the bacterial transposon Tn*phoA* has been widely used to identify genes with membrane-spanning and signal sequences and has been employed in studies ranging from determination of membrane protein topology to identification of genes relevant to bacterial virulence (32). Tn*phoA* carries the *E. coli* AP structural gene *phoA*, but not its promoter or signal sequence. Insertions of Tn*phoA* into genes containing membrane-spanning or signal sequences, in the correct orientation and reading frame, may result in enzymatically active fusions between the products of the target genes and AP. *E. coli* colonies expressing AP are blue because of their activity on XP (5-bromo-3-chloro-indolyl phosphate).

Because our interests center on surface proteins and virulence factors of spirochetes for which genetic exchange has not been developed, we developed a plasmid cloning vector, pMG (7), to provide an alternative to Tn*phoA* for analysis of virulence in situations where Tn*phoA* cannot be used. pMG was designed to allow identification of fusions between putative export signals and a *phoA* gene without a signal sequence. Functional pMG AP fusions have included those with signal peptidase I cleavage sites, those with signal peptidase II cleavage sites, which are acylated by *E. coli*, and sequences predicted to be membrane-spanning alpha-helices (7). Functional AP fusions with signal peptidase II cleavage sites (lipoproteins) were readily identified by virtue of their acylation and consequent hydrophobicity (7). In this report, we demonstrate that fusion proteins derived from cytoplasmic membrane proteins can be distinguished from those more likely to be of relevance to virulence, namely outer membrane and secreted proteins, by use of the "blue halo" assay first described by Strauch and Beckwith (44).

Strauch and Beckwith (44) utilized the leaky outer membrane of *E. coli* with the *lpp-5508* mutation to develop an assay for mutants defective in general proteolysis. Certain AP fusion proteins which spanned the cytoplasmic membrane were known to be degraded by periplasmic proteolytic activity, liberating the enzymatically active AP domain of the fusion protein. Extracellular leakage of AP resulted, and the cells formed blue colonies with blue halos on XP agar. By Tn*5* mutagenesis, a mutation, designated *degP4*::Tn*5*, which reduced the periplasmic proteolytic activity was found. AP fusion proteins otherwise subjected to degradation, and hence blue halo formation, no longer formed blue halos in the presence of the *degP4* mutation.

* Corresponding author.

We reasoned that *E. coli* KS330r$^-$ (*lpp-5508 degP4*:Tn*5*) could provide a suitable host strain for assessing whether AP fusion proteins were processed by leader peptidase I (wholly exported) or were anchored to the cytoplasmic membrane by an alpha-helical membrane-spanning segment. It was expected that colonies of the former would exhibit blue halos and that colonies of the latter would be blue without halos if the *degP4* mutation was effective in preventing degradation of AP fusions which spanned the cytoplasmic membrane. Indeed, in this report, we demonstrate that fusions between inner membrane proteins and AP that are predicted to remain anchored to the cytoplasmic membrane result in blue colonies without halos. In contrast, fusions between periplasmic or outer membrane proteins and AP cleaved by signal peptidase I result in blue colonies with blue halos. The potential utility of this approach was illustrated by screening a library constructed from a 9.0-kb plasmid isolated from a virulent strain of *Borrelia burgdorferi* in *E. coli* KS330r$^-$. From one of the blue halo colonies, we isolated the 5′ end of a gene with a signal sequence and a leader peptidase I recognition site.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* KS330 (44) was provided by K. Strauch, Harvard Medical School. KS330 was made restriction minus (KS330r$^-$) by J. Jessee (Bethesda Research Laboratories, Gaithersburg, Md.). KS330r$^-$ and *E. coli* DH5α, used as recipients for the pMG vectors, were grown in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) at 32 and 37°C, respectively. Virulent *B. burgdorferi* B31 was obtained from S. Barthold, Yale University, as a low-passage isolate from infant rats; infectivity was confirmed with C3H mice as previously described (41) (data not shown). A multi-passaged, avirulent *B. burgdorferi* strain, B31, was obtained from the American Type Culture Collection and grown in BSK II medium (3).

Plate assays for AP activity. *E. coli* DH5α and KS330r$^-$ recombinants were spread onto LB plates containing 100 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml, 100 μM isopropylthiogalactopyranoside (IPTG; Sigma Chemical Co.), and 40 μg of XP (Sigma Chemical Co.) per ml. AP-expressing recombinants were identified as blue colonies.

Blue halos in KS330r$^-$ were demonstrated as follows. KS330r$^-$ recombinants were grown in LB broth containing 100 μg of ampicillin per ml and 100 μM IPTG at 32°C for 8 to 12 h. After incubation, the optical density at a wavelength of 600 nm was measured, and 1.5 ml of each culture was pelleted and resuspended in 10 to 50 μl of LB broth proportionally to the measured optical density at 600 nm to yield final suspensions with similar concentrations. A sample (2.5 μl) of each cell suspension was spotted onto LB plates containing 100 μg of ampicillin per ml, 1 mM IPTG, and 200 mg of XP per ml, followed by incubation at 32°C for 12 to 20 h.

Plasmids. The construction of pMG from pCH58 (23), provided by A. Wright, Tufts University, and pMMB66HE (16) has been described (7). The β-lactamase (*bla*) and the tetracycline resistance (Tc^r) genes from plasmid pBR322 (Bethesda Research Laboratories), the leader peptidase gene from plasmid pRD8 (13), the *secY* gene from plasmid pKY6 (42), the *ompA* gene from plasmid pTRC-omp9 (12) (provided by W. Wickner, UCLA), and a polymerase chain reaction (PCR) product (provided by E. Wager, UCLA) containing the first 664 bp of the *Chlamydia trachomatis* serovar L2 MOMP gene (43) were used for the creation of β-lactamase–AP, Tc^r-AP, leader peptidase-AP, SecY-AP, OmpA-AP, and MOMP-AP fusion proteins, respectively. *Treponema pallidum*-AP fusions Tp 9, TmpA-AP, Tp 75, and Tp 70 were constructed in pMG and have been described previously (7). Briefly, Tp 9 is a *T. pallidum*-AP fusion protein with a signal peptide and a leader peptidase I recognition site. TmpA-AP and Tp 75 are lipoprotein-AP fusions, and Tp 70 is an AP fusion protein whose predicted topology includes a membrane-spanning domain of a cytoplasmic membrane protein.

General techniques for recombinant DNA analysis. All restriction endonucleases and enzymes for DNA cloning were used in accordance with the specifications of the manufacturers (Bethesda Research Laboratories; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; New England Biolabs, Beverly, Mass.; Stratagene, La Jolla, Calif.) unless otherwise specified. Transformation of strain DH5α was done by the standard transformation method of Hanahan (20) and that of strain KS330r$^-$ was done by either the calcium chloride procedure of Mandel and Higa (29) or by a modification of the Hanahan method as follows. One milliliter of an overnight culture of KS330r$^-$ was inoculated into 50 ml of SOB medium (20) and incubated at 32°C with agitation to an optical density at 550 nm of 0.35 to 0.50. Cells were pelleted and resuspended twice, first in 15 ml of standard transformation buffer (TFB) (20) and then in 3.5 ml of TFB, followed by incubation on ice for at least 1 h. The cells were incubated with DNA for 30 min and then were heat shocked as previously described (20). Small-scale preparation of plasmid DNA was done by the alkaline lysis method of Birnboim and Doly (6). Large-scale preparation of plasmid DNA and purification in CsCl-ethidium bromide gradients were conducted as described previously (37). Southern blotting was performed as previously described (37).

Two oligonucleotides corresponding to each of the first 40 nucleotides of the *ospA* and *ospB* genes of *B. burgdorferi* (5) were synthesized (Don Glitz, Biological Chemistry, UCLA) to be used as probes. The oligonucleotides were end-labelled with [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) by using T4 polynucleotide kinase as described previously (45). The unincorporated isotopes were removed by using a Bio-Spin 30 column (Bio-Rad Laboratories, Richmond, Calif.) according to the directions of the manufacturer. In order to identify the OspA-AP fusion recombinants, colony hybridization was performed as previously described (7).

Preparation of *B. burgdorferi* DNA. Avirulent *B. burgdorferi* B31 cells (9×10^{10}) grown in 400 ml of BSK II medium (3) were used to prepare genomic DNA essentially as described previously for *T. pallidum* (7). A virulent *B. burgdorferi* strain, B31, passaged four times in vitro was used to prepare the supercoiled plasmid fraction as described by Barbour and Garon (4). The DNA was electrophoresed on a 0.8% low-melting-temperature agarose gel, and the smallest visible band, which comigrated with the 6.7-kb fragment of the λ *Hin*dIII size marker, was excised from the gel and purified. This supercoiled plasmid measured 9.0 kb after linearization with *Eco*RI (data not shown) and was designated the 9.0-kb plasmid.

Creation of a fusion between β-lactamase and AP in pMG. *Mse*I digest of pBR322 resulted in 15 fragments. A 372-bp fragment containing the *bla* gene promoter and the first 211 nucleotides of the gene encompassing its signal sequence was isolated. An *Ssp*I digest divided this fragment into a 145-bp *Mse*I-*Ssp*I fragment containing the *bla* promoter and a 227-bp *Ssp*I-*Mse*I fragment containing the *bla* signal sequence. The 372-bp *Mse*I-*Mse*I fragment and the 277-bp

*Ssp*I-*Mse*I fragment were individually ligated to each of the three reading frames of *Nde*I-digested and *Sma*I-*Nde*I-digested pMG, respectively, and were designated pBla372 and pBla227, respectively.

Creation of fusion between the *B. burgdorferi* lipoprotein OspA and AP in pMG. *B. burgdorferi* genomic DNA of highly passaged avirulent strain B31 was partially digested with *Mse*I to a mean size of about 3.0 kb, ligated to *Nde*I-digested pMG in three reading frames, and transformed into DH5α. The *Mse*I enzyme was chosen for its AT-rich recognition sequence (TTAA) in view of the low GC content (27 to 30%) reported for *B. burgdorferi* (40). Blue colonies were hybridized at high stringency with a 40-mer oligonucleotide probe corresponding to nucleotides 1 to 40 of the *ospA* gene, thereby encompassing the signal sequence of this gene. The results of the colony hybridization were confirmed by releasing the insert with a *Bam*HI-*Not*I digest, Southern blotting, and rehybridization with the *ospA* probe. In addition, DNA sequence analysis of this clone was performed in order to determine the sequence of the *ospA*/*phoA* junction.

Creation of fusions between the *C. trachomatis* major outer membrane protein MOMP and AP in pMG. A PCR product containing the first 664 bp of the MOMP-encoding gene *ompl* (9), flanked by *Bam*HI sites on each end, was digested with *Bst*YI. The *Bam*HI-*Bst*YI fragment encompassing the first 135 bp of *ompl* was ligated to *Bam*HI-digested pMG 3.29, which, on the basis of the known sequences of *ompl* and the *phoA* genes, was predicted to result in an in-frame fusion between the two genes and was designated pMOMP135.

Creation of fusions between the *E. coli* outer membrane protein OmpA and AP in pMG. A *Sau*3A digest of plasmid pTRC-omp9 (12) yielded a 533-bp fragment containing the first 452 bp of the *ompA* gene. The fragment was cloned into *Bam*HI-digested pMG 2.7, the reading frame predicted to result in a functional AP fusion protein, and was designated pOmpA16.

Creation of fusions between inner membrane proteins and AP in pMG. An *Eco*RV digest of plasmid pRD8 (13) yielded a 531-bp fragment containing the first 384 nucleotides of the leader peptidase gene *lep*. This fragment was cloned into *Sma*I-digested pMG in three reading frames and was designated pLpB.

Two fusion proteins between the SecY protein and AP were constructed as follows. (i) A *Hin*dIII-*Eco*RI digest of plasmid pKY6 (42) yielded a 1,114-bp fragment containing the first 1,063 bp of the *secY* gene. This fragment was isolated, filled-in with the large fragment of DNA polymerase I, ligated to *Sma*I-digested pMG in three reading frames, and designated pSecY2a. (ii) Fragment SecY2a was digested with *Mse*I, yielding a 241-bp fragment containing the first 190 bp of the *secY* gene. This fragment was cloned into *Sma*I-*Nde*I-digested pMG 2.7, the predicted reading frame to result in a functional fusion protein, and was designated pSecYD2.

Three fusion genes between the Tc^r gene and *phoA* were constructed as follows. (i) pBR322 was digested with *Mse*I, yielding a 1,664-bp fragment which was isolated, filled-in, and digested with *Bam*HI. The resulting 319-bp, blunt-ended *Bam*HI fragment, containing the first 289 nucleotides of the Tc^r gene, was ligated into pMG digested with *Sma*I and *Bam*HI and was designated pTetG. (ii) The *Ssp*I-*Eco*RV digest of plasmid pBR322 released a 378-bp fragment containing the first 99 nucleotides of the Tc^r gene, which was ligated into pMG digested with *Sma*I and was designated pTetH. (iii) An *Eco*RV-*Bam*HI digest of plasmid pBR322 yielded a 190-bp fragment, containing nucleotides 99 to 289 of the Tc^r gene, which was ligated into pMG digested with *Sma*I and *Bam*HI and was designated pTetJ.

Identification of lipoprotein-AP and *B. burgdorferi*-AP fusion proteins. Lipoprotein-AP fusions were identified by tritiated glycerol and palmitate intrinsic radiolabelling, as described previously (7). The *B. burgdorferi* fusion proteins were identified by slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with monoclonal antibody specific for AP, as previously described (7).

DNA sequencing. The polylinker region and DNA inserts in pMG were sequenced by using the dideoxynucleotide chain termination method of Sanger et al. (38) and [α-^{35}S]dATP (specific activity, 1,000 Ci/mmol; Amersham Corp.). Nineteen- and 20-mer primers corresponding to sequences approximately 40 bp upstream and downstream of the pMG polylinker were synthesized (Genosys Biotechnologies, Inc., The Woodlands, Tex.) for sequencing. Sequencing reactions were performed for both stands in accordance with the protocol recommended by Pharmacia LKB, Inc., Piscataway, N.J.

Construction of pLeader containing the signal sequence of pBb244. Two oligonucleotides with the sequences 5′-CGCG GATCCATGAGAAAAATAAGCCTA-3′ and 5′-CGCGGA TCCAGCACTTAAATCAATGCT-3′, corresponding to opposite strands of the signal peptide encoded by pBb244, were synthesized (Anita Rambo, Department of Microbiology and Immunology, UCLA) and used for PCR (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's suggestions. The PCR product was purified, digested with *Bam*HI, subcloned in pMG 3.29, and transformed into both DH5α and KS330r$^-$. In addition, the construct was sequenced in order to determine whether any PCR mistakes had been incorporated. The clone was designated pLeader.

Topological predictions. Nucleotide and amino acid sequence data analysis were performed with the program DNA Strider, version 1.0 (33).

RESULTS

Expression of a β-lactamase–AP fusion protein in *E. coli* DH5α is under control of the pMG *tac* promoter. The pMG vector (7) is composed of the following elements: (i) the parental broad-host-range expression vector pMMB66HE (16), including its *tac* promoter and the *lac* repressor (*lacI*^{q}); (ii) the *phoA* gene lacking its promoter, signal sequence, and the sequence encoding the first 13 residues of the mature protein; and (iii) a newly designed polylinker, which replaced the original polylinker of pMMB66HE between the promoter and the *phoA* gene to allow insertion of DNA in three reading frames (pMG 1.C, pMG 2.7, and pMG 3.29, respectively). Figure 1 shows the polylinker nucleotide sequence.

pBla372, containing the *bla* gene promoter and signal sequence in pMG, was expressed in DH5α. Blue colonies appeared on the XP-containing plates, with or without IPTG, in only the second reading frame (pMG 2.7), as predicted from consideration of the relevant pMG and *bla* sequences. pBla227 transformants, containing the *bla* leader sequence without its promoter, once again appeared only in the second reading frame, but only when IPTG was present, indicating that the Bla-AP fusion protein synthesis was under the control of the pMG *tac* promoter.

Expression of a *B. burgdorferi* OspA-AP fusion protein in *E. coli* DH5α. The *ospA* and *ospB* genes of *B. burgdorferi* encode two abundant proteins with signal peptides charac-

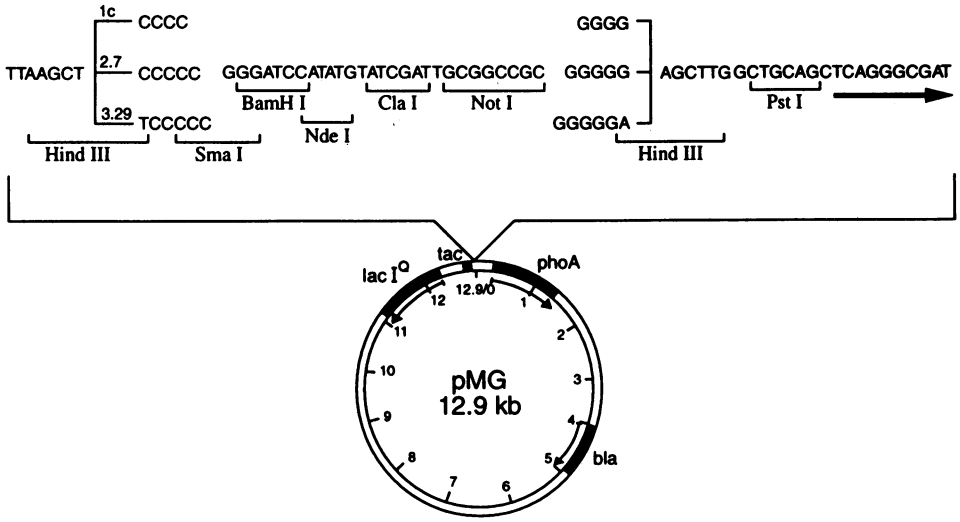

FIG. 1. Nucleotide sequence of the pMG polylinker in the three reading frames (pMG 1.C, pMG 2.7, pMG 3.29), including the region of fusion with the truncated *phoA* gene. The *Hin*dIII sites on both sides of the polylinker were reconstituted after adding the *Sma*I linker, but only in the third reading frame. All restriction sites in the polylinker, except for *Cla*I, are unique in pMG. The arrow represents the direction and sequence of the *phoA* gene.

teristic of prokaryotic lipoproteins (5). The two genes are present on a 49-kb linear plasmid and are transcribed together as one transcriptional unit through a promoter upstream from the *ospA* gene (5, 24). A genomic library of avirulent *B. burgdorferi* DNA was constructed in pMG and transformed into DH5α. One hundred forty blue colonies out of a total of 10,000 transformants (1.4%) appeared on XP-IPTG plates. An *ospA* probe containing the leader sequence of this gene identified one blue colony, which had a 6.8-kb insert of *B. burgdorferi* DNA and was designated pBb340. pBb340 failed to hybridize with an *ospB* 40-mer probe corresponding to nucleotides 982 to 1021 of the *ospA-ospB* unit and containing the *ospB* signal sequence, indicating that *ospB* was not present and therefore that the fusion occurred with *ospA* and not *ospB*. Immunoblot analysis of pBb340 probed with anti-AP monoclonal antibodies or polyclonal anti-B31 *B. burgdorferi* serum identified a 70-kDa fusion protein (data not shown). The 70-kDa fusion protein size is consistent with the molecular mass of the truncated AP, calculated to be 45.9 kDa, together with 24.1 kDa of the OspA portion encoded by 657 bp of DNA. Indeed, an *Mse*I site was present at position 667 of the *ospA* gene (5). In addition, DNA sequence analysis of the *ospA/phoA* junction was performed, and the sequence was shown to be identical to that of *ospA* (data not shown). Like the native protein, the OspA-AP fusion protein was acylated in *E. coli*, as demonstrated by incorporation of [^{3}H]palmitate and [^{3}H]glycerol (data not shown).

Expression of outer membrane protein-AP fusions in *E. coli* DH5α. The *C. trachomatis* major outer membrane protein, MOMP (9), is a surface exposed membrane protein that contains a 22-residue signal peptide which is cleaved after export and before insertion of the protein into the outer membrane (26). OmpA is one of the abundant proteins of the *E. coli* outer membrane. It consists of an amino-terminus membrane region that crosses the outer membrane eight times and a periplasmic carboxyl terminus (48).

pMomp135, containing the leader sequence of *ompl* (the MOMP-encoding gene) and encoding a fusion protein between residue 45 of the MOMP protein and AP, and pOmpA16, containing the leader sequence of *ompA* and encoding a fusion protein between residue 150 of the OmpA protein and AP, were individually transformed into DH5α. Approximately 50% of these outer membrane protein-AP recombinants turned blue in the predicted reading frames of pMG on XP-IPTG-containing plates.

Expression of inner membrane protein-AP fusions in *E. coli* DH5α. The *E. coli* leader peptidase is a 323-residue inner membrane protein responsible for the cleavage of signal peptides from exported proteins with leader peptidase I recognition sites (50). The enzyme has two hydrophobic transmembrane domains, separated by a cytoplasmic domain, and a large periplasmic domain (39). We constructed a leader peptidase-AP fusion, pLpB, at residue 85 of leader peptidase, placing the AP in the periplasmic domain (Fig. 2).

The *E. coli* inner membrane protein, SecY, is essential for translocation of proteins across the cytoplasmic membrane (2, 42). It is embedded in the cytoplasmic membrane by its 10

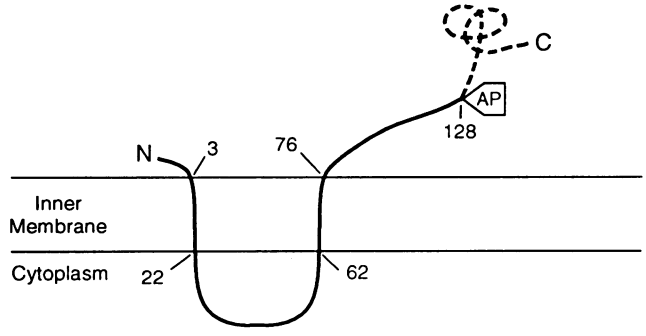

FIG. 2. Topological diagram of the leader peptidase-AP fusion protein pLpB2 (based on reference 38). Numbers indicate amino acid residues. N, amino terminus; C, carboxy terminus. The solid line represents the amino-terminal part of leader peptidase, and the dashed line represents the carboxy-terminal part, which is missing in this fusion protein. It is not known whether the N-terminus part assumes the topology shown.

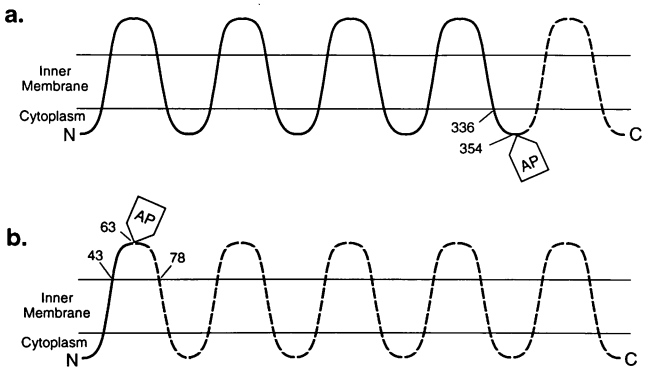

FIG. 3. Topological diagram of the SecY-AP fusion proteins (based on references 1 and 25). (a) pSecY2a; (b) pSecYD2. Numbers represent amino acid residues. It is not known whether the N-terminus part assumes the topology shown.

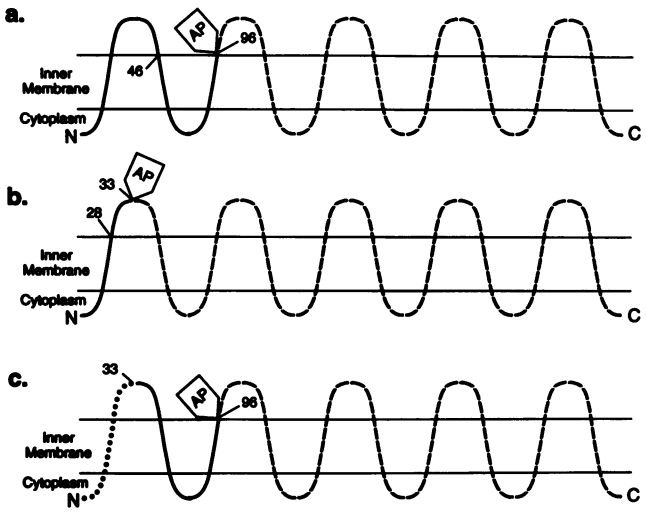

FIG. 4. Topology diagram of the Tc^r-AP fusion proteins (based on reference 22). Numbers represent amino acid residues. The solid lines represents the amino termini (a and b) and the inner membrane segment (c) of the Tc^r protein used for the construction of the fusion proteins. The dashed lines represent the carboxy termini, which are missing in these fusion proteins, and the dots (c) represent the amino terminus of Tc^r, which is missing from this construct. It is not known whether the N-terminus part assumes the topology shown. (a) pTetG; (b) pTetH; (c) pTetJ.

transmembrane segments (1, 25). Two SecY-AP fusion proteins were constructed. On the basis of the known sequence and topology of SecY, the first fusion protein, SecY2a-AP, was predicted to result in fusion of AP to residue 354 of the SecY protein, placing the AP in the cytoplasmic domain (Fig. 3a). The second SecY-AP construct, SecYD2-AP, was predicted to have an AP fusion after residue 63 of the SecY protein, placing the AP in the periplasmic domain (Fig. 3b).

The product of the *E. coli* Tc^r gene is an inner membrane protein that decreases the intracellular accumulation of tetracycline by active efflux of the antibiotic across the cell membrane (34). On the basis of the hydropathic profile of the Tc^r protein, it was predicted to have 12 membrane-spanning alpha-helices (22). Three Tc^r-AP fusions were constructed in pMG. On the basis of the putative topology of the Tc^r protein, the AP portion of the first fusion protein, TetG-AP, was predicted to be located after residue 96, in the second periplasmic domain, between the third and the fourth transmembrane segments (Fig. 4a). The second construct, TetH-AP, was predicted to result in a fusion protein that would place the AP after residue 33, in the first periplasmic domain, between the first and the second transmembrane segments of the Tc^r protein (Fig. 4b). The third fusion protein, TetJ-AP, contained the second and third transmembrane segments (residues 33 to 96) of the Tc^r protein. The location of the AP part of the fusion protein was the same as for TetG; however, TetJ was missing the first transmembrane segment (Fig. 4c).

All the above inner membrane protein-AP fusion recombinants in which the AP was located in the periplasmic space, namely, pLpB, pSecYD2, pTetG, pTetH, and pTetJ, were identified as blue colonies on XP-IPTG-containing plates. In contrast, no blue colonies were identified among the 10,000 SecY2a recombinants (in which the AP portion was situated in the cytoplasm) that were screened in each reading frame.

Behavior of pMG clones expressing functional AP fusion proteins in the blue halo assay. The pMG-based fusions described above, as well as several *T. pallidum*-AP fusions reported recently (7), were used to transform the *degP4*::Tn*5* *lpp-5508 E. coli* strain KS330r$^-$. AP fusion proteins containing signal peptidase I cleavage sites, including the outer membrane proteins MOMP (encoded by pMomp135) and OmpA (pOmpA16), the periplasmic β-lactamase protein (pBla372), and the *T. pallidum* recombinant Tp 9, resulted in blue colonies with blue halos when spotted on plates containing XP and IPTG (Fig. 5). In contrast, no halo formation was observed with AP fusions of inner membrane proteins, including leader peptidase (pLpB), SecY (pSecYD2), and those derived from the Tc^r gene (pTetG, pTetH, and pTetJ). In addition, Tp 70, a *T. pallidum* recombinant containing a predicted hydrophobic spanning domain (7), also failed to generate a blue halo colony. By comparison, fusions between AP and lipoproteins containing signal peptides with leader peptidase II cleavage recognition sites, including OspA (pBb340), Tp 75, and TmpA (see Materials and Methods), gave variable halo results; the halos produced were significantly smaller than those produced by the periplasmic or outer membrane fusion proteins.

pMG library construction with the *B. burgdorferi* 9.0-kb plasmid. We applied the pMG cloning vector approach to a 9.0-kb plasmid isolated from virulent *B. burgdorferi* B31. A similar plasmid, described by Schwan et al. for *B. burgdorferi* Sh-2-82, was lost in parallel to loss of virulence in white-footed mice during in vitro cultivation (41). Similarly, the 9.0-kb plasmid of virulent *B. burgdorferi* B31 was also lost after multiple passages (data not shown). The 9.0-kb plasmid was partially digested with *Mse*I, cloned into *Nde*I-digested pMG in all three reading frames, and transformed into DH5α. Approximately 20,000 recombinant clones were screened on XP-IPTG-containing plates, and 2,000 clones were screened on XP plates without IPTG, yielding approximately 1% blue colonies. Plasmid DNA was individually prepared from the AP-expressing clones and transformed into KS330r$^-$, resulting in blue colonies, some of which showed blue halo formation. One such clone showing a blue halo (Fig. 5), designated pBb244, was chosen for further study. This clone contained an 800-bp insert in pMG 3.29. Nucleic and deduced amino acid sequences revealed a single open reading frame with typical characteristics of a signal peptide: an amino terminus beginning with methionine and

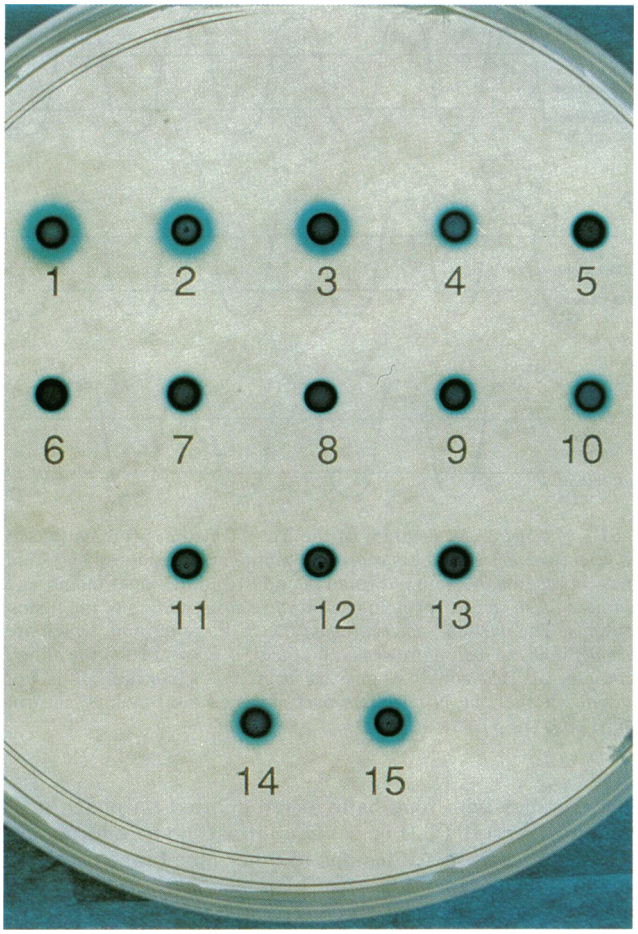

FIG. 5. Blue halo assay for AP fusion recombinants in KS330r$^-$. Concentrated cultures of KS330r$^-$ recombinants were individually spotted onto LB plates containing 100 μg of ampicillin per ml, 1 mM IPTG, and 200 μg of XP per ml. Numbers above the colonies represent the various clones as follows: 1, pMomp135; 2, Tp 9; 3, pBla372; 4, pOmpA16; 5, pTetG; 6, pTetH; 7, pTetJ; 8, pSecYD2; 9, pBb340; 10, TmpA; 11, Tp75; 12, pLpB2; 13, Tp 70; 14, pBb244; 15, pLeader.

a.

```
ATG AGA AAA ATA AGC CTA TTG TTA TTT TTA TTA TTT ATG TTA AGC ATT GAT TTA AGT GCT
met arg lys ile ser leu leu leu phe leu leu phe met leu ser ile asp leu ser ala

TTT ATG
phe met
```

b.

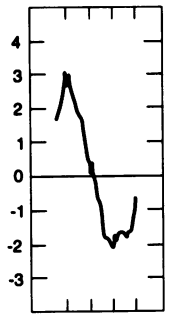

FIG. 6. (a) Nucleotide and deduced amino acid sequence of the amino terminus of pBb244. The putative leader peptidase I recognition site is underlined. (b) Kyte and Doolittle hydropathy analysis of the deduced amino acid sequence of pBb244.

basic charged residues, a hydrophobic core, and a putative leader peptidase I recognition site, Leu-X-Ala (Fig. 6). In addition, a Shine-Dalgarno ribosome binding site was identified upstream from the putative ATG start codon, as well as the −35 and −10 promoter regions, which were similar to the putative *ospA* promoter described by Bergstrom et al. (5) (data not shown).

Leader peptidase I cleavage of pLeader. In order to demonstrate processing of the pBb244 signal peptide by *E. coli*, we generated, by PCR, the signal sequence of pBb244 and subcloned it back into pMG 3.29. After transformation into DH5α and KS330r$^-$, the cells were plated in the presence of XP and IPTG as previously described. As with pBb244, transformation with pLeader resulted in blue colonies in DH5α and blue halo formation in KS330r$^-$ (Fig. 5), indicating the presence of enzymatic *phoA* activity in the periplasm.

DISCUSSION

The use of *phoA* as a reporter gene has been a major part of molecular biology relating to the understanding of membrane proteins (32). AP fusion hybrids have been utilized in two types of systems. The first is Tn*phoA* (1), which has been used for identification of bacterial virulence genes (14, 27, 35, 46), identification of genes encoding membrane-associated proteins (8, 17, 28), and analysis of membrane protein topology (31). However, there are several limitations to this approach. (i) Tn*phoA* can be applied only to microorganisms compatible with this transposon. (ii) Expression of the fusion protein is dependent upon transcription from the promoter of the target gene (8, 31). (iii) There is an inability to identify membrane-associated proteins if insertional inactivation of these genes results in lethality.

A second AP fusion system has been utilized in a series of plasmid vectors. These plasmids have been constructed to allow fusions between the target genes and *phoA* by using predefined endpoints in both *phoA* and the gene under study (11, 23, 36, 47). However, they were not designed for a random cloning approach, nor were they equipped with the elements necessary for versatile cloning and expression of foreign DNA.

The pMG plasmid vector was designed to overcome the disadvantages of the AP fusion systems mentioned above. We have created in vitro AP fusions in pMG and successfully cloned and expressed fusion genes derived from *E. coli*, *B. burgdorferi*, *T. pallidum*, and *C. trachomatis* as well as those from *Leptospira interrogans* (18). These AP fusions were created with the amino termini of proteins from different categories: those having leader peptidase I recognition sites, lipoproteins with leader peptidase II recognition sites, and cytoplasmic membrane proteins. As we demonstrated with the β-lactamase–AP fusions, the pMG fusion protein synthesis is under the control of an inducible *tac* promoter and therefore might allow expression of proteins which are under tight regulation in their original host. Also, pMG minimizes concerns about potential toxicity from cloned bacterial outer membrane proteins, as has been shown in the cases of OmpA (15) and gonococcal protein I (10). This is due to the need to clone only a small fragment of the amino terminus of a gene to obtain a hybrid protein with enzymat-

ically active AP. Indeed, we have found that 14 and 19 residues of the amino terminus were sufficient to produce blue colonies in two *B. burgdorferi* AP fusions that have been sequenced (data not shown). Furthermore, pMG should identify essential virulence genes, membrane proteins, for example, whose inactivation by Tn*phoA* might lead to lethality. To avoid introduction of any potential bias against cloning genes on the basis of the use of restriction enzymes, sonicated repaired DNA can be alternatively used.

The recent work of Strauch and Beckwith (44) describing *E. coli* KS330 has provided us with an approach to further classify *phoA* positive recombinants in pMG into three groups. The first group comprises fusion proteins that were exported beyond the cytoplasmic membrane, namely the periplasmic β-lactamase and the outer membrane proteins MOMP and OmpA. Tp 9, having a cleavable signal peptide with a leader peptidase I recognition site, also belongs to this group. The leaky membrane of KS330r$^-$ has resulted in the diffusion of these fusion proteins beyond the outer membrane with AP activity appearing as blue halos around the colonies. Proteins that are secreted beyond the cell envelope (e.g., toxins) should also be included in this group, although we did not provide an example of such a protein in this study.

The second group consists of inner membrane protein-AP fusions. The *degP4* mutation in KS330r$^-$ stabilizes the inner membrane protein-AP fusions and protects them from proteolysis (44). Such fusion hybrid proteins therefore remained anchored to the cytoplasmic membrane, and the resulting colonies appeared blue but without halo formation. The leader peptidase-AP, SecY-AP, Tc^r-AP, and Tp 70 fusion proteins, all of which did not generate blue halos, belong to this category. As with Tn*phoA*, colonies turned blue only when the AP was located in the periplasm, whereas only white colonies were observed with clone SecY-2a, in which the AP was facing the cytoplasm. While blue halos were not observed from this set of inner membrane protein-*phoA* fusions, it is important to note that certain inner membrane AP fusions could undergo proteolytic cleavage even in a *degP4* background. In addition, although it is conceivable that certain inner membrane proteins may be targets of leader peptidase I, this is extremely rare.

Lipoproteins having leader peptidase II cleavage sites represent a third group of membrane proteins. These proteins are anchored to either inner or outer membranes of gram-negative bacteria by their lipid moieties (21, 49). pMG recombinants of such proteins in DH5α resulted in blue colonies. This was demonstrated by the *B. burgdorferi* OspA-AP fusion in this study and the *T. pallidum* lipoprotein-AP fusions Tp 75 and TmpA-AP, which were described recently by Blanco et al. (7). However, cloning of these fusion proteins into KS330r$^-$ gave variable results in the blue halo assay. The OspA-AP, Tp 75, and TmpA-AP fusions produced blue colonies with halos. Although the small halos produced by these fusion proteins were distinguishable from the larger halos of the periplasmic and outer membrane fusion proteins, other lipoprotein-AP fusions should be studied before definitive conclusions can be made. Potential difficulty in categorizing lipoproteins into a halo or non-halo group can be overcome, however, by identifying the lipoproteins by tritiated fatty acid intrinsic radiolabelling, as demonstrated in this study and the study by Blanco et al. (7).

The identification of the *B. burgdorferi* clone pBb244 as a blue colony with a blue halo serves as an example of the application of this system to an organism not amenable to Tn*phoA* mutagenesis. Because the pBb244 gene was cloned from a *B. burgdorferi* plasmid which has been correlated with virulence (41), the presence of a leader peptidase I recognition site makes pBb244 a potential virulence factor candidate. It is important to note that the leader peptidase I cleavage site of pBb244 is identical to that recently reported for an outer membrane protein of the pathogenic spirochete *Leptospira alstoni* (19).

The pMG/KS330r$^-$ genetic approach can identify the amino termini of genes encoding exported proteins and distinguish this group of proteins from cytoplasmic proteins; this approach can also distinguish these proteins from lipoproteins, if fatty acid radiolabelling is used. This system can serve as a first step in the identification of genes encoding virulence factors in various pathogens, particularly those not amenable to Tn*phoA* mutagenesis.

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