INFECTION AND IMMUNITY, Jan. 1984, p. 108–114
0019-9567/84/010108-07$02.00/0

Vol. 43, No. 1

Characterization of Common Virulence Plasmids in *Yersinia* Species and Their Role in the Expression of Outer Membrane Proteins

DANIEL A. PORTNOY,[1]† HANS WOLF-WATZ,[2] INGRID BOLIN,[2] ANN B. BEEDER,[1] AND STANLEY FALKOW[1]*

Department of Medical Microbiology, School of Medicine, Stanford University, Stanford, California 94305[1]; and National Defense Research Institute S-901 82 Umeå, Sweden[2]

Received 9 June 1983/Accepted 10 October 1983

The virulence plasmids pYV019, pYV8081, and pIB1 from *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*, respectively, were characterized by restriction endonuclease analysis. The three plasmids exhibited a region of common DNA previously shown to encode determinants which confer Ca^{2+} dependence. The plasmids from *Y. pestis* and *Y. pseudotuberculosis* were similar throughout their genomes. In contrast, a region of the plasmid from *Y. enterocolitica* which contained an origin of replication differed from the other two plasmids as determined by DNA homology and replication properties. Plasmid-associated outer membrane proteins from all three species of *Yersinia* were characterized by polyacrylamide gel electrophoresis. There were no differences in the outer membrane protein profiles between plasmid-containing and homogenic strains lacking the plasmid after growth at 28°C. After growth at 37°C, both *Y. enterocolitica* and *Y. pseudotuberculosis* showed at least four major plasmid-associated outer membrane proteins. *Y. pestis* did not show any discernible changes after growth at 37°C. It was shown by using *E. coli* minicell analysis that the plasmid DNA from all three species of *Yersinia* contained the coding capacity for production of the novel outer membrane proteins.

The genus *Yersinia* is composed of three pathogenic species: *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis*, all of which are capable of causing invasive disease in humans and other mammals (12). *Y. pestis* and *Y. pseudotuberculosis* show 90% DNA sequence homology, whereas both share about 50% DNA sequence homology with *Y. enterocolitica* (6, 10). All three bacterial species have been shown to harbor a related plasmid that is associated with virulence and Ca^{2+}-dependent in vitro growth at 37°C (Ca^{2+} dependence) (13–15, 28, 29, 32). We have shown that transfer of a 72-kilobase (kb) plasmid from *Y. pestis* by P1 transduction to a plasmid-free, avirulent strain of *Y. pestis* restores the strain to full virulence and Ca^{2+} dependence (27). Furthermore, Tn*5* insertions in appropriate regions of the plasmid abolish both Ca^{2+} dependence and mouse virulence of the *Y. pestis* strain. Therefore, Ca^{2+} dependence is a virulence-associated phenomenon, although its role in pathogenesis is unknown.

All three pathogenic species of *Yersinia* express a number of plasmid-associated polypeptides at 37°C. One of these, the V antigen, has recently been shown to be plasmid encoded (27). Previous reports have described the outer membrane protein profile of *Yersinia* with and without the virulence plasmid and during growth at different temperatures. Portnoy et al. (29) reported that *Y. enterocolitica* expresses at least three novel plasmid-associated polypeptides during growth in broth at 37°C. Straley and Brubaker (31) reported similar results for *Y. enterocolitica* and *Y. pseudotuberculosis* but reported that *Y. pestis* had no plasmid-associated differences in their outer membrane proteins. Bölin et al. (7) have recently demonstrated that *Y. pseudotuberculosis* and *Y. enterocolitica* possess a plasmid-associated outer membrane protein of approximately 140,000 daltons, which they termed protein 1. However, they could not reproduce the results of other reports which described a number of lower-molecular-weight plasmid-associated outer membrane proteins (29, 31). Although the precise role of the outer membrane proteins is unknown, they may confer serum resistance upon the bacteria (23, 25).

In this report, we compare the plasmid DNA from the three species of *Yersinia*. We show that the three plasmids have the coding capacity for expression of the novel 37°C-specific outer membrane proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in these studies are listed in Table 1. *Yersinia* species were grown on brain heart infusion agar and broth (Difco Laboratories, Detroit, Mich.) at 28°C. *Escherichia coli* strains were grown on brain heart infusion agar or L agar and broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride) (Difco Laboratories). Stock cultures were kept as suspensions of cells at −20°C in 50% glycerol–50% L broth.

Isolation of plasmid DNA, restriction endonuclease digestion, and agarose gel electrophoresis. Plasmid DNA was isolated from *Yersinia* species as previously described (28). The restriction endonucleases *Bam*HI, *Sal*I, *Xba*I, and *Bgl*II were used under conditions recommended by the supplier (Bethesda Research Laboratories, Rockville, Md.). Approximately 0.2 μg of restricted plasmid DNA was subjected to electrophoresis in either a horizontal 0.4-cm Tris-acetate-buffered 0.7% agarose gel (29) for 17 h at 50 V or alternatively, a vertical 0.25-cm Tris-borate-buffered 0.7% agarose gel (24) for 2 h at 100 V.

Restriction endonuclease mapping of plasmid DNA. Plasmid DNA from *Y. pestis* O19(pYV019) was previously mapped (28). Plasmid DNA from *Y. enterocolitica* 8081(pYV8081) was mapped by the following strategy: *Sal*I and *Xba*I sites were mapped relative to one another by the analysis of single and double digestion patterns. To locate *Bam*HI restriction sites, fragments produced by *Sal*I-*Xba*I

* Corresponding author.
† Present address: Department of Cellular Physiology and Immunology, Rockefeller University, New York, NY 10021.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant properties	Reference
Y. enterocolitica 8081 (pYV8081)	Virulent, Ca^{2+} dependent	29
Y. enterocolitica 8081-c	Avirulent, Ca^{2+} independent	29
Y. pseudotuberculosis YPIII(pIB1)	Virulent, Ca^{2+} dependent	7
Y. pseudotuberculosis YPIII	Avirulent, Ca^{2+} independent	7
Y. pestis EV76(pYV019)	Avirulent (*pgm*), Ca^{2+} dependent	28
Y. pestis EV76-6	Ca^{2+} independent	28
E. coli LE392	Restriction minus (*hsdR*)	27
E. coli SF800	DNA polymerase minus (*pol A*)	16
E. coli P678-54	Minicell-producing *E. coli*	3
pYV8081::Tn*5*		This study
pIB1::Tn*5*		8
pYV019::Tn*5*		27

double digestion were isolated from an agarose gel by electroelution and subsequently digested with *Bam*HI. Mapping was also facilitated by the analysis of *Bam*HI-*Bgl*II double digests. Plasmid DNA from *Y. pseudotuberculosis* YPIII(pIB1) was mapped by the analysis of overlapping cosmid clones. The details are reported elsewhere (8). The three restriction maps are shown in Fig. 1.

Transformation of plasmid DNA. *E. coli* strains were transformed with plasmid DNA by the method of Brown et al. (11).

Localization of plasmid origin of replication. The probable location of the origin of replication from pYV019 has been described previously (28).

*Bam*HI restriction fragments 1 to 9 from pYV8081 were individually cloned into plasmid pBR325 (9), using T4 DNA ligase purchased from New England Biolabs, Beverly, Mass. Plasmid pBR325 was derived from ColE1 and requires DNA polymerase I for replication (20). Plasmid DNA from chloramphenicol- and ampicillin-resistant, tetracycline-sensitive clones was analyzed by digestion with *Bam*HI. Only recombinant plasmids containing *Bam*HI restriction endonuclease fragment 3 (*Bam*HI-3) were capable of transforming *E. coli* SF800 (*polA*) to chloramphenicol resistance, and these colonies appeared only after incubation at 28°C and not 37°C. Therefore, it was concluded that *Bam*HI-3 must contain sufficient sequences to permit replication in a *polA* host. The *Bam*HI-3 was subcloned, and the putative origin of replication was localized to a 4-kb *Bam*HI-*Sal*I restriction fragment.

*Bam*HI restriction fragments from pIB1 have been cloned into pBR322 (8). By using the same strategy as described above, an origin of replication was localized to *Bam*HI-4.

Introduction of Tn*5* into *Yersinia* plasmids. The introduction of Tn*5* into pYV019 and characterization of the insertion mutants have been described previously (27). The particular Tn*5* insertion used in this study is adjacent to the Ca^{2+} dependence locus within *Bam*HI-1 and causes no loss of virulence in *Y. pestis* strains harboring this plasmid. The introduction of Tn*5* into pIB1 is described elsewhere (8). The particular Tn*5* insertion used in this study resides within *Bam*HI-10 and has no effect on Ca^{2+} dependence or the virulence of *Y. pseudotuberculosis*.

Cotransformation (21) was used to introduce pYV8081

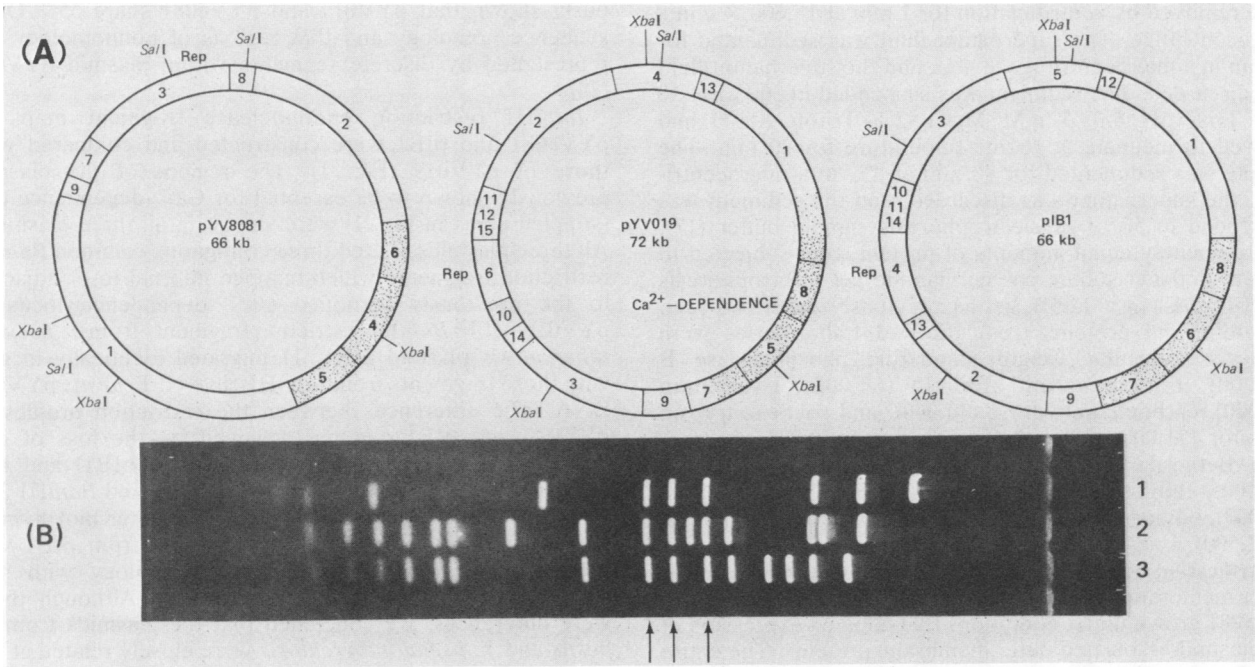

FIG. 1. (A) Restriction fragment maps from *Yersinia* virulence plasmids. The sizes of *Bam*HI restriction fragments (in kilobases) are: (pYV8081, fragments 1 to 9, respectively) 24.5, 13.7, 9.8, 5.3, 4.4, 4.0, 2.5, 1.1, and 0.7; (pYV019, fragments 1 to 15, respectively) 14.5, 10.7, 9.7, 5.8, 5.3, 4.9, 4.4, 4.0, 2.9, 2.2, 1.7, 1.6, 1.3, 1.1, and 0.9; (pIB1, fragments 1 to 14, respectively) 12.0, 9.7, 7.8, 6.6, 5.8, 5.3, 4.4, 4.0, 2.9, 1.7, 1.6, 1.3, 1.1, and 0.9. The stippled area of pYV019 represents the Ca^{2+} dependence locus (27). The stippled areas in pYV8081 and pIB1 refer to three common *Bam*HI fragments in the Ca^{2+} dependence locus. (B) *Bam*HI restriction endonuclease digestion patterns of plasmid DNA. Lane 1, pYV8081; lane 2, pYV019; lane 3, pIB1. Electrophoresis was for 17 h at 50 V in Tris-acetate buffer (29). Arrows point out three common *Bam*HI restriction fragments.

into *E. coli* LE392. The cotransforming plasmid was pMR5, a temperature-sensitive derivative of RP1 which encodes resistance to ampicillin. kanamycin, and tetracycline (30). A 3-ng amount of pMR5 DNA was mixed with 1 μg of pYV8081 DNA, transformed into *E. coli* LE392, and plated at 28°C on L agar supplemented with kanamycin. Kanamycin-resistant colonies (100) were transferred to a nitrocellulose filter, and the colonies containing pYV8081 were identified by a DNA-colony hybridization technique (18), using ^{32}P-labeled pYV8081 DNA as a probe. Hybridization-positive colonies (3/100) were shown to contain pMR5 and pYV8081 by agarose gel electrophoresis. After passage at 42°C, so that 50% of the cells had lost pMR5, 30% of the kanamycin-sensitive cells still retained pYV8081. *E. coli* LE392(pYV8081) was infected with bacteriophage P1::Tn*5* (22) at 28°C. Plasmid DNA was isolated from the lawn of cells that arose on L agar supplemented with kanamycin and used to transform *E. coli* LE392 to kanamycin resistance. Eight kanamycin-resistant colonies were all shown to contain pYV8081::Tn*5* by analysis of *Bam*HI restriction digestion patterns of the plasmid DNA.

Isolation of bacterial outer membranes and SDS-PAGE. Whole membranes and Triton X-100-insoluble outer membranes were isolated by a modification of the method of Achtman et al. (1). A 10-ml volume of the bacterial cultures was grown at 28 and 37°C to stationary phase in a medium consisting of 1% tryptone, 0.5% yeast extract, 0.02% sodium oxalate, and Higuchi salts, pH 7.2 (19). The tryptone, yeast extract, and sodium oxalate were mixed together as a 10× stock, boiled, and filter sterilized. This medium was optimal for the expression of Ca^{2+} dependence. The cultures were subjected to centrifugation and the bacterial sediment was suspended in 1.5 ml of 10 mM Tris–5 mM $MgCl_2$, pH 7.5. The bacteria were lysed by sonication, and unbroken cells were removed by centrifugation for 1 min at 15,600 × *g* in a microcentrifuge. The supernatant fluid was sedimented for 30 min in a microcentrifuge at 4°C, and the supernatant fluid was discarded. The sediment was suspended in 300 μl of 10 mM Tris (pH 8.0)–5 mM $MgCl_2$–2% Triton X-100 and allowed to incubate at room temperature for 20 min. The sample was sedimented for 30 min at 4°C in a microcentrifuge, the supernatant was discarded, and the sediment was suspended in 50 μl of electrophoresis sample buffer (17). Approximately equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% gel as previously described (29). The following proteins from Bio-Rad Laboratories were used as molecular weight standards: phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (21,000); other molecular weight standards were from Bethesda Research Laboratories: beta-lactoglobulin (18,400), alpha-chymotrypsinogen (25,700), ovalbumin (43,000), bovine serum albumin (68,000), and phosphorylase b (92,500).

Purification of the 24,000-dalton outer membrane protein. Outer membranes were isolated from 200 ml of *Y. enterocolitica* 8081 grown under conditions that allowed expression of the plasmid-associated outer membrane proteins. The entire sample was applied to a preparative SDS-PAGE gel. After electrophoresis, the gel was stained with Coomassie blue G for 2 min and immediately rinsed in water, at which time the 24,000-dalton polypeptide was visible. The stained band was excised from the gel and removed by electroelution into dialysis tubing. The sample was dialyzed against distilled water. The amount of protein was determined by using the Bio-Rad protein assay kit, with bovine serum albumin as a standard.

Minicell analysis of outer membrane proteins. The Tn*5*-labeled *Yersinia* plasmids were transformed into the minicell-producing *E. coli* strain P678-54 (3). Labeling conditions and sample preparation have been described before (17), with the modification that strains harboring pYV8081 were grown at 28°C. Approximately 100,000 cpm of acetone-precipitable material was applied to a 12.5% SDS-polyacrylamide gel. The gel was stained and examined by fluorography, using En^{3}Hance (New England Nuclear Corp., Boston, Mass.).

Immunoprecipitations were performed by lysing the minicells in a boiling 1% SDS solution for 5 min. This was followed by mixing of the sample with an antiserum directed against 37°C-grown virulent *Y. enterocolitica* 9576 (29) and protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). After incubation for 2 h, the sedimented sample was washed, mixed, with electrophoresis sample buffer, boiled, and applied to the gel. For competition experiments, 2 μg of the purified 24,000-dalton outer membrane protein was added to the sample along with antiserum and protein A. The antiserum was a generous gift from R. J. Martinez (23).

RESULTS

Comparison of plasmid restriction maps from *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. The three pathogenic species of the genus *Yersinia*, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, each harbor plasmids of similar size. These plasmids are associated with Ca^{2+} dependence and virulence (7, 13–15, 29, 32) and are represented by pYV019, pYV8081, and pIB1, respectively. We have previously shown that pYV019 and pYV8081 share 55% DNA sequence homology and that regions of nonhomology are represented by discrete segments within plasmid pYV019 (28).

*Bam*HI restriction endonuclease fragment maps of pYV8081 and pIB1 were constructed and compared with those of pYV019 (Fig. 1). The regions of the plasmid previously shown to be essential for Ca^{2+} dependence (27) (stippled areas in Fig. 1) were similar in all three plasmids. All three plasmids shared three contiguous common *Bam*HI restriction fragments which mapped internal to or adjacent to the previously identified Ca^{2+} dependence locus of pYV019. Of 14 *Bam*HI restriction fragments from *Y. pseudotuberculosis* plasmid pIB1, 11 migrated identically in gels with those fragments from *Bam*HI-cleaved *Y. pestis* pYV019 DNA. The differences between the restriction profiles of pYV019 and pIB1 can be explained by the loss of one *Bam*HI site in pIB1 (within *Bam*HI-4 of pIB1) and two insertions within pYV019 (within *Bam*HI-1 and *Bam*HI-2 of pYV019). Moreover, using nitrocellulose filter blot hybridization, each *Bam*HI restriction fragment from pIB1 was shown to contain significant DNA homology with ^{32}P-labeled pYV019 DNA (data not shown). Although there were differences, we concluded that the plasmids from *Y. pestis* and *Y. pseudotuberculosis* were closely related at the molecular level.

Plasmid pYV8081 from *Y. enterocolitica* shared three of nine *Bam*HI restriction fragments with the other two plasmids. The three common bands encompassed DNA sequences on the plasmid previously shown to be associated with Ca^{2+} dependence (27). Although it is not possible to determine the extent of DNA sequence homology between

any two plasmid DNA molecules based on a comparison of restriction endonuclease cleavage patterns, the fact that plasmid pYV8081 from *Y. enterocolitica* only shares three common bands with the other two plasmids was consistent with the observed 55% DNA sequence homology between pYV019 and pYV8081 (28).

Previously, we localized the origin of replication of plasmid pYV019 to *Bam*HI-6 DNA by analysis of spontaneous Ca^{2+}-independent deletion mutants of *Y. pestis* EV76 (28). We located the analogous region within pYV8081 and pIB1 by constructing recombinant plasmids between *Bam*HI restriction fragments from the *Yersinia* plasmids with *Bam*HI-cleaved cloning vehicles pBR325 and pBR322 (9). These cloning vehicles were derived from plasmid ColE1, which requires DNA polymerase 1 for replication (20). The recombinant plasmids were transformed into *E. coli* SF800 (16), and the DNA fragment of pYV8081 which would allow propagation of the cloning vehicle within the *polA* host was localized to a 4-kb *Bam*HI-*Sal*I restriction fragment within *Bam*HI-3. By using nitrocellulose filter hybridization, it was shown that *Bam*HI-3 from pYV8081 shared little or no DNA homology with the *Y. pestis* plasmid pYV019 (data not shown). Also, *Bam*HI-6, which contains an origin of replication from the *Y. pestis* plasmid pYV019, shares little DNA homology with pYV8081 (28). Therefore, it appeared that those regions of the plasmids from *Y. pestis* and *Y. enterocolitica* which contained an origin of replication were dissimilar. An origin of replication of pIB1 was located within the 6.6-kb *Bam*HI-4 (Fig. 1). As expected, this appeared to map to the same site as the origin of replication from the *Y. pestis* plasmid.

E. coli SF800 harboring recombinant clones containing the origin of replication from the *Y. enterocolitica* plasmid could form colonies on antibiotic-containing media at 28°C but not at 37°C. *E. coli* SF800 harboring recombinant clones containing the origin of replication from the *Y. pseudotuberculosis* plasmid grew well at both temperatures on antibiotic-containing media. Thus, the observed difference in the DNA sequence of the origins of plasmid replication was further reflected by their replication properties at different temperatures. However, these data must be interpreted with caution since it is possible that the plasmids use other sequences as their origin of replication within the normal host bacterium.

Plasmid-associated expression of outer membrane proteins. Reports concerning the plasmid-associated expression of outer membrane proteins in various *Yersinia* species have not been uniform (7, 29, 31). Consequently, we directly compared the outer membrane proteins of all three species with and without the plasmid after growth at 28 and 37°C (Fig. 2). Both of the plasmid-containing strains of *Y. enterocolitica* and *Y. pseudotuberculosis* expressed a high-molecular-weight protein (protein 1 [7]) and at least three additional outer membrane polypeptides as compared with the plasmid-free strains or the plasmid-containing strains grown at 28°C. In contrast, *Y. pestis* showed no apparent plasmid-associated changes in its outer membrane protein profile after growth at either temperature.

The appearance of plasmid-associated outer membrane polypeptides in *Y. enterocolitica* and *Y. pseudotuberculosis* was growth medium dependent as well as temperature dependent. Protein 1 was expressed in all media tested. The lower-molecular-weight outer membrane polypeptides were expressed in brain heart infusion broth and in an oxalated tryptone broth (see above) but were absent from cultures grown in a minimal medium E plus Casamino Acids (7), L broth, and in tryptone soya broth (data not shown). Therefore, the difference in plasmid-mediated protein expression reported previously probably reflects differences in growth media. Although we have not identified the specific growth requirement(s) for expression of the outer membrane proteins, it may be related to Ca^{2+} dependence because Ca^{2+} added to brain heart infusion prevents expression of the outer membrane proteins (29), and they are fully expressed in an oxalated medium which is optimal for the expression of Ca^{2+} dependence. It is clear that *Y. enterocolitica* and *Y. pseudotuberculosis* have the potential to dramatically alter their outer membrane profiles in response to growth at 37°C. Furthermore, all but one of the four plasmid-associated outer membrane proteins from *Y. enterocolitica* and *Y. pseudotuberculosis* appear to be identical in molecular mass (Fig. 2).

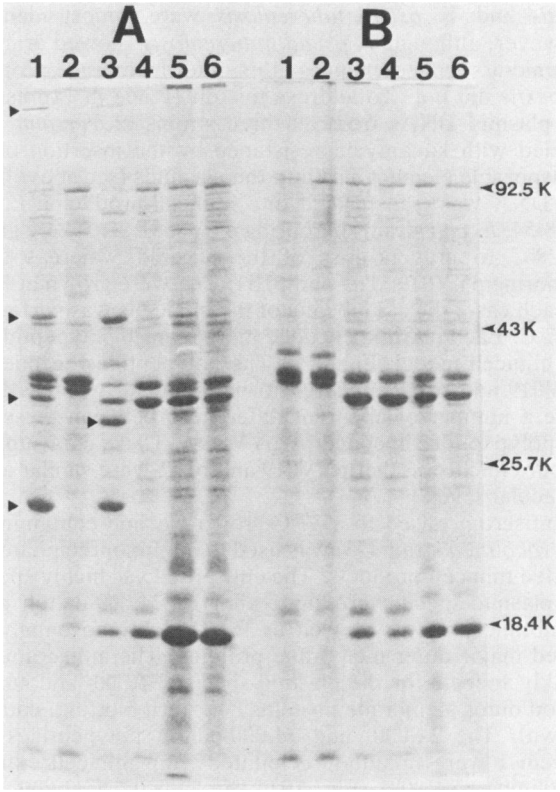

FIG. 2. SDS-PAGE of *Yersinia* outer membrane polypeptides. (A) *Yersinia* strains grown at 37°C; (B) strains grown at 28°C. Lanes: 1, *Y. enterocolitica* 8081; 2, *Y. enterocolitica* 8081-c; 3, *Y. pseudotuberculosis* YPIII(pIB1); 4, *Y. pseudotuberculosis* YPIII; 5, *Y. pestis* EV76; 6, *Y. pestis* EV76-6. The arrowheads designate the major plasmid-associated outer membrane proteins.

The strains of *Y. enterocolitica* and *Y. pseudotuberculosis* used in these studies were fully virulent (7, 29), whereas the *Y. pestis* strain, because of a pigmentation mutation, was not (27). Therefore, to exclude the possibility that the pigmentation mutation which affects virulence had an effect on the expression of plasmid-associated outer membrane proteins, we analyzed the outer membrane protein profile of *Y. pestis* 195-P, a fully virulent strain (27). The fully virulent strain *Y. pestis* 195-P, like *Y. pestis* EV76, showed no evidence of plasmid-associated outer membrane proteins (data not shown).

Minicell analysis of plasmid-encoded outer membrane proteins. By the criteria we applied, the plasmid DNAs from *Y.*

pestis and *Y. pseudotuberculosis* were almost identical. However, although *Y. pseudotuberculosis* showed dramatic plasmid-associated changes in its outer membrane proteins, *Y. pestis* did not. To address this difference in expression, the plasmid DNAs from all three strains of *Yersinia* were labeled with kanamycin resistance by the insertion of the transposable element Tn*5* into the plasmids (see above), and the DNA was transformed into minicell-producing *E. coli* P678-54. *E. coli* strains harboring pYV8081::Tn*5* were grown at 28°C to prevent loss of the plasmid, whereas those harboring pYV019::Tn*5* and pIB1::Tn*5* were grown at 37°C. In each case, the ^{35}S labeling of the minicells was performed at 37°C. Each plasmid encoded more than 20 polypeptides in the minicell model (Fig. 3A). The polypeptides encoded by pYV019 and pIB1 appeared to be identical, whereas there were a number of apparent differences between these and the polypeptides encoded by pYV8081. These data supported our conclusion that pYV019 and pIB1 were similar at the molecular level.

Antiserum raised to a 37°C-grown plasmid-containing *Y. enterocolitica* strain (23) was used to immunoprecipitate ^{35}S-labeled minicell products. The antiserum was highly specific for plasmid-specific protein 1 and the 24,000-dalton outer membrane protein, as well as for the chromosomally encoded major outer membrane proteins. The antiserum was weakly specific for the plasmid-specific 34,000- and 46,000-dalton outer membrane proteins (Western blotting, data not shown). The 24,000- and 34,000-dalton polypeptides and protein 1 were immunoprecipitated from minicell extracts containing pYV8081 (Fig. 3B). The 24,000-, a 32,000-, and the 46,000-dalton polypeptides were immunoprecipitated from minicells containing pYV019 or pIB1. To show that the 24,000-dalton polypeptide recognized by the antiserum in all plasmid-containing minicell extracts was the same polypeptide made in *Y. enterocolitica*, purified unlabeled protein was added to the immunoprecipitation. The protein had been isolated from a preparative SDS-polyacrylamide gel. The results (Fig. 3C) showed that the purified 24,000-dalton protein competed with the minicell extract for antibody, thus indicating its identity with the 24,000-dalton outer membrane protein from *Y. enterocolitica*. These data indicated that the plasmids from *Y. pestis* and *Y. pseudotuberculosis* had the coding potential for expression of outer membrane proteins immunologically related to those produced by *Y. enterocolitica*. Therefore, although *Y. pestis* does not appear to express these proteins in its outer membrane, the plasmid isolated from *Y. pestis* does have the coding capacity to do so under appropriate but undefined conditions in *E. coli*.

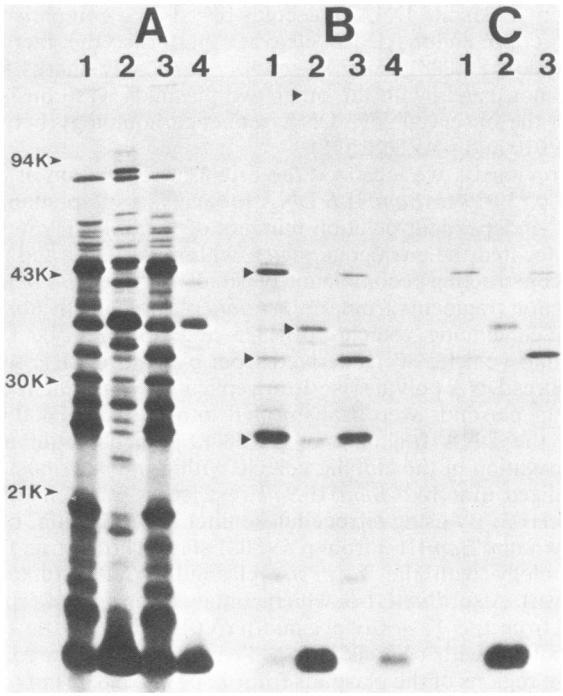

FIG. 3. Detection of outer membrane proteins in *E. coli* minicells. (A) Autoradiograph of ^{35}S-labeled products; (B) immunoprecipitate of same samples with antiserum directed against surface of *Y. enterocolitica* 9576 grown at 37°C; (C) identical to B, except that 2 μg of purified 24,000-dalton outer membrane protein from *Y. enterocolitica* was included in the reaction. The arrowheads designate the plasmid-associated outer membrane proteins which are also shown in Fig. 2. Lanes: 1, pYV019::Tn*5*; 2, pYV8081:Tn*5*; 3, pIB1::Tn*5*; 4, minicells alone.

DISCUSSION

The results of this study show that there is a common virulence plasmid in yersiniae which mediates Ca^{2+} dependence and encodes a number of outer membrane proteins. The plasmids from *Y. pestis* and *Y. pseudotuberculosis* appear to be extremely similar. The plasmid from *Y. enterocolitica* is similar to the others in the region concerned with Ca^{2+} dependence, yet it appears to be significantly different in a region of the plasmid necessary for maintenance and other essential plasmid functions. This hypothesis is based on DNA homology studies (28) and on the stability of the plasmid at 37°C in *E. coli* K-12. Plasmid pYV8081::Tn*5* from *Y. enterocolitica* was rapidly lost at 37°C, whereas pYV019::Tn*5* from *Y. pestis* and pIB1::Tn*5* from *Y. pseudotuberculosis* were stable even after repeated passage at 37°C. This observation may also explain the high rate of plasmid curing (about 1%) (5) in *Y. enterocolitica*. This is in contrast to *Y. pestis*, where the plasmid is lost at a frequency of less than 10^{-4} per cell per generation (19, 29). The observed difference in the replication regions from the different plasmids is consistent with the hypothesis that there was a recombinational event between two ancestral plasmids, one encompassing the replication region and another encompassing the Ca^{2+} dependence region.

The three *Yersinia* species all possess plasmids with the coding capacity for the expression of unique outer membrane proteins at 37°C under calcium limitation, yet *Y. pestis* does not express the proteins under similar growth conditions. *Y. pestis* and *Y. pseudotuberculosis* share about 90% overall DNA sequence homology, whereas each only shares about 50% homology with *Y. enterocolitica* (6, 10). Thus, although the plasmids from *Y. pestis* and *Y. pseudotuberculosis* are similar and both species are highly related, *Y. pestis* fails to express the unique plasmid-encoded outer membrane proteins. The expression of the outer membrane proteins in *Y. enterocolitica* and *Y. pseudotuberculosis* is growth medium dependent. Several explanations could exist for these differences in expression. There could be some unknown factor in the growth medium which represses the expression of the outer membrane proteins in *Y. pestis*, or an essential factor could be lacking in the growth medium. Alternatively, the lack of expression by *Y. pestis* may reflect a difference in cellular physiology. For example, a different outer membrane composition might exist. R. Darveau and W. Charnetsky have recently shown (personal communication) that *Y. pestis* alters its LPS composition at different

temperatures. There is also evidence that *Y. enterocolitica* alters its lipopolysaccharide composition with temperature (2; Portnoy and Darveau, unpublished data). Therefore, the expression of the outer membrane proteins may be dependent on a specific outer membrane environment which is not present in *Y. pestis*, at least in vitro. This might also account for the fact that these outer membrane proteins are expressed only at 37°C in *Y. enterocolitica* and *Y. pseudotuberculosis*. This hypothesis is consistent with an observation of Beher and Schnaitman that the OmpA protein was not expressed in certain LPS mutants of *E. coli* K-12 (4).

Pai and DeStephano have recently shown that a virulent strain of *Y. enterocolitica* grown at 37°C but not at 25°C was resistant to the bactericidal action of normal human serum (25). Martinez has corroborated this finding and further correlated serum resistance with the presence of the outer membrane proteins (23). Furthermore, Martinez has shown that all of the plasmid-associated outer membranes are externally exposed in *Y. enterocolitica* and react specifically with human serum from a patient convalescing from yersiniosis. Together, these data suggest that these proteins are made by *Y. enterocolitica* in vivo and afford the bacteria altered surface properties which provide them with resistance to the bactericidal activity of normal human serum and possibly other host defense mechanisms. It is unknown whether *Y. pestis* expresses the plasmid-encoded outer membrane proteins in vivo; if not, this may be consistent with the fact that *Y. pestis* is constitutively serum resistant (26).

It is clear that a family of plasmids is essential for the pathogenicity of *Yersinia* species. Although it has been recognized for some time that Ca^{2+} dependence is associated with virulence (12), only recently has it been genetically shown that Ca^{2+} dependence is essential for virulence (27), although its precise role remains unknown. A role for the plasmid-encoded outer membrane proteins and the V antigen awaits isolation of specific mutations in the various genes, followed by testing these strains for virulence in an appropriate animal model.

ACKNOWLEDGMENTS

We thank Rafael Martinez for anti-*Y. enterocolitica* 9576 antiserum and for helpful discussions.

This work was supported by grants from the National Science Foundation (PCM 8015722) and from the Swedish Medical Research Council (B84-16V-6758-01).

LITERATURE CITED

1. **Achtman, M., S. Schwuchow, R. Helmuth, G. Morelli, and P. A. Manning.** 1978. Cell-cell interactions in conjugating *Escherichia coli*: con$^-$ mutants and stabilization of mating aggregates. Mol. Gen. Genet. **164**:171–183.
2. **Acker, G., W. Knapp, K. Wartenberg, and H. Mayer.** 1981. Localization of enterobacterial common antigen in *Yersinia enterocolitica* by the immunoferritin technique. J. Bacteriol. **147**:602–611.
3. **Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree.** 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. U.S.A. **57**:321–326.
4. **Beher, M. G., and C. A. Schnaitman.** 1981. Regulation of the OmpA outer membrane protein of *Escherichia coli*. J. Bacteriol. **147**:972–985.
5. **Berche, P. A., and P. B. Carter.** 1982. Calcium requirement and virulence of *Yersinia enterocolitica*. J. Med. Microbiol. **15**:277–284.
6. **Bercovier, H., H. H. Mollaret, J. M. Alonso, J. Brault, G. R. Fanning, A. G. Steigerwalt, and D. J. Brenner.** 1980. Intra- and interspecies relatedness of *Yersinia pestis* by DNA hybridization and its relationship to *Yersinia pseudotuberculosis*. Curr. Microbiol. **4**:225–229.
7. **Bolin, I., L. Norlander, and H. Wolf-Watz.** 1982. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. Infect. Immun. **37**:506–512.
8. **Bolin, I., and H. Wolf-Watz.** 1984. Molecular cloning of the temperature-inducible outer membrane protein 1 of *Yersinia pseudotuberculosis*. Infect. Immun. **43**:72–78.
9. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI-generated recombinant DNA molecules. Gene **4**:121–136.
10. **Brenner, D. J., A. G. Steigerwalt, D. P. Falcao, R. E. Weaver, and G. R. Fanning.** 1976. Characterization of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by deoxryibonucleic acid hybridization and by biochemical reactions. Int. J. Syst. Bacteriol. **26**:180–194.
11. **Brown, M. G. M., A. Weston, J. R. Saunders, and G. O. Humphreys.** 1979. Transformation of *Escherichia coli* c600 by plasmid DNA at different phases of growth. FEMS Microbiol. Lett. **5**:219–222.
12. **Brubaker, R. R.** 1979. Expression of virulence in *Yersiniae*, p. 168–171. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
13. **Ferber, D. M., and R. R. Brubaker.** 1981. Plasmids in *Yersinia pestis*. Infect. Immun. **31**:839–841.
14. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27**:682–685.
15. **Gemski, P., J. R. Lazere, T. Casey, and J. A. Wohlhieter.** 1980. Presence of a virulence-associated plasmid in *Yersinia pseudotuberculosis*. Infect. Immun. **28**:1044–1047.
16. **Gill, R., F. Heffron, G. Dougan, and S. Falkow.** 1978. Analysis of sequences transposed by complementation of two classes of transposition-deficient mutants of Tn*3*. J. Bacteriol. **136**:742–756.
17. **Gill, R. E., F. Heffron, and S. Falkow.** 1979. Identification of the protein encoded by the transposable element Tn*3* which is required for its transposition. Nature (London) **282**:797–801.
18. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. **77**:3961–3965.
19. **Higuchi, K., and J. L. Smith.** 1961. Studies on the nutrition and physiology of *Pasteurella pestis*. IV. A differential plating medium for the estimation of the mutation rate to avirulence. J. Bacteriol. **81**:605–608.
20. **Kingsbury, D. T., and D. R. Helinski.** 1970. DNA polymerase as a requirement for the maintenance of the bacterial colicinogenic factor Col E1. Biochem. Biophys. Res. Commun. **41**:538–544.
21. **Kretschmer, P. J., A. C. Y. Chang, and S. N. Cohen.** 1975. Indirect selection of bacterial plasmids lacking identifiable phenotypic properties. J. Bacteriol. **124**:225–231.
22. **Kuner, J. M., L. Avery, D. E. Berg, and D. Kaiser.** 1981. Uses of transposon Tn*5* in the genetic analysis of *Myxococcus xanthus*, p. 128–132. *In* D. Schlessinger (ed.), Microbiology—1981. American Society for Microbiology, Washington, D.C.
23. **Martinez, R. J.** 1983. Plasmid-mediated and temperature-regulated surface properties of *Yersinia enterocolitica*. Infect. Immun. **41**:921–930.
24. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoresis method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
25. **Pai, C. H., and L. DeStephano.** 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. Infect. Immun. **35**:605–611.
26. **Perry, R. D., and R. R. Brubaker** 1983. Vwa$^+$ phenotype of *Yersinia enterocolitica*. Infect. Immun. **40**:166–171.
27. **Portnoy, D. A., H. F. Blank, D. T. Kingsbury, and S. Falkow.**

1983. Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. J. Infect. Dis. **148**:297–304.
28. **Portnoy, D. A., and S. Falkow.** 1981. Virulence-associated plasmids from *Yersinia enterocolitica* and *Yersinia pestis*. J. Bacteriol. **148**:877–883.
29. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
30. **Robinson, M. K., P. M. Bennett, S. Falkow, and H. M. Dodd.** 1980. Isolation of a temperature-sensitive derivative of RP1. Plasmid **3**:343–347.
31. **Straley, S. C., and R. R. Brubaker.** 1981. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc. Natl. Acad. Sci. U.S.A. **78**:1224–1228.
32. **Zink, D. L., J. C. Feeley, J. G. Wells, C. Vanderzant, J. C. Vickery, W. D. Roof, and G. A. O'Donovan.** 1980. Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*. Nature (London) **283**:224–226.