

Molecular Cloning of the Temperature-Inducible Outer Membrane Protein 1 of *Yersinia pseudotuberculosis*

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When *Yersinia pseudotuberculosis* is shifted from growth at 26°C to growth at 37°C, the synthesis of a plasmid-associated outer membrane protein, protein 1, is induced (Bölin et al., *Infect. Immun.* 37:506-512). The structural gene of this protein was found to be located on the virulence plasmid pIB1 of *Y. pseudotuberculosis*. One cosmid hybrid plasmid pBW8 was studied which carried a region of the virulence plasmid. This hybrid plasmid expressed in *Escherichia coli* K-12 a novel temperature-inducible outer membrane protein which is immunologically related to and has the same molecular weight as protein 1. Protein 1 was purified to homogeneity, and 14 amino acids of the N-terminal end were determined. From this sequence, the tentative corresponding DNA sequence was deduced, and a set of 11-nucleotide-long DNA probes was chemically synthesized. By using these probes in Southern blotting experiments, the genetic location of the N-terminal end of protein 1 was established. By introducing the transposon Tn5 into the virulence plasmid pIB1, mutants were obtained that did not express protein 1. One class of these mutants was still Ca²⁺ dependent and virulent, suggesting that protein 1 is not a major virulence determinant. Tn5-derived insertion mutants were also obtained which were Ca²⁺ independent. Such mutants were found to be avirulent. One Ca²⁺-independent mutant still expressed protein 1, indicating that the regulatory expression of protein 1 is not linked to Ca²⁺ dependence.

All three species of pathogenic *Yersinia* (*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*) carry virulence plasmids (1, 3, 13-15, 21a, 22, 23, 28). These plasmids show a high degree of DNA homology, especially the plasmids of *Y. pestis* and *Y. pseudotuberculosis*, which are structurally almost identical. One region of the plasmids seems to be fully conserved and associated with Ca²⁺ dependence (D. A. Portnoy, H. Wolf-Watz, I. Bölin, A. B. Beeder, and S. Falkow, manuscript in preparation).

At 26°C, the pathogens can form colonies on agar medium lacking Ca²⁺, but at 37°C, only bacteria devoid of the plasmid or having a mutation within the plasmid can form colonies (Ca²⁺ independent) (1, 13-15, 17, 21a, 22, 23). Ca²⁺-independent mutants of *Y. pestis* which carry insertion mutations in the plasmid are no longer virulent (21a), emphasizing the earlier observations of the important correlation between the unknown mechanism giving rise to Ca²⁺ dependence and the virulence of the pathogen (4-6). The region of the virulence plasmid which has been shown to be involved in the Ca²⁺ dependence is large and comprises about one-third of the plasmid (21a).

The synthesis of the V and W antigens has been associated with the virulence of *Yersinia* (7-10, 20). These two antigens are the only gene products described so far to be associated with the virulence plasmid of *Y. pestis* (13, 24). Plasmid-containing strains of *Y. enterocolitica* and *Y. pseudotuberculosis* express at least four different temperature-inducible outer membrane polypeptides in addition to the V and W antigens (23; Portnoy et al., manuscript in preparation). One of the plasmid-associated proteins is a high-molecular-weight outer membrane protein designated protein 1 (3). The expression of this protein responds very rapidly to a shift in temperature from 26 to 37°C and is only moderately affected by the concentration of Ca²⁺ in the growth medium, in

contrast to the other plasmid-associated outer membrane proteins. In addition, this protein is expressed in several different growth media (3).

We show in this report that the outer membrane protein 1 of *Y. pseudotuberculosis* YPIII(pIB1) is encoded by the virulence plasmid. We further show that Tn5-derived insertions probably located within the structural gene of protein 1 do not affect the virulence of the pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth medium. The growth conditions were as described earlier (3). The strains and plasmids used are described in Table 1.

Plasmid DNA isolation. Plasmid DNA was isolated as previously described (22).

Restriction digests. Restriction endonucleases were used under conditions recommended by the suppliers (Boehringer Mannheim Corp. and New England Biolabs). Restricted DNA was subjected to electrophoresis on a horizontal 0.7% agarose gel in Tris-acetate buffer (0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA [pH 8.0]).

Test for Ca²⁺ dependence. Single colonies were suspended in 0.9% sodium chloride, diluted, and plated on blood agar base or magnesium oxalate agar (MOX), which consisted of blood agar base (Oxoid Ltd.), 20 mM MgCl₂ and 20 mM sodium oxalate (17). Ca²⁺-independent strains gave a 100% viable count on MOX plates at 37°C as compared with the same dilution on blood agar base plates at 26°C. Ca²⁺-dependent strains gave less than a 0.1% viable count on MOX plates at 37°C.

Synthesis and labeling of probe DNA. A mixture of 16 oligonucleotides (see Fig. 4) was purchased from Kabigene AB and synthesized as previously described (11). The oligonucleotides were labeled at the 5' end by transfer of ³²P from [γ -³²P]ATP (Amersham Corp.), using bacteriophage T4 polynucleotide kinase (Boehringer Mannheim Corp.) to a specific activity of 10⁷ to 10⁸ cpm/ μ g of probe.

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TABLE 1. Strains and plasmids used

Strain or plasmid	Comments	Reference
Strain		
YPIII(pIB1)	Wild-type <i>Y. pseudotuberculosis</i> serotype III	3, 15
YPIII	Plasmid-cured derivative of <i>Y. pseudotuberculosis</i> YPIII(pIB1)	3
DH1	<i>E. coli</i> K-12 <i>recA supE hsdR endA</i> host strain for cosmid cloning	18
294	<i>E. coli</i> K-12 <i>endol hsdR hsdM thi</i>	2
Plasmid		
pIB1	Wild-type virulence plasmid of strain YPIII(pIB1)	3
pHC79	Cosmid cloning vector	18
pIB101	Tn5 inserted into <i>Bam</i> HI fragment 10 of pIB1	This study
pIB102	Tn5 inserted into <i>Bam</i> HI fragment 10 of pIB1	This study
pIB71	Tn5 inserted into <i>Bam</i> HI fragment 7 of pIB1	This study
pIB540	Hybrid plasmid between pBR322 and <i>Bam</i> HI fragment 10 of pIB1	This study
pBW8	Cosmid hybrid plasmid between pHC79 and partially <i>Sau</i> 3A-digested pIB1 (see Fig. 7)	This study

DNA filter hybridization. After agarose gel electrophoresis, the DNA was transferred to nitrocellulose filters (Gene Screen, New England Nuclear Corp.) by electroblotting in a Trans-Blot apparatus (Bio-Rad Laboratories) as described by the manufacturer.

Hybridization with the ³²P-labeled 11-mer oligonucleotide mix was performed as previously described (25) with a probe concentration of 16 ng/ml. Briefly, the filter was hybridized with the ³²P-labeled probe in 6× NET (1× NET = 0.16 M NaCl, 0.015 Tris-hydrochloride [pH 7.5], 1 mM EDTA), 5× Denhardt solution, and 0.5% sodium dodecyl sulfate (SDS) at 14°C for 24 h. No dextran sulfate was added. The filter was then washed at 20°C in 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate [pH 7.2]), dried, and exposed to Cronex-4 X-ray film (DuPont Co.) between two intensifier screens (Cronex Hi-Plus BF, DuPont Co.) for at least 24 h.

Determination of the N-terminal amino acid sequence. The N-terminal sequence of purified protein 1 was determined by the procedures of Trädgårdh et al. (27) and Eriksson et al. (12).

Construction of recombinant plasmids. Plasmid pIB1 was digested with *Sau*3A and ligated to a *Bam*HI-digested cosmid vector pHC79 (18) with T4 ligase as described by the manufacturer (New England Biolabs). The ligated DNA was then packaged in vitro with the phage λ system as previously described (18) and transduced into *Escherichia coli* DH1. A *Bam*HI digest of pIB1 was cloned into pBR322 and transformed to *E. coli* 294.

Transposition. Tn5 was inserted into strain YPIII(pIB1) as described elsewhere (21a). The cells were grown overnight at 26°C in NB medium containing 10 mM MgCl₂ and 5 mM CaCl₂. A 1-ml volume of this culture was infected with a phage stock of P1_{cm1}::Tn5 *clr*100 to give a multiplicity of about 1. After an adsorption period of 30 min the cells were diluted into 10 ml of NB medium and incubated for 1 h at 37°C. Ca²⁺-independent pIB1::Tn5-derivatives were isolated by plating on MOX plates containing 20 μg of kanamycin per ml and incubated at 37°C. To obtain Ca²⁺-dependent

pIB1::Tn5 derivatives, the cells were first plated on blood agar base plates containing 10 mM Ca²⁺ and 20 μg of kanamycin per ml. The resulting clones were then screened for Ca²⁺ dependence by plating on MOX agar at 37°C. Ca²⁺-dependent clones which lost kanamycin resistance after one passage on MOX agar at 37°C were further investigated by plasmid restriction analysis of the master Ca²⁺-dependent clone.

Preparation of outer membranes. For the preparation of membranes, 50 ml of culture was centrifuged at 4°C. The bacterial pellet was suspended in 5 ml of a solution containing 10 mM Tris-hydrochloride (pH 7.8), 5 mM EDTA (pH 7.8), and 1 mM β-mercaptoethanol. The cells were then disrupted by four 15-s bursts at the full power of a Branson ultrasonic disintegrator. Cell debris was removed by low speed centrifugation, and membranes were pelleted from the supernatant fraction by centrifugation at 100,000 × g for 1 h. The membrane pellet was then suspended in 5 ml of a solution containing 0.5% Sarkosyl and 1 mM β-mercaptoethanol in distilled water. The total membrane fraction was incubated for at least 30 min at 4°C (in most cases it was convenient to incubate overnight), and then the suspension was centrifuged at 100,000 × g for 1 h. The final outer membrane-containing pellet was suspended in 100 μl of sample buffer (62.5 mM Tris-hydrochloride [pH 6.8], 1% SDS, 0.5% β-mercaptoethanol, and 10% glycerol) (3).

SDS-polyacrylamide gel electrophoresis. The proteins were analyzed in SDS-polyacrylamide gels essentially by the procedure by Laemmli (19). To separate the outer membrane proteins, we used slab gels containing 16% acrylamide and 0.094% bisacrylamide or gradient gels (17.5 to 10% acrylamide and 0.46 to 0.20% bisacrylamide). Proteins were fixed

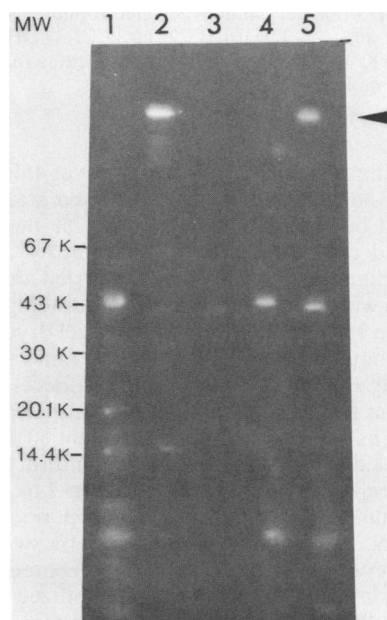


FIG. 1. Western blotting of strains expressing or not expressing protein 1. The strains were first grown in minimal medium at 26°C. At an optical density of 0.15 at 450 nm, the cultures were shifted to 37°C and incubated overnight. Outer membranes were isolated and used in Western blotting. The antiserum was prepared from rabbits immunized with purified protein 1. Lanes: 1, YPIII; 2, YPIII(pIB1); 3, YPIII(pIB102); 4, YPIII(pIB101); 5, YPIII(pIB71). The arrowhead indicates the position of protein 1. MW, molecular weight.

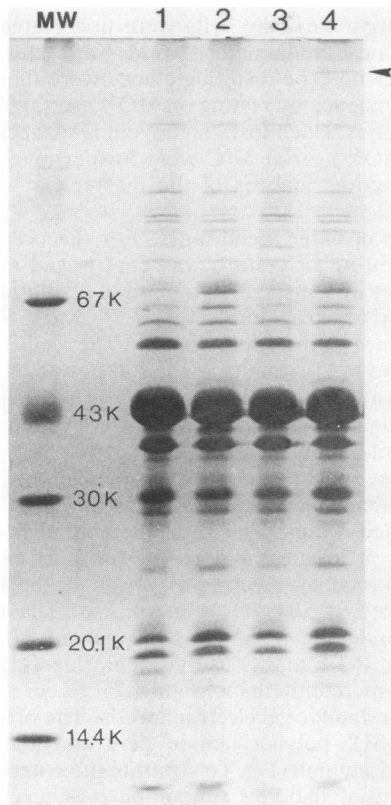


FIG. 2. Outer membrane protein profiles of *E. coli* K-12 strains DH1(pBW8) and DH1(pHC79) grown at 26 and 37°C. The strains were grown on NA plates containing 50 µg of ampicillin per ml overnight at either 26 or 37°C. Bacteria were harvested from the plates, and the outer membrane was isolated. The proteins were separated by SDS-polyacrylamide gel electrophoresis. Lanes: 1, DH1(pHC79) at 26°C; 2, DH1(pHC79) at 37°C; 3, DH1(pBW8) at 26°C; DH1(pBW8) at 37°C. The arrowhead indicates the position of protein 1. MW, molecular weight.

by immersing the gel for 10 min in a mixture of 45% methanol and 9% acetic acid. The proteins were then stained for 30 min in 0.25% Coomassie brilliant blue in 7% methanol–5% acetic acid and destained in 7% methanol–5% acetic acid with several changes. All steps were carried out at 37°C. When proteins were to be visualized by autoradiography, the gel was dried in an LKB Instruments slab gel dryer and then put in direct contact with Cronex-4 X-ray film. After exposure for 48 h at –70°C, the film was developed.

Purification of protein 1. Cells of strain YPIII(pIB1) were grown in 100 ml of NB medium overnight at 26°C. This culture was used to inoculate a 2-liter culture of minimal medium. The cells were incubated for about 2 to 4 h at 26°C on a rotary shaker. After this incubation period, the flask was transferred to 37°C, and the bacteria were grown overnight. The outer membrane was prepared, and the resulting outer membrane pellet was solubilized in 1 ml of SDS sample buffer and heated to 95°C for 5 min. The entire sample was applied to a preparative SDS-polyacrylamide gel. After electrophoresis overnight, the gel was stained with 0.25 M KCl as described by Hager and Burgess (16). After 5 min of staining, the protein 1 band was clearly visible. It was excised from the gel and eluted with elution buffer (0.1% SDS, 0.05 M Tris-hydrochloride [pH 7.9], 0.1 mM EDTA, 5 mM dithiothreitol, 0.2 M NaCl), usually overnight. After

centrifugation to remove the preparative gel material, the supernatant containing protein 1 was precipitated with 5 volumes of acetone and incubated for 1 h at –20°C. The tubes were then centrifuged for 10 min at 10,000 rpm, and the protein 1-containing pellet was dried under vacuum. In general, about 1 mg of protein 1 was recovered per liter of bacterial culture.

Western blotting. Western blotting after SDS-PAGE of outer membrane proteins was performed as described by Swanson et al. (26). Undiluted protein 1 rabbit antiserum (enzyme-linked immunosorbent assay titer, 1:1,000) was used.

Oral infection of Swiss albino mice. Oral infection of Swiss albino mice with *Y. pseudotuberculosis* was essentially carried out as described by Gemski et al. (15). Groups of five Swiss albino mice weighing 17 to 20 g were deprived of water for 18 h and then allowed to drink freely from a 50-ml water suspension containing 10^9 bacteria per ml of each strain.

RESULTS

Molecular cloning of protein 1 gene. When plasmid-containing cells of *Y. pseudotuberculosis* are grown in minimal medium at 26°C and then shifted to 37°C, the synthesis of large amounts of the outer membrane protein 1 is induced (3). This protein is not expressed in bacteria which lack the virulence plasmid (Fig. 1) (3). This result suggested that the structural gene of protein 1 was located on the virulence plasmid. To answer this question, purified plasmid DNA was cloned into pHC79. The resulting transductants all carried hybrid molecules of pHC79 and pIB1. These clones were screened for production of outer membrane protein 1 at

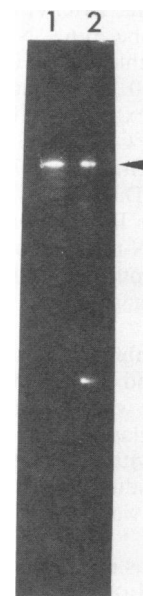


FIG. 3. Western blotting of purified protein 1 and outer membrane of *E. coli* K-12 strain DH1(pBW8), using protein 1 antiserum. Strain DH1(pBW8) was grown on NA plates containing 50 µg of ampicillin per ml at 37°C. The outer membrane was isolated from these cells and thereafter used in a Western blotting experiment. The antiserum was prepared from rabbits immunized with purified protein 1. Lanes: 1, purified protein 1; 2, outer membrane of strain DH1(pBW8). The arrowhead indicates the position of protein 1.

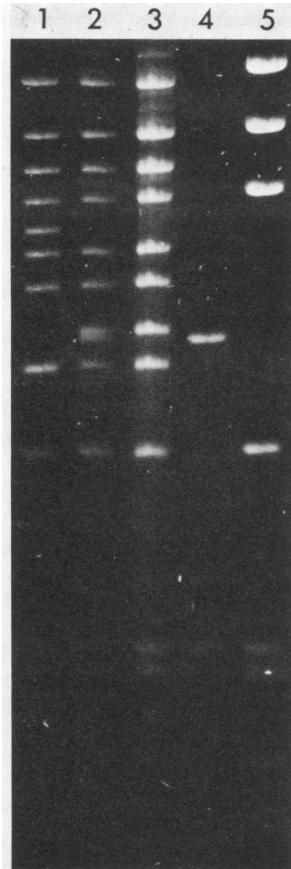


FIG. 4. Agarose gel electrophoresis of different plasmids digested with the restriction endonuclease *Bam*HI. Lanes: 1, pIB71; 2, pIB101; 3, pIB1; 4, pIB540; 5, pBW8. Plasmid pIB102 gave almost the same restriction digest pattern as that of pIB101. Plasmid pIB1 contains 15 *Bam*HI fragments (denoted 1 to 15) with the following sizes in kilobases: 12.0, 9.8, 7.8, 6.6, 5.8, 5.3, 4.4, 4.0, 2.9, 1.7, 1.6, 1.3, 1.1, 0.9, and 0.7.

37°C. A clone of *E. coli* K-12 strain DH1 carrying the hybrid plasmid pBW8 was found to express a novel outer membrane protein which showed the same molecular weight as that of protein 1 (Fig. 2 and 3). This protein was also found to be temperature inducible in *E. coli* K-12 (Fig. 2). Furthermore, an immunological relationship between protein 1 of *Y. pseudotuberculosis* and the temperature-inducible outer membrane protein of strain DH1(pBW8) was established by using Western blotting analysis with rabbit antiserum directed against purified protein 1 of *Y. pseudotuberculosis* YPIII (Fig. 3).

These results clearly indicate that the structural gene of protein 1 is located on the virulence plasmid. It was found, however, that protein 1 was not stably expressed in the *E. coli* K-12 strain DH1(pBW8). When 20 independent single-cell clones derived from a single clone known to express protein 1 were studied, about 10% of the clones no longer showed the ability to synthesize protein 1. However, when such negative clones were restreaked on selective plates and 20 individual single-cell clones were assayed for the ability to express protein 1, 10% of these clones now showed the capacity to express protein 1. Furthermore, the *Bam*HI restriction endonuclease digestion patterns of pBW8 from both positive and negative clones were identical. It was found that plasmid pBW8 contained the *Bam*HI fragments 2, 4, 9, 10, 11, 13, and 14 of plasmid pIB1 (Fig. 4).

Construction of DNA probes for the localization of the structural gene of protein 1. Outer membrane protein 1 was purified, and its N-terminal amino acid sequence was deduced. The N-terminal sequence of 14 amino acids was obtained (Fig. 5). The information from the amino acid sequence was thereafter used to design a set of DNA probes which could be used to localize the N-terminal end of the structural gene of protein 1 on the virulence plasmid pIB1 by hybridization.

A mixture of 16 different 11-mers, as indicated in Fig. 5, was labeled in vitro with [γ -³²P]ATP and used as a DNA probe in Southern blotting experiments. It was found that the probe hybridized to one *Bam*HI fragment of plasmid

Amino acid sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
	?	glu	pro	glu	asp	gly	asn	asp	gly	ile	pro	arg	leu	lys	
Possible codons	5'	GAA	CCU	GAA	GAU	GGU	AAU	GAU	GGU	AUU	CCU	AGA	UUA	AAA	3'
		G	C	G	C	C	C	C	C	C	C	G	G	G	
			A		A			A	A	A			CCU		
			G		G			G		G	G		C		
													A		
													G		
Probe		5'	CCT	GAA	GAT	GG	3'								
			C	G	C										
			A												
			G												

FIG. 5. NH₂-terminal amino acid sequence of protein 1. Protein was purified, and the N-terminal end was sequenced. From this sequence, the corresponding DNA sequence was deduced, and 16 different 11-mer oligonucleotides were chemically synthesized.

pIB1 (Fig. 6). In addition, plasmid pIB540, which was incorporated in the experiment as marker for *Bam*HI fragment 10 of pIB1, showed that the probe mixture recognized this *Bam*HI fragment, indicating that the N-terminal end of the structural gene of protein 1 was within *Bam*HI fragment 10 of the virulence plasmid (Fig. 4 and 6).

pIB1::Tn5 insertion mutants of *Y. pseudotuberculosis* YPIII. To determine whether protein 1 was associated with the Ca²⁺ dependence of strain YPIII(pIB1), the transposon Tn5 was introduced into plasmid pIB1 by the use of phage P1*clm*::Tn5 *clr*100. This phage has previously been found to work as an excellent vehicle to introduce the transposon Tn5 into *Y. pestis* (21a). Accordingly, we isolated Ca²⁺-independent Tn5-derived mutants by selection on Mg²⁺ oxalate plates containing 20 µg of kanamycin per ml at 37°C. These mutants were examined for expression of protein 1. Two different classes of Ca²⁺-independent mutants were obtained. The first class did not express protein 1, whereas the other class did. One representative strain of each class was chosen for further study. These strains were denoted YPIII(pIB101) and YPIII(pIB71), respectively. A third strain, YPIII(pIB102), showing Ca²⁺ dependency was also studied. This strain did not express protein 1. These strains were examined for Ca²⁺ dependence, expression of protein 1, virulence, and location of Tn5.

The location of the Tn5 insertions within the plasmids was analyzed by digestion with the restriction enzyme *Bam*HI (Fig. 4). It was found that plasmid pIB101 had Tn5 inserted into *Bam*HI fragment 10 of plasmid pIB1. Bacteria carrying this plasmid were Ca²⁺ independent and did not express protein 1 (Fig. 1 and Table 2). Plasmid pIB102 also carried Tn5 in *Bam*HI fragment 10. Bacteria carrying this plasmid were Ca²⁺ dependent but did not express protein 1 (Fig. 1 and Table 2). Plasmid pIB71 and Tn5 inserted into *Bam*HI fragment 7 (Fig. 4) and was Ca²⁺ independent but did

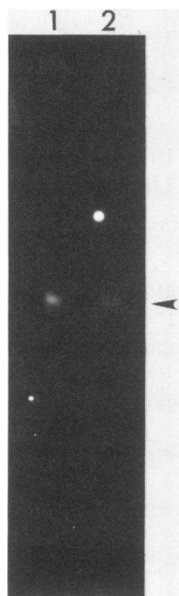


FIG. 6. Southern blotting with *Bam*HI-digested plasmid DNA, using a ³²P-labeled mixture of 11-mer oligonucleotides as a probe. Lanes: 1, pIB1; 2, pIB540. The arrowhead indicates the position of *Bam*HI fragment 10 (see Fig. 4).

TABLE 2. Phenotypical behavior of three different characters expressed in strains carrying different plasmids

Strain	Ca ²⁺ dependence ^a	Expression of protein 1 ^b	Virulence ^c
YPIII(pIB1)	+	+	10/10
YPIII(pIB71)	-	+	0/10
YPIII(pIB101)	-	-	0/10
YPIII(pIB102)	+	-	10/10
YPIII	-	-	0/10

^a The response to Ca²⁺ was tested on magnesium oxalate plates at 37°C. +, Ca²⁺ dependent; -, Ca²⁺ independent.

^b The protein profile of different strains grown at 37°C in minimal medium was analyzed by SDS-PAGE. +, Expression of protein 1; -, no expression of protein 1.

^c Ten Swiss albino mice were challenged orally with about 10¹⁰ bacteria per mouse, and the fate of the infection was monitored (number dead/number challenged).

express protein 1 (Fig. 1 and Table 2). These results indicate that there may be two different loci of plasmid pIB1 which are involved in the Ca²⁺ response of *Y. pseudotuberculosis*, one of which also affects the expression of protein 1.

These strains were tested for their ability to cause a lethal infection in mice after oral challenge. The parental strain as well as YPIII(pIB102) caused a lethal infection in mice (Table 2). However, both YPIII(pIB101) and YPIII(pIB71) were avirulent in this test (Table 2).

DISCUSSION

In a recent report, we suggested that the structural gene of the plasmid-associated temperature-inducible outer membrane protein protein 1 of *Y. pseudotuberculosis* was located on the virulence plasmid pIB1 (3). We have demonstrated in this study that a protein showing the same molecular weight and immunological relatedness to protein 1 is expressed in a strain of *E. coli* K-12 harboring pBW8, a plasmid which is a hybrid between pHC79 and pIB1. This argues strongly for the assumption that protein 1 is plasmid encoded. This protein was also found to be temperature inducible in the *E. coli* K-12 strain DH1(pBW8), indicating that the regulatory system involved in the temperature-dependent expression of protein 1 is also plasmid associated.

DNA probes specifically designed to recognize the N-terminal end of the structural gene corresponding to protein 1 were found to hybridize specifically to *Bam*HI fragment 10 of plasmid pIB1, indicating that the N-terminal end of this gene is to be found within this *Bam*HI fragment. This hypothesis is also supported by the results obtained with plasmids pIB101 and pIB102. These plasmids have Tn5 inserted into *Bam*HI fragment 10 of plasmid pIB1. Strains carrying plasmids pIB101 and pIB102 were found to be unable to express protein 1, in contrast to the wild-type strains or other plasmid-carrying strains, which have Tn5 inserted into other regions of the plasmid pIB1. These data suggest that the transposons inserted into *Bam*HI fragment 10 have hit the structural gene of protein 1.

Protein 1 has a molecular weight of about 140,000 (3), which implies that the structural gene should be about 4 kilobases. It is therefore reasonable to suggest that the gene is located within a 8-kilobase DNA region having the N-terminal part within *Bam*HI fragment 10. Therefore, it is most likely that the structural gene of protein 1 is located on plasmid pIB1 as suggested in Fig. 7.

The plasmid-containing wild-type strain YPIII(pIB1) is

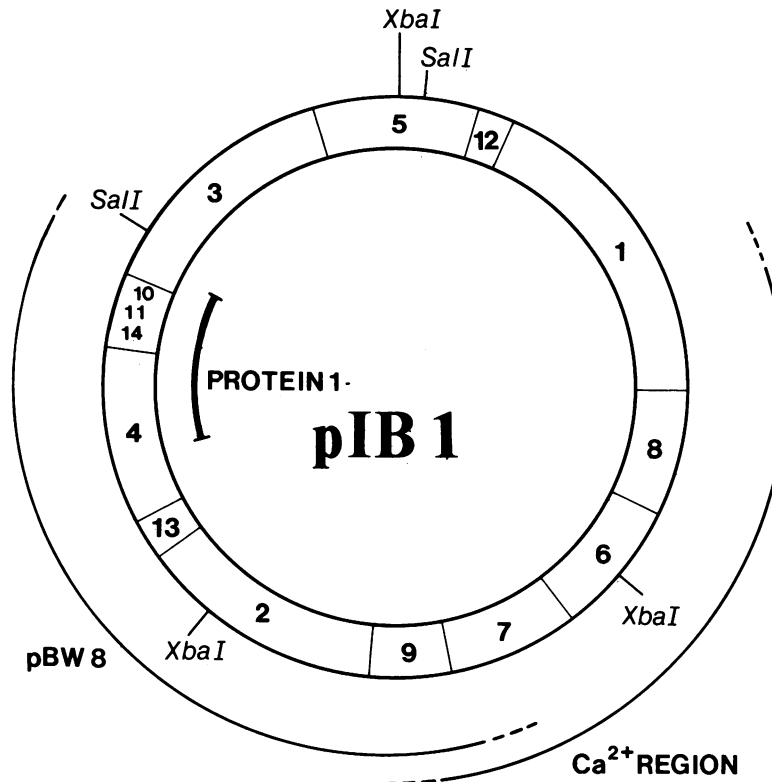


FIG. 7. Restriction endonuclease *Bam*HI map of plasmid pIB1. Plasmid pBW8 was cut with different restriction enzymes, and a restriction map was deduced. Only the portion generated from plasmid pIB1 of plasmid pBW8 is shown. The Ca^{2+} region indicates the region of the virulence plasmid pYV019 of *Y. pestis* which seems to be involved in the Ca^{2+} response of that strain. Data were obtained from Portnoy et al. (in press). The symbol |—| shows the suggested genetic location of protein 1 on plasmid pIB1.

highly virulent for Swiss albino mice after oral challenge (3, 15). This was also found to be true for Ca^{2+} -dependent strain YPIII(pIB102) which did not express protein 1, indicating that protein 1 is not required as a virulence determinant of *Y. pseudotuberculosis* to cause a lethal infection in mice. In contrast, the strain YPIII(pIB101) was found to be avirulent. This strain is also unable to express protein 1 but, furthermore, is Ca^{2+} independent. Therefore, the avirulent behavior of this latter strain is more likely accounted for by the correlation with Ca^{2+} independence rather than with incapability to express protein 1.

Plasmid pIB101 has Tn5 inserted into *Bam*HI fragment 10 of pIB1. Since this *Bam*HI fragment is located far from fragment 7 (Fig. 7) and since the transposon probably is inserted into or is located very close to the structural gene for protein 1, it cannot be excluded that expression of protein 1 is coregulated with Ca^{2+} dependence. However, it is possible either that another mutation simultaneously caused Ca^{2+} independence, since this was the basis for selection, or that Tn5 mediates polar effects on distal genes.

Plasmid pIB71 seems to support the latter possibilities since a strain carrying this plasmid was found to be Ca^{2+} independent but able to express protein 1. Thus, the results obtained with plasmids pIB102 and pIB71 show that the expression of protein 1 is not obligatorily linked to Ca^{2+} dependence. However, strains YPIII(pIB101) and YPIII(pIB71), respectively, were found to be avirulent after oral challenge, confirming results obtained from work with *Y. pestis* showing that Ca^{2+} -independent Tn5-derived insertion mutants are avirulent (21a).

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