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Thermoregulation-Dependent Expression of *Yersinia enterocolitica* Protein 1 Imparts Serum Resistance to *Escherichia coli* K-12

RAFAEL J. MARTINEZ

Department of Microbiology, College of Letters and Science, University of California, Los Angeles, California 90024

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Resistance to the bactericidal action of normal human serum is one of the characteristics of virulent *Yersinia enterocolitica*. This property is attributable to the virulence plasmid harbored by pathogenic strains of the species. Serum resistance in *Y. enterocolitica* is thermoregulated, and its expression correlates well with the presence of virulence plasmid-encoded outer membrane proteins. To further examine the biochemical basis underlying resistance, we cloned a large segment (ca. 30 kilobases) of virulence plasmid DNA and studied the expression of plasmid-encoded outer membrane proteins in a serum-sensitive strain of *Escherichia coli*. The presence of the 160-kilodalton *Y. enterocolitica*-derived outer membrane protein 1 on *E. coli* transformants conferred a high degree of hydrophobicity, autoagglutinability, and resistance to serum killing. All of these properties were thermoregulated in *E. coli* with fidelity, suggesting that a functional thermoregulatory element was present in the cloned DNA. Elimination of protein 1 from the outer membrane of *E. coli* transformants by insertional inactivation of the structural gene with a Kan^r gene cassette abrogated all of these properties and returned the serum-sensitive phenotype.

Yersinia enterocolitica, an invasive enteric pathogen of humans and animals, owes its pathogenic potential in part to the expression of determinants encoded on a 70-kilobase-pair (kb) virulence plasmid (10, 25). Several reports (2, 15, 21, 24, 31) have presented evidence supporting the notion that virulence plasmid-encoded outer membrane proteins (OMPs) of *Y. enterocolitica* may be responsible for the resistance of this species to serum. Only cells with the plasmid-encoded OMPs exposed on their outer surface tolerate complement. Removal of these OMPs by a variety of means renders the cells sensitive to the bactericides in normal human serum. Recently, Balligand et al. (2) suggested that the 240-kilodalton (kDa) plasmid-encoded OMP of *Y. enterocolitica* W22708 serogroup O:9 "is a necessary but not sufficient condition for serum resistance." Presumably this 240-kDa protein is the counterpart of other high-molecular-mass proteins (>100 kDa) seen in sodium dodecyl sulfate (SDS)-polyacrylamide gels of outer membranes of different virulent strains of this species (9). Bolin et al. (5) referred to the corresponding protein with an apparent molecular mass of 140 kDa from *Y. pseudotuberculosis* (pIB1) and two strains of *Y. enterocolitica* as protein 1. I will follow that nomenclature.

In this communication I present evidence demonstrating that a recombinant plasmid (pRM1), composed of 31 kb of *Y. enterocolitica*(pYV8081) virulence plasmid DNA and the plasmid vector pACYC184, confers, among other characteristics, serum resistance on *Escherichia coli* MM294 transformants. This strain, like other K-12 derivatives (22), is sensitive to complement-mediated killing. Several lines of evidence suggest that expression of protein 1 in the outer membrane is responsible for the serum resistance seen in *E. coli* MM294(pRM1). Interruption of the structural gene coding for protein 1 by insertion of a kanamycin resistance gene cassette in pRM1 DNA eliminated protein 1 from the outer membranes and simultaneously abolished serum resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1.

The following media were routinely used: M9 salts (19) without the calcium, supplemented with 0.2% glucose (M9 minimal medium) and 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) (M9-CA); LB (19); and brain heart infusion broth (Difco) supplemented with 20 mM $MgSO_4$. Cultures were grown aerobically either with shaking in a water bath or static in an incubator at the desired temperature (usually 10 ml of medium in a 125-ml Erlenmeyer flask). Plasmids expressing antibiotic resistance markers were selected on M9 or LB plates containing the following concentrations of the desired antibiotics: chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; ampicillin, 100 μg/ml; nalidixic acid, 50 μg/ml.

DNA methods. Restriction enzymes, Klenow fragment of DNA polymerase, BAL 31, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or from New England BioLabs Inc., Beverly, Mass., and were used as specified by the suppliers. Calf intestinal phosphatase was purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany, and used as suggested by the manufacturer. Plasmid DNA was isolated essentially as described by Birnboim and Doly (3). When necessary, plasmid DNA was further purified by cesium chloride-ethidium bromide density gradient centrifugation (19). DNA fragments generated by restriction endonuclease digestion were separated by agarose gel electrophoresis with Tris-borate-EDTA buffer (19). When required, overhanging 5′ ends of DNA were filled in by using Klenow fragment to generate blunt ends as described by Maniatis et al. (19). DNA fragments were purified by electroelution from agarose (19).

Construction of recombinant plasmids. A *Bam*HI partial digest of pYV8081 DNA was ligated into the *Bam*HI site of the vector plasmid pACYC184 (Cam^r, Tet^r), interrupting the

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
Y. enterocolitica (pYV8081)	Nal^r, serotype O:8	26
E. coli MM294	*thi pro*	1
E. coli SM10 λ *pir*	*thi thr leu* Tra$^+$ RP4	23
Plasmids		
pACYC184	Cam^r Tet^r	8
pJM703.1	*oriR6K mobRP4* Amp^r	23
pRM221	pJM703.1 with the pUC18 polylinker inserted at the *Eco*RV site	This study
pUC18	Amp^r	36
pUC4K	Amp^r Kan^r	35
pRM1	pYV8081 *Bam*HI fragments 1 and 5 ligated into pACYC184, Cam^r	This study
pRM109	9.5-kb *Bam*HI-*Xba*I fragment of pRM1 ligated with pRM221, Amp^r	This study
pRM309	Kan^r gene inserted in *Pst*I site of pRM109	This study
pRM301	Kan^r gene recombinant at *Pst*I site of protein 1 sequences in pRM1	This study

tet gene, and the resultant recombinant plasmids were introduced into *E. coli* MM294 by transformation (19). Colonies of Cam^r Tet^s bacteria were picked and grown for plasmid isolation. A stable recombinant plasmid, pRM1, was used for subsequent studies. This plasmid was shown by restriction enzyme analysis to contain contiguous *Bam*HI fragments 1 and 5 of pYV8081 (27) (Fig. 1). Several approaches were used to generate the restriction map of pRM1. Initially, purified restriction fragments were end labeled (19) and either completely or partially digested with selected restriction enzymes. A second restriction enzyme was then used to orient the internal fragments relative to each other and to the end fragments. These data generated the basic map, which was expanded by standard single and double restriction enzyme digestions.

The vector plasmid pRM221 was constructed by ligating the 322-base-pair *Pvu*II fragment containing the polylinker from pUC18 into the *Eco*RV site of the recently described wide-host-range, mobilizable suicide plasmid pJM703.1 (23).

Many attempts to generate stable mutations in genes of interest by transposition mutagenesis of pRM1 (or pYV8081) by using several transposons and delivery systems were unsuccessful. Extensive rearrangement of pRM1 DNA was observed in all cases in which transposition was demonstrated, regardless of the *recA* genotype of the host. In some instances, interesting phenotypes were observed, but it was impossible to determine with any degree of certainty whether the phenotypic changes were the result of the DNA rearrangements or the result of the transposition event. To circumvent the plasmid DNA instability problem, an insertion of the Kan^r gene into the putative structural gene for protein 1 was isolated as follows. The 9.5-kb *Bam*HI-*Xba*I fragment of pRM1 was subcloned into the *Sma*I site of pRM221. This recombinant plasmid, replicated in *E. coli* SM10 λ*pir*, was called pRM109. Bolin et al. (4) mapped the structural gene for protein 1 in pYV8081 to this *Bam*HI-*Xba*I fragment and localized two *Pst*I sites in this fragment to DNA sequences within the structural gene for protein 1. Therefore, to inactivate the protein 1 structural gene, the Kan^r gene cassette from pUC4K, isolated from a *Pst*I digest, was ligated with pRM109 partially digested with *Pst*I. Several Amp^r Kan^r clones showing predicted restriction endonuclease profiles were isolated. One, pRM309, was selected for further study and was propagated in *E. coli* SM10 λ*pir*. Restriction enzyme analyses localized the Kan^r gene in pRM309 to the *Pst*I site in map positions 2380/2480 of pRM1 and showed that the 1-kb segment spanning the two *Pst*I sites had been deleted during ligation and had been replaced by the Kan^r gene cassette. To promote marker exchange (30) between the Kan^r-inactivated gene in pRM309 and its wild-type counterpart in pRM1, *E. coli* SM10 λ*pir*- (pRM309) (*thi thr leu*) was mated with *E. coli* MM294(pRM1) (*thi pro*) and Kan^r Cam^r colonies were selected on M9 minimal medium supplemented with proline. Stable Kan^r Cam^r Amp^s exconjugants, which arose by a double recombinational event between homologous regions of pRM1 and pRM309, were grown for plasmid isolation and characterization. The location of the Kan^r gene in DNA specifying protein 1 was confirmed by restriction analysis of a kanamycin-resistant derivative of pRM1, pRM301 (Fig. 1).

Expression of pRM1-encoded protein 1 in *E. coli*. *E. coli* MM294(pRM1) transformants were examined for expression of the *Y. enterocolitica*(pYV8081)-encoded protein 1 by performing immunoblots essentially as previously described (21). Total cell protein or Triton X-100-insoluble outer membrane preparations were electrophoresed on SDS–7.5 to 15% polyacrylamide linear gradient gels. After separation and transfer to nitrocellulose, the proteins bound to nitrocellulose sheets were probed with antiserum directed against

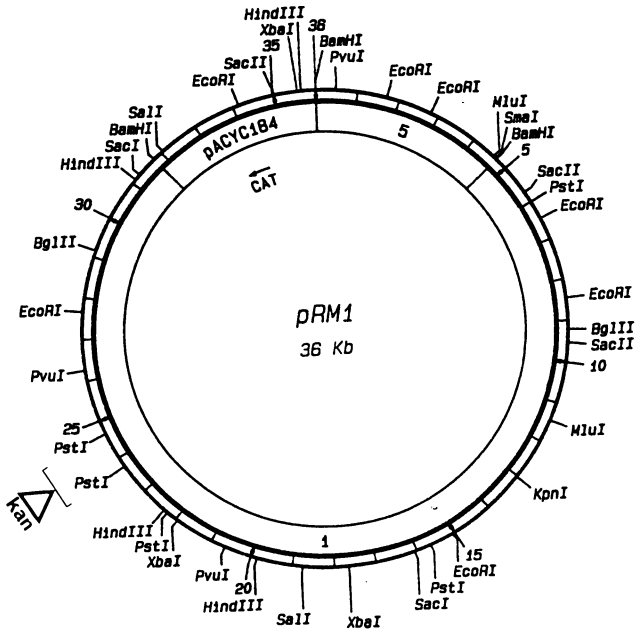

FIG. 1. Restriction map of pRM1. The DNA sequence of pACYC184 has been published previously (28). The bracketed *Pst*I 1-kb fragment was deleted and replaced by a Kan^r gene cassette in pRM301. Map distances are given in kilobases. Many restriction site positions which I had assigned were independently verified or corrected.

protein 1 or against outer membranes prepared from *Y. enterocolitica*(pYV8081) cells grown at 37°C. Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was purchased from Cappel Laboratories, Cochranville, Pa.

Preparation of Triton X-100–insoluble outer membranes, electrophoresis, and immunoblot analyses. Triton X-100-insoluble outer membranes were prepared as previously described (21, 31). SDS-acrylamide gel electrophoresis and immunoblotting were done essentially as reported previously (21). Antiserum to protein 1 was prepared by cutting out the band from a 1.5-mm preparative SDS-polyacrylamide gel. Rabbits were injected with the homogenized gel suspended in complete Freund adjuvant and boosted 4 weeks later. Antisera to the pYV8081-encoded OMPs were prepared by injecting the rabbits with about 100 μg of Triton X-100–insoluble outer membranes from *Y. enterocolitica*(pYV8081) grown at 37°C, in complete Freund adjuvant, with one booster injection 1 month later. All sera were absorbed with Formalin-fixed cells of *Y. enterocolitica* 8081c and with *E. coli* MM294, both grown at 37°C.

Densitometric analyses of photographs of Coomassie blue R250-stained acrylamide gels were performed by using a Bio-Rad densitometer (Bio-Rad Laboratories, Richmond, Calif.). By taking into account the differences in molecular mass, the relative concentration of each polypeptide in the protein profile was estimated. The simplifying assumption was made that Coomassie blue bound in the same quantitative relation to all proteins.

LPS isolation. Lipopolysaccharide (LPS) was isolated from bacterial strains by a small-scale procedure developed by R. P. Darveau and D. A. Portnoy (personal communication) as a modification of the large-scale method of Darveau and Hancock (12). With permission, the method is briefly described here. Outer envelopes were isolated from 5 to 10 ml of overnight cultures. The sedimented envelopes were suspended in 200 μl of 0.1 M tetrasodium EDTA–2% SDS–10 mM Tris (pH 10 to 11) and heated to 80°C for 1 min. After they had cooled to room temperature, pronase (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 200 μg/ml, and the samples were incubated at 37°C for 2 h. More pronase was added (200 μg/ml), and incubation was continued overnight. Two volumes of 95% ethanol containing 0.375 M $MgCl_2$ were added, and the tubes were placed in a dry-ice–ethanol bath for 5 min or at −20°C overnight. The tubes were centrifuged in a table-top high-speed centrifuge (Brinkman Instruments, Inc., Westbury, N.Y.) for 10 min. The supernatant fluids were aspirated, and the sedimented LPS was suspended in 500 μl of sample buffer (0.1 M Tris [pH 6.8], 2% SDS, 40 mM tetrasodium EDTA [pH 6.8], 10% sucrose, and the desired amount of bromphenol blue). Usually 0.5 to 5 μl was electrophoresed in SDS–15% polyacrylamide gels, and the LPS profiles were visualized after silver staining (34). LPS isolated from *Salmonella typhimurium* LT2 was used for comparison.

RESULTS

Construction of pRM1. *E. coli* MM294 was transformed with a plasmid library consisting of DNA fragments from pYV8081 partially digested with *Bam*HI and ligated into the *Bam*HI site of pACYC184. The resultant Cam^r MM294 colonies were grown, and lysates were screened by immunoblot analysis for production of proteins that cross-reacted with anti-pYV8081 OMP antiserum or anti-protein 1 antiserum. A stable clone that showed extensive autoagglutination when grown in M9-CA at 37°C but grew as dispersed single cells at 21°C was selected for further examination. Restriction endonuclease analyses of the recombinant plasmid harbored by this clone revealed an element of about 36 kb consisting of the pYV8081 contiguous *Bam*HI fragments 1 (ca. 26 kb) and 5 (4.7 kb) ligated into pACYC184 (pRM1; Fig. 1). The insert DNA cloned in pRM1 was shown by restriction mapping to be colinear with pYV8081 (27) (Fig. 1).

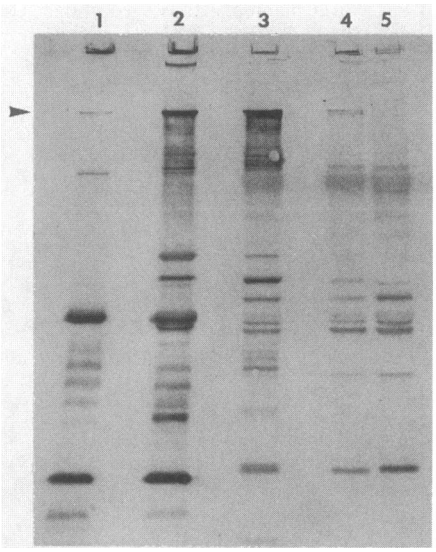

FIG. 2. Immunoblot of *E. coli* MM294(pRM1) cultures grown at 21 and 37°C. The designated cultures (20 μl) were precipitated with acetone and electrophoresed, and the separated protein bands were transferred to nitrocellulose. The papers were probed with antiserum directed against pYV8081-encoded OMPs. Lanes: 1, *Y. enterocolitica* 8081 (21°C) outer membrane standard; 2, *Y. enterocolitica* (37°C) outer membrane standard; 3, *E. coli* MM294(pRM1) culture grown at 37°C; 4, *E. coli* MM294(pRM1) culture grown at 21°C; 5, *E. coli* MM294 culture grown at 37°C. The bands seen in lanes 3 to 5 resisted absorption with *E. coli* MM294 grown at 37°C.

Expression of pYV8081-encoded protein 1 by *E. coli* MM294(pRM1). The virulence plasmid-encoded proteins present in the Triton X-100-insoluble outer membrane of *Y. enterolitica*(pYV8081) have been described previously (20, 26, 27). Expression of the OMP structural genes encoded on the virulence plasmid of *Y. enterocolitica* is regulated by temperature (9, 10, 25); i.e., outer membranes prepared from plasmid-bearing cultures grown at 21°C exhibited only traces of these OMPs (Fig. 2, lane 1), whereas the proteins are phenotypically expressed in large amounts by cultures grown at 37°C (Fig. 2, lane 2). Figure 2 is an immunoblot of total cell proteins of *E. coli* MM294(pRM1), showing that MM294(pRM1) (lane 3) synthesized a protein that comigrated with protein 1 of *Y. enterocolitica*(pYV8081) (lane 2) and reacted with antiserum directed against the pYV8081-encoded OMPs. Furthermore, synthesis of this protein showed thermoregulation in a fashion analogous to that seen for *Y. enterocolitica* (Fig. 3, lanes 1 and 2); i.e., the protein was made in *E. coli* in copious quantities at 37°C (Fig. 2, lane 3), but a 21°C (Fig. 2, lane 4) only traces were seen on the immunoblot.

To determine the location of the 160-kDa protein in *E. coli* MM294(pRM1), Triton X-100-insoluble outer membranes were prepared from cultures of MM294 and transformants carrying pRM1, and the protein profiles of these preparations were compared with the OMPs of *Y. enterocoli-*

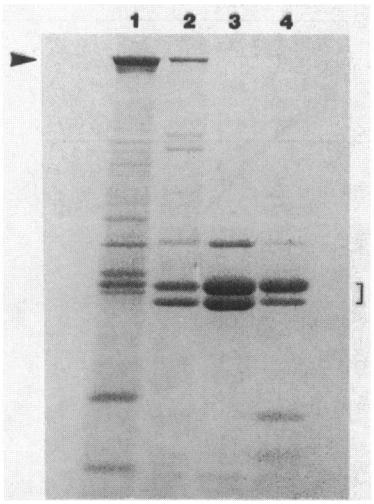

FIG. 3. SDS-polyacrylamide gel electrophoretic protein profile of Triton X-100-insoluble outer membrane preparations of *E. coli* MM294(pRM1) grown at 21 and 37°C. Lanes: 1, *Y. enterocolitica* 8081 (37°C) standard; 2, *E. coli* MM294(pRM1) grown at 37°C; 3, *E. coli* MM294(pRM1) grown at 21°C; 4, *E. coli* MM294 (37°C) standard. The protein 1 band in lane 1 shows a faster leading edge than that in lane 2 owing to the higher protein concentration. This artifact is clearly seen by comparing the mobilities of the major OMP bands (brackets) in lanes 2 and 3. Lane 3 was purposely overloaded to show unambiguously the effect of protein load on mobility: the higher the protein load, the faster the leading edge of the band.

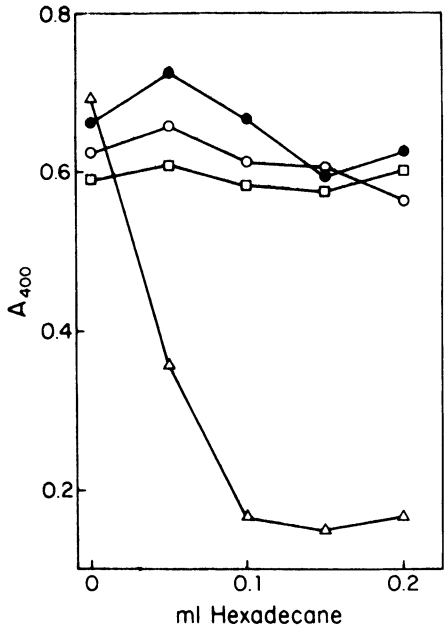

FIG. 4. Effect of protein 1 on the hydrophobicity of *E. coli* MM294(pRM1). *E. coli* strains were grown in M9-CA at 21 or 37°C; 0.2 ml of the cultures was sedimented and suspended in 1.2 ml of PUM buffer (29). Hexadecane was added to the tubes as indicated, and the mixtures were vortexed for 30 s. The phases were allowed to separate, and the optical density at 400 nm (A_{400}) of the aqueous phase was recorded. A reduction in optical density indicates partitioning of the cells into the hydrocarbon phase because of a hydrophobic cell surface. Symbols: ●, *E. coli* MM294 grown at 37°C; ○, *E. coli* MM294(pRM1) grown at 21°C; △, *E. coli* MM294(pRM1) grown at 37°C; □, *E. coli* MM294(pRM301) grown at 37°C.

tica(pYV8081) (Fig. 3). The 160-kDa protein produced by *E. coli* MM294(pRM1) (lane 2) fractionated with the Triton X-100-insoluble outer membrane, as it did in *Y. enterocolitica* (lane 1). Further support for location of the 160-kDa protein on the externally exposed surface of *E. coli* MM294(pRM1) was the observed susceptibility of this protein to proteolysis on exposure of intact cells to pronase (data not shown) (21).

Characterization of the cloned *Y. enterocolitica* protein 1 in *E. coli*. Expression of protein 1 on the surface of different strains of *Y. enterocolitica* imparts some unique phenotypic characteristics to the bacteria. The presence of the protein in the outer membrane of *Y. enterocolitica* and *Y. pseudotuberculosis* strains from several serogroups is associated with the phenomenon of autoagglutination (2, 9, 17, 25, 32). Cells of *Yersinia* species having this protein on their outer membrane also have a hydrophobic surface (2, 21, 32). Cells of *E. coli* MM294(pRM1) grown at 37°C in M9-CA partitioned to the hydrocarbon phase in the hydrophobicity assay of Rosenberg et al. (29) (Fig. 4). In addition, extensive autoagglutination was observed in these cultures. If grown under static conditions, a pellicle formed that subsequently settled to the bottom of the flask; when aerated by shaking, a rim of cells was deposited on the glass surface just above the liquid level. In contrast, *E. coli* MM294(pRM1) grown at room temperature showed a hydrophilic cell surface (Fig. 4), and the cells from these cultures showed no evidence of agglutination, either visually or microscopically. These data further support the correlation of autoagglutination and protein 1 expression on the outer membrane of *Y. enterocolitica*.

It has been reported (38) that protein 1 of a serotype O:3 strain of *Y. enterocolitica* is an oligomer with an apparent molecular mass of 200 kDa in SDS-acrylamide gels. Heating the outer membrane preparations in Laemmli sample buffer (18) containing 10 M urea dissociated the 200-kDa protein into monomeric subunits that migrated with an apparent molecular mass of ca. 47 kDa. To determine whether protein 1 from *Y. enterocolitica*(pYV8081) and from *E. coli* MM294(pRM1) behaved in a similar fashion, outer membrane preparations were denatured in SDS sample buffer containing 10 M urea. Samples were heated either at 37 or at 100°C for 5 min prior to electrophoresis. The 160-kDa protein of *Y. enterocolitica*(pYV8081) (Fig. 5, lane 5) and *E. coli* MM294(pRM1) (Fig. 5, lane 9) migrated with an apparent molecular mass of about 45 kDa when the outer membrane samples were denatured at 100°C in 10 M urea. These polypeptides reacted with antiserum prepared against protein 1 of *Y. enterocolitica*, further supporting their relatedness (data not shown).

The data presented above strongly support the contention that the 160-kDa protein present in outer membranes of *E. coli* MM294(pRM1) grown at 37°C is the same as the protein 1 on the externally exposed surface of *Y. enterocolitica*(pYV8081).

Complement-mediated killing. To explore the possibility that protein 1 is involved in resistance to complement-mediated killing (2), *E. coli* MM294(pRM1) was grown at 21 or 37°C in M9-CA. Between 10^8 and 10^9 cells were incubated at 37°C in phosphate-buffered saline with 10% normal human serum. Samples were removed at intervals, and survivors were determined by their viable count. Owing to the extensive agglutination seen in cultures of this strain grown at 37°C, reproducibility among replicates in any dilution was less than desirable, thus requiring numerous confirmations. Figure 6 and its inset present the results of two such

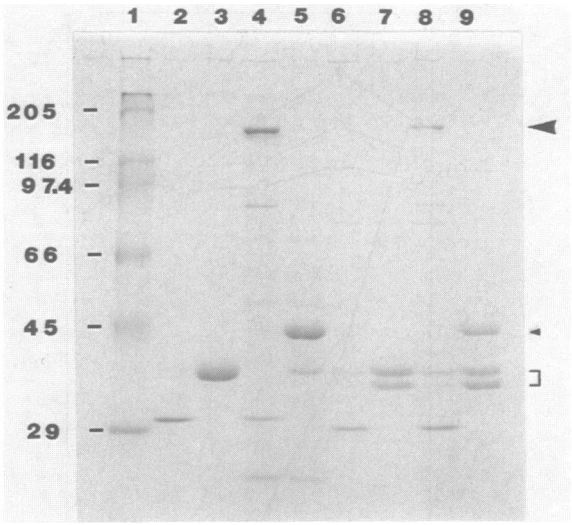

FIG. 5. Effect of 10 M urea on the mobility of protein 1 in SDS-polyacrylamide gel electrophoresis. Outer membrane preparations from *Y. enterocolitica* and *E. coli* strains grown at 37°C were suspended in Laemmli sample buffer (18) containing 10 M urea and held at 37°C or heated at 100°C for 5 min. Lanes: 1, high-molecular-mass protein standards (Sigma); 2, *Y. enterocolitica* 8081c held at 37°C; 3, *Y. enterocolitica* 8081c heated at 100°C; 4, *Y. enterocolitica* 8081 held at 37°C; *Y. enterocolitica* 8081 heated at 100°C; 6, *E. coli* MM294 held at 37°C; 7, *E. coli* MM294 heated at 100°C; 8, *E. coli* MM294(pRM1) held at 37°C; 9, *E. coli* MM294(pRM1) heated at 100°C. Lanes 2, 3, 6, and 7 are controls showing the effect of heat on the mobility of the major OMPs (bracket). The molecular masses of the protein standards are indicated in kilodaltons. The large arrow indicates the 160-kDa oligomeric protein 1; the small arrow indicates the ca. 45-kDa monomer of protein 1.

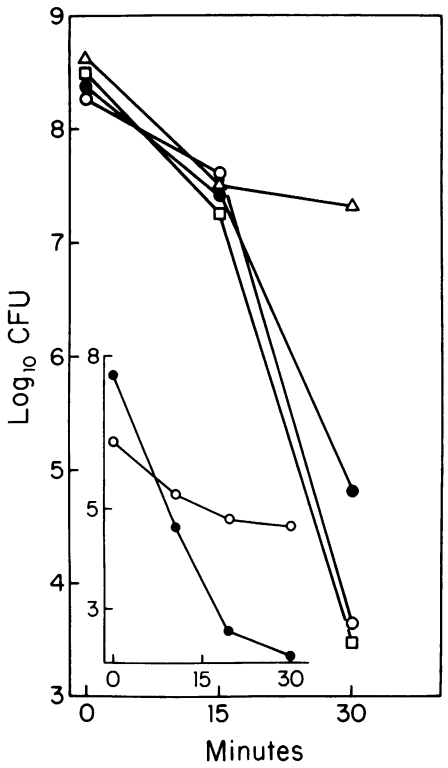

FIG. 6. Bactericidal effect of 10% normal human serum on *E. coli* MM294 and derivatives. Bacteria were grown on M9-CA at either 21 or 37°C to saturation. The sedimented cells were suspended in phosphate-buffered saline containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}. Bactericidal assays were performed as previously described (20). All samples were examined in duplicate or triplicate. Serum heated at 56°C for 30 min to inactivate the complement system was used as a control and showed no decrease in CFU. Symbols: ●, *E. coli* MM294 grown at 37°C; ○, *E. coli* MM294(pRM1) grown at 21°C; △, *E. coli* MM294(pRM1) grown at 37°C; □, *E. coli* MM294(pRM301) grown at 37°C. The inset shows the result of an independent experiment. Symbols: ○, *E. coli* MM294(pRM1) grown at 37°C; ●, *E. coli* MM294(pRM1) grown at 21°C.

experiments. *E. coli* MM294(pRM1) having protein 1 in its outer membrane was quite resistant to complement-mediated killing, usually showing a reduction in $\log_{10}$ CFU of 1 or less in 10% serum. However, *E. coli* MM294(pRM1) grown at 21°C, devoid of protein 1, was as sensitive to serum killing as the parental MM294 was, i.e., a reduction in $\log_{10}$ CFU of 5 to 6 in 30 min.

LPS is a major gram-negative bacterial cell surface component that is involved in serum killing (16). Because of the large number of pYV8081 sequences encoded on pRM1, it was possible that plasmid genes affecting the structure of LPS were responsible for the resistance to serum killing seen in *E. coli* MM294(pRM1). If this were the case, the O side chains of LPS isolated from MM294(pRM1) grown at 37°C might be expected to be longer than those from cells grown at 21°C (14, 16). To examine this possibility, LPS from MM294 cultures grown at 21 and 37°C and LPS from transformants carrying pRM1 were analyzed on SDS-polyacrylamide gels. All LPS samples, regardless of growth temperature or plasmid content of the strains, were free of O side chains in SDS-polyacrylamide gels (data not shown), making the involvement of LPS in serum resistance in MM294(pRM1) unlikely.

If the expression of protein 1 was indeed responsible for conferring resistance to complement-mediated killing, prevention of this expression in *E. coli* MM294(pRM1) by insertional inactivation of the structural gene should result in a serum-sensitive phenotype. By using the restriction map reported by Bolin et al. (4), which localizes the structural gene for protein 1 in pYV8081, this gene was insertionally inactivated in pRM109 by ligating a Kan^r gene cassette within its coding sequence, generating pRM309. This mutation was introduced into *E. coli* MM294(pRM1) by marker exchange (30) as described in Materials and Methods. pRM301 is a derivative of pRM1 with a Kan^r gene replacing the 1-kb *Pst*I fragment (Fig. 1). *E. coli* MM294(pRM301) did not express protein 1 on its outer membrane (Fig. 7, lane 4). Other polypeptide bands detected in OMP profiles of *E. coli* MM294(pRM1) (lane 3) were seen in those from MM294(pRM301), suggesting that the Kan^r insertion resulted in the loss of only protein 1 in the mutant.

Protein 1 appears to impart a hydrophobic character to the bacterial cell surface (2, 32) (Fig. 4). *E. coli* MM294(pRM301), which was free of protein 1, did not partition to the hexadecane phase (Fig. 4), reaffirming the notion that the presence of protein 1 was responsible for conferring a hydrophobic character on the outer membrane. Lastly, incubation of MM294(pRM301) in 10% serum resulted in a dramatic loss of viability (Fig. 6 and inset), indicating that expression of protein 1 and resistance to complement-mediated killing were closely related in *E. coli*.

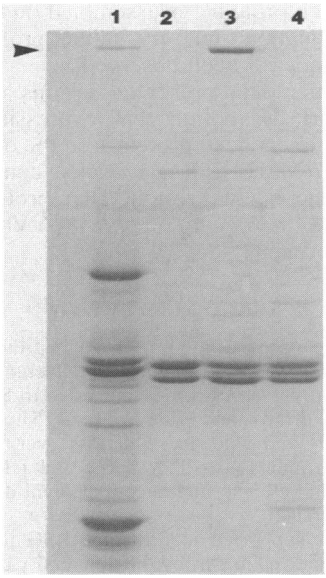

FIG. 7. Absence of protein 1 in the outer membrane of *E. coli* MM294(pRM301). *E. coli* strains were grown at 37°C. Triton X-100-insoluble outer membranes were prepared and electrophoresed as described in Materials and Methods. Lanes: 1, *Y. enterocolitica*(pYV8081) outer membrane standard; 2, *E. coli* MM294; 3, *E. coli* MM294(pRM1); 4, *E. coli* MM294(pRM301).

DISCUSSION

Pathogenic *Yersinia* spp. must harbor and express a virulence plasmid to cause disease; loss of the virulence plasmid invariably results in loss of virulence (9, 25). Likewise, mutants defective in the expression of one of several of the plasmid-encoded OMPs have proven avirulent in animal models (7, 13, 33). Thus, there is convincing evidence that the plasmid-encoded OMPs of *Yersinia* spp. are essential virulence determinants. What is lacking is an understanding of the biochemical mechanisms by which these proteins allow *Yersinia* spp. to overcome host defense mechanisms and replicate.

To examine the role of the plasmid-encoded OMPs in the process of pathogenesis, specifically the relation between protein 1 and resistance of *Y. enterocolitica*(pYV8081) to complement-mediated killing, a library of large DNA fragments from the virulence plasmid pYV8081 was constructed. The goal was to clone a fragment of pYV8081 DNA which expressed protein 1 in *E. coli* and was responsive to the same regulatory controls in *E. coli* as observed in *Y. enterocolitica*(pYV8081). Such a clone, derived from the *Y. pseudotuberculosis* virulence plasmid pIB1, was reported by Bolin and Wolf-Watz (6). However, the level of expression of protein 1 in *E. coli* outer membranes was quite low. In addition, the authors mention that there is an instability of protein 1 expression in their *E. coli* strain.

Protein 1 was implicated in serum resistance by the work of Balligand et al. (2). They demonstrated that serotype O:9 *Y. enterocolitica* W22708 cells, defective in protein 1 production, no longer showed autoagglutination and were sensitive to serum killing. However, expression of protein 1 alone in the outer membrane did not impart resistance to *Y. enterocolitica* W22708. In contrast, in *E. coli* MM294(pRM1), expression of this protein on the outer membrane appears to be sufficient for conferring the serum resistance trait (Fig. 6). I believe that this discrepancy can be reconciled by considering the relative levels of expression of protein 1 in the outer membrane preparations of *Y. enterocolitica* W22708(pGB08) (2) and *E. coli* MM294(pRM1). I believe that had the time after the temperature shift to 37°C been extended, autoaggregation and serum resistance would have been observed. Densitometric analyses of Coomassie blue-stained outer membrane preparations from *E. coli* MM294(pRM1) show that the cells do not attain maximum resistance to serum killing until the level of protein 1 is well above 10% of the total protein on the outer membrane. This takes more than 2 h of growth after the temperature shift to 37°C.

From the original pYV8081 *Bam*HI partial digest and subsequent subclonings, clones making up more than 95% of the virulence plasmid DNA in large fragments were constructed in pACYC184, pBR322, or other plasmid vectors R. J. Martinez, unpublished data). Only *E. coli* transformants which contained the contiguous pYV8081 *Bam*HI fragments 1 (ca. 26 kb) and 5 (ca. 4.7 kb), or BAL 31 deletion derivatives of this clone (J. Pepe, unpublished data), have revealed any of the plasmid-encoded OMPs in immunoblot analyses. Physical separation of fragments 1 and 5 by digestion of pRM1 DNA with *Bam*HI, followed by subcloning of the individual fragments, resulted in loss of expression from either subclone alone or when complementing each other in *trans* (Martinez, unpublished). A reasonable explanation is that physical separation of fragments 1 and 5 disrupts an element required for expression of the *omp* structural gene for protein 1 and possibly other genes on the plasmid.

Recently, a regulatory element (*virF*) from the virulence plasmid pYVe22708 of serogroup O:9 *Y. enterocolitica* was cloned and thoroughly analyzed by Cornelis et al. (10). The evidence clearly indicates that *virF* plays a major controlling role in the thermoregulation of the 50-kDa protein observed in *Y. enterocolitica* and, by inference, in other *Yersinia* spp. as well. Taking into account the close homology seen in the calcium response region (9, 10) among all serogroups of *Y. enterocolitica*, this element would appear to map in *Bam*HI fragment 5 of pRM1. Unpublished sequence analyses (J. Pepe, D. Johnson, J. Ohmen, and R. Martinez, unpublished data) of the first 1,750 base pairs into *Bam*HI fragment 5 of pRM1 starting from the *Bam*HI 1-5 junction (Fig. 1) show that the last 200 base pairs has 99% homology (198 of 200 base pairs) with the carboxy terminus of the published sequence of *vir*F (10). Furthermore, Kan^r gene insertions in the 1.1-kb *Eco*RI fragment in the middle of *Bam*HI fragment 5 abolished the expression of protein 1 by *E. coli* transformants (Martinez, unpublished). All of this evidence, and many published data, support the notion that the expression of protein 1 is regulated by elements located at or adjacent to the calcium response region. This is consistent with the gene map location of *virF* and verifies the results of Cornelis et al. (10). These data provide further corroborative evidence that the Kan^r gene cassette in pRM301 did interrupt the structural gene for protein 1 rather than its regulatory element, and they verify the gene location identified by Bolin et al. (4) and Cornelis et al. (11).

The first 1 kb of the *Bam*HI fragment 5 sequence contains an open reading frame which is interrupted by scission of the *Bam*HI 1-5 junction. This area corresponds to the approximate map location of *virC* (10). Preliminary data suggest that this putative *virC* gene plays a major role in the thermoregulation of protein 1 expression (Martinez, unpublished).

Expression of the pYV8081-encoded *omp* genes was thermoregulated in *E. coli* MM294(pRM1) (Fig. 2 and 3), as it

was in *Y. enterocolitica* (Fig. 2). In addition, of the pYV8081-encoded OMPs expressed in *E. coli* MM294 (pRM1) (Fig. 2), only the 160-kDa OMP was translocated to the outer membrane (Fig. 3 and 7). This novel 160-kDa protein produced by *E. coli* MM294(pRM1) shared several properties with protein 1 of *Y. enterocolitica*, including a similar if not identical apparent molecular mass in SDS-polyacrylamide gradient gels (Fig. 2, 3, 5, and 7); cross-reaction with anti-pYV8081-encoded OMP antiserum (Fig. 2), as well as with anti-protein 1 antiserum (data not shown); dissociation to subunits with similar molecular masses in Laemmli sample buffer containing 10 M urea (Fig. 5); relationship between the presence of protein 1 on the *E. coli* cell surface and an increased hydrophobicity of the bacteria (Fig. 4); and, lastly, resistance to serum killing of *E. coli* MM294(pRM1) expressing protein 1 as an OMP (Fig. 6).

The amount of protein 1 present in the outer membrane of *E. coli* MM294(pRM1) was substantial. From densitometric analyses, protein 1 was estimated to make up roughly 5 to 25% of the total protein of the outer membrane, depending on the growth conditions. Unless there was a dramatic reduction in the amounts of the major OMPs produced by *E. coli* MM294(pRM1) at 37°C, and the gels did not reveal this, it is apparent that the outer membrane of *E. coli* can accommodate large quantities of a foreign protein. A comparison of the OMP profiles of virulent *Y. enterocolitica* grown at low and high temperatures (Fig. 2) also suggests that the protein content of outer membranes can indeed vary significantly. These data challenge the commonly accepted notion that the protein concentration of the outer membrane is fixed.

The fact that protein 1 is an oligomeric protein (38) (Fig. 5) may help to explain the differences in molecular mass reported for protein 1 of different *Y. enterocolitica* species (9). This may also account for the large number of minor protein bands migrating below the 160-kDa band that were coexpressed with protein 1 both in *Y. enterocolitica* and in *E. coli* (Fig. 2, lanes 2 and 3).

It cannot yet be ruled out that a protein whose expression or transport is dependent on protein 1 is responsible for the serum resistance observed in *E. coli* MM294(pRM1). There are, however, several lines of evidence suggesting that protein 1 may indeed be responsible for imparting serum resistance to *E. coli*. First, production of protein 1 by *E. coli* is dependent on the presence of plasmid pRM1 with coding sequences for protein 1. Second, *E. coli* MM294(pRM1), like *Y. enterocolitica*(pYV8081), is sensitive to serum killing when grown at room temperature but is resistant when grown at 37°C (Fig. 6 and inset). This correlates well with the thermoregulated character of protein 1 (Fig. 2). The thermoregulated gene expression seen in *E. coli* MM294(pRM1) grossly mimics that seen in *Y. enterocolitica*(pYV8081); i.e., at low temperatures protein 1 is produced only in trace amounts or not at all, whereas at 37°C the plasmid-encoded OMPs become major components of the outer membrane. Third, insertional inactivation of the structural gene for protein 1 with a Kan^r gene cassette eliminates protein 1 from the outer membrane (Fig. 7), and these cells lose the hydrophobicity seen in *E. coli* MM294(pRM1) (Fig. 4) and become serum sensitive (Fig. 6). Fourth, LPS isolated from MM294(pRM1) grown at either low or high temperature comigrated with LPS from the non-plasmid-bearing parent. O side chains were not seen in the silver-stained gel (data not shown). Thus, the simplest inference is that pRM1 does not encode O-side-chain glycosyl transferases which might impart resistance to complement-mediated killing. The physical form in which protein 1 is assembled on the cell surface is under examination, but crystalline protein arrays, or S-layers, have not yet been observed.

In totality, the data presented in this communication strongly support the notion that expression of *Yersinia* virulence plasmid-encoded protein 1 on the outer membrane protects *E. coli* MM294(pRM1) against complement-mediated killing. One might presume that protein 1 serves a similar function in *Y. enterocolitica*(pYV8081), but this remains to be proven.

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