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Identification of Specific Outer Membrane Polypeptides Associated with Virulent *Yersinia enterocolitica*

MING TU CHANG AND MICHAEL P. DOYLE*

Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

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An antiserum (WA-SAA) that agglutinates specifically with mouse virulent but not avirulent strains of *Yersinia enterocolitica* was used to identify virulence-associated factors by Western blot techniques. Several outer membrane polypeptides were identified only in the virulent strains, which included serotypes O:8, O:3, O:9, O:4,32, O:5,27, and O:21. These included three, and possibly four, major outer membrane polypeptides. The prominent high-molecular-weight species was demonstrated by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot, whereas the others were only revealed by the Western blot technique. Expression of these polypeptides correlated with antiserum agglutination reaction and the presence of a 42- and/or 82-megadalton plasmid. These polypeptides were highly temperature dependent and only slightly affected by the inclusion of 10 mM Ca^{2+} in the growth medium. These polypeptides were produced during both the logarithmic and stationary phases of growth at 37°C. We suggest that the production of these specific polypeptides and calcium dependency may be coded for by the plasmid(s) but are regulated by independent mechanisms. These polypeptides appear to be novel markers specific for virulent strains of *Y. enterocolitica* and may be important to the pathogenicity of this organism.

Yersinia enterocolotica causes a variety of infections in humans, including enterocolitis, acute mesenteric lymphadenitis, terminal ileitis, and occasional septicemia (4, 16). Although ubiquitous in the environment, only certain biochemical and serological types are consistently shown to be pathogenic in humans. We have therefore developed a method utilizing a specific antiserum that is diagnostic in identifying virulent strains of this organism (8). This antiserum, WA-SAA, agglutinates with virulent isolates of *Y. enterocolitica* that caused diarrhea or death in mice.

In this study, we identified specific polypeptides that react with WA-SAA and examined their correlation with virulence-associated plasmids among the virulent and avirulent isolates of this organism.

MATERIALS AND METHODS

Cultures. Strains of *Y. enterocolitica* used for this study were detailed in our previous report (8). *Y. enterocolitica* WA 20^+ was obtained from N. Stern (15).

Bacterial membrane isolation and SDS-PAGE. Bacteria were grown in Trypticase soy broth (TSB; BBL Microbiology Systems), in brain heart infusion broth (Difco Laboratories), or in E medium (18) supplemented with 0.2% glucose and 0.2% Casamino Acids. Triton-insoluble outer membrane was isolated as described by Achtman et al. (1). Trasylol was obtained from Mobay Chemical Corp., FBA Pharmaceuticals, and was used as a protease inhibitor at 1 to 2% (vol/vol) in all solutions for outer membrane isolation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (12) using 7.5% running gels. The sample preparation of outer membrane for SDS-PAGE was as described by Portnoy et al. (13). Staining and destaining of the gels were done as described previously (6). Molecular weight markers and reagents for SDS-PAGE were obtained from BioRad. The following proteins were used as molecular weight standards: myosin (200,000), β-galactosidase (116,000), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000).

* Corresponding author.

Antibody-binding assay. The preparation of specific absorbed antiserum to strain WA of *Y. enterocolitica* (WA-SAA) and agglutination assay have been described previously (8). Western blotting from an SDS gel was accomplished by diffusion transfer and assayed by the method of Chang et al. (6) with the following modifications. After primary antibody reaction and washings, the nitrocellulose sheet was reacted with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Miles). The developing reagent was naphthol AS-MX phosphate (Sigma; at 0.32 mg/ml) in 0.75 M aminoethyl propanediol (Sigma) (pH 10.0), embedded in 1.2% agar (L. Kahan, personal communication). The substrate agar was overlaid onto nitrocellulose paper. WA-SAA-specific antigens were identified by in situ fluorescence to a long wavelength UV source (UV product, UVGL-58) and photographed with a Tiffen no. 58 green filter and type 57 Polaroid film.

Selection of mutants and plasmid DNA isolation. Spontaneous mutants that were independent of calcium for growth at 37°C were isolated as large colonies on magnesium oxalate agar (10) and streaked on Trypticase soy agar plates to ensure clonality. Plasmid DNA was isolated by the method of Birnboim and Doly (2) and resuspended in Tris-borate buffer. Electrophoresis was carried out on an LKB multiphore unit at 4°C.

RESULTS

Analysis of outer membrane polypeptides by SDS-PAGE. We reported previously an antiserum (WA-SAA) that agglutinates specifically with virulent strains of *Y. enterocolitica* (8). This differential reaction suggests differences in the composition of surface antigens. Analysis of outer membrane polypeptides from log-phase cells revealed one major polypeptide with an apparent molecular weight of 160,000 present in strain WA, but absent in the isogenic, avirulent strain WA-ETBR (Fig. 1, top panel, lanes 3 and 4). As judged by staining intensity, this polypeptide was also expressed in a temperature-dependent manner (Fig. 1, lanes 1 and 3), and inclusion of 10 mM Ca^{2+} in the growth medium only slightly reduced the production of this polypeptide (Fig.

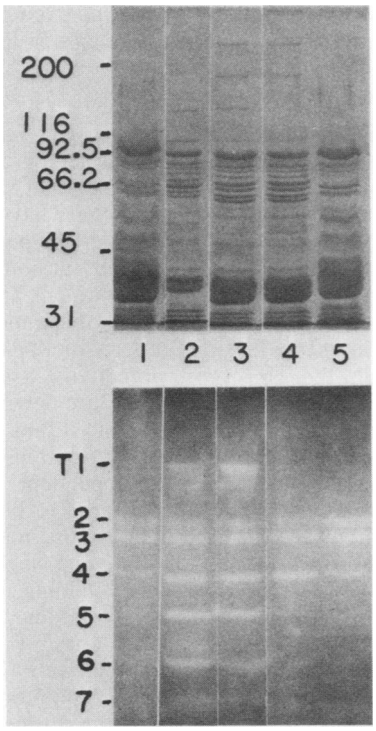

FIG. 1. Identification of specific outer membrane polypeptides associated with virulent *Y. enterocolitica* WA. Outer membrane polypeptides of cultures grown in TSB for 18 h were analyzed by SDS-PAGE (150 μg per lane) and stained by Coomassie blue (top panel) or by Western blot with WA-SAA (bottom panel). The migration of molecular weight markers is indicated on the top panel. Molecular size is expressed in kilodaltons. Lane 1, strain WA grown at 25°C in TSB; lane 2, strain WA grown at 37°C in TSB with 10 mM Ca^{2+}; lane 3, strain WA grown at 37°C in TSB; lane 4, strain WA-ETBR grown at 37°C in TSB; lane 5, strain WA-ETBR grown at 25°C in TSB.

1, lanes 2 and 3). Two other temperature-dependent polypeptides of apparent molecular weights of 250,000 and 192,000, respectively, were observed in both strains (Fig. 1, lanes 3 and 4). We have also compared the membrane of brain heart infusion broth-grown cells with that of TSB-grown cells. No qualitative difference was observed, regardless of the growth temperature, nor was there an appreciable difference in agglutination titers with WA-SAA.

Analysis of outer membrane polypeptides by Western blot using WA-SAA. Cells of strain WA grown at 37°C produced higher agglutination titers than did cells grown at 25°C; sometimes as much as a 40-fold difference was observed. Specific antigens responsible for high agglutination titer were revealed by Western blot techniques (Fig. 1, bottom panel). Three major polypeptide regions, T1, T5, and T6, were highly active in antibody binding to WA-SAA. T1 polypeptide, the major antigenic determinant reacting with WA-SAA, corresponded to the WA-specific, temperature-dependent polypeptide in the high-molecular-weight region ranging from 140,000 to 160,000. There were at least two antibody-binding bands in the T1 region. T5 and T6, with molecular weights of 47,000 and 35,000, respectively, were also found to be WA-specific and highly temperature dependent (Fig. 1, bottom panel, lanes 1 and 3). Inclusion of 10 mM Ca^{2+} in the growth medium (TSB) did not appreciably change the ability of these polypeptides to bind with WA-SAA (Fig. 1, bottom panel, lanes 2 and 3). The three polypeptides in the middle molecular weight range (T2, T3, and T4) showed binding activities to WA-SAA that were common to both strains WA and WA-ETBR. Using the Western blot technique, we found that all of the virulence-associated polypeptides were produced during both the logarithmic and stationary phases of growth at 37°C. Once produced, these polypeptides remained in intact membrane for at least 3 weeks of storage at 4°C (data not shown).

Effect of growth medium on outer membrane polypeptides. There was about an eightfold reduction in agglutination titer of *Y. enterocolitica* WA grown in medium E from that grown in TSB (1:15,360 to 1:122,880). Analysis of outer membrane polypeptides by SDS gel electrophoresis showed differences in the electrophoretic mobilities in the major polypeptides (Fig. 2, lanes A and B). The general pattern and staining intensities of E medium-produced polypeptides appeared to be similar but shifted slightly toward higher molecular weights than TSB-produced polypeptides. The only exception was the E1 polypeptide, which had electrophoretic mobility similar to that of the T1 polypeptide (Fig. 2, lanes A and B). Increased staining intensity suggested that production of the E1 polypeptide is slightly amplified in medium E. E1 appeared to be similar to T1 in antibody binding to WA-SAA (Fig. 2, lanes C and D). E1 and E5 were both specific for virulent strain WA and were highly temperature dependent when analyzed by the Western blot technique to WA-SAA (Fig. 3). The apparent molecular weight of E5 was 50,000. Three common polypeptides, i.e., E2, E3, and E4, appeared to be similar to T2, T3, and T4 in antibody-binding abilities.

Identification of virulence-associated polypeptides among *Y. enterocolitica* of different serotypes. We have shown previously (8) that WA-SAA agglutinates with several virulent isolates of *Y. enterocolitica* regardless of serotype. Outer membrane materials prepared from several of these isolates

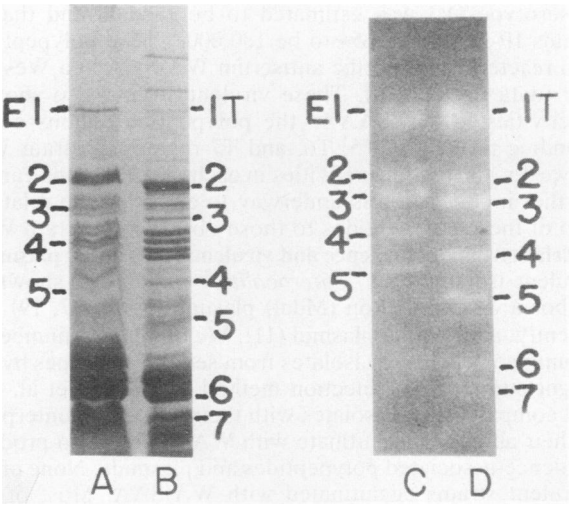

FIG. 2. Comparison of virulence-associated polypeptides in cultures of *Y. enterocolitica* WA grown in TSB and E medium. Outer membrane polypeptides of cultures grown in E medium (lanes A and C) and TSB medium (lanes B and D) were compared by SDS-PAGE (lanes A and B) and by Western blot (lanes C and D). Cultures grown in E medium were harvested after the temperature was shifted from 25 to 37°C, as described by Bölin et al. (3).

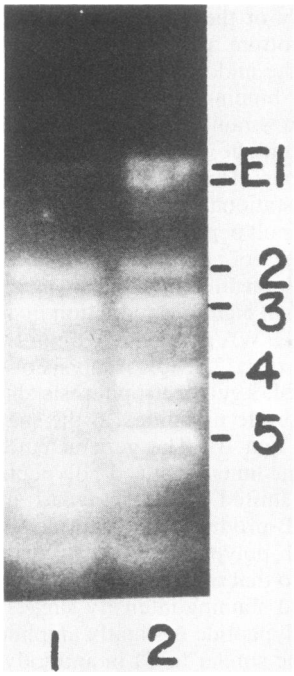

FIG. 3. Comparison of virulence-associated polypeptides of *Y. enterocolitica* WA grown at 25 and 37°C. Cultures were grown in E medium at 25°C and then split, with half shifted to 37°C as described by Bölin et al. (3). Outer membrane polypeptides of *Y. enterocolitica* grown at 25°C (lane 1) and at 37°C (lane 2) were analyzed by Western blot with WA-SAA.

and their equivalent avirulent counterparts were analyzed by SDS-PAGE. An additional polypeptide of high molecular weight (HMP) could be detected in virulent strains IP-383 (serotype O:9), C122-76 and E-675 (both serotype O:3), and E-654 (serotype O:5,27) (Fig. 4, lanes 1, 3, 4, and 6, respectively). The molecular weight of HMP in both strains of serotype O:3 was estimated to be 186,000 and that of strains IP-383 and E-654 to be 180,000. These polypeptides also reacted with specific antiserum WA-SAA in a Western blot (data not shown). These virulent strains also showed reactivities to WA-SAA in the polypeptide regions corresponding to the T1, T5, T6, and T7 regions of strain WA. However, the binding activities in each of the regions varied. Further investigation is underway to elucidate the relationship of these polypeptides to those observed in strain WA.

Relationship of virulence and virulence-associated plasmids. Virulent isolates of *Y. enterocolitica* have been shown to harbor a 42-megadalton (Mdal) plasmid (9, 14, 17, 19) and recently, an 82-Mdal plasmid (11). We obtained a number of spontaneous avirulent isolates from several serotypes by the magnesium oxalate selection method of Higuchi et al. (10) and compared these isolates with their virulent counterparts in their ability to agglutinate with WA-SAA and to produce virulence-associated polypeptides and plasmids. None of the avirulent strains agglutinated with WA-SAA. Most of the virulent isolates examined harbored both 42- and 82-Mdal plasmids, with the former being the prominent species (Fig. 5). Using the method of Birnboim and Doly (2) improved recovery of the 82-Mdal plasmid compared with our previous method (7); however, we could not demonstrate unambiquiously the presence of this plasmid in virulent strain C122-76 (Fig. 5, lane 7). Avirulent isolates consistently lacked both plasmids regardless of serotype, as exemplified by an avirulent isolate of strain CDC-A2635 (Fig. 5, lane 2).

DISCUSSION

Our analysis of the outer membrane of *Y. enterocolitica* WA by SDS-PAGE revealed several temperature-dependent, high-molecular-weight polypeptides that were not observed previously, possibly because others have used higher-percentage gels (13). Two of these polypeptides, i.e., 250,000- and 192,000-dalton polypeptides, although temperature dependent, were found in both virulent and avirulent strains (WA and WA-ETBR). Perhaps these molecules are related to the in vitro adhesion property of *Y. enterocolitica* as observed in the HeLa cell infectivity assay (3, 14). Expression of this property is temperature dependent; however, both virulent and avirulent isogenic strains cured of the virulence plasmid(s) possess this trait. Within the limit of resolution, the major temperature-dependent polypeptide (T1; molecular weight, 160,000) appeared to be specific to strain WA and was also identified as the major antigen reacting with WA-SAA. Although only one single band was seen occasionally by Coomassie blue staining, at least two bands were detected by the antibody-binding assay. This may represent microheterogeneity or possibly degradative products of the major species, although we have routinely included 1 to 2% Trasylol as a protease inhibitor in preparation procedures to minimize degradation artifacts. Alternatively, there may be at least two polypeptide species that bind with WA-SAA because the antibody-binding assay is a more sensitive detection method than Coomassie blue staining.

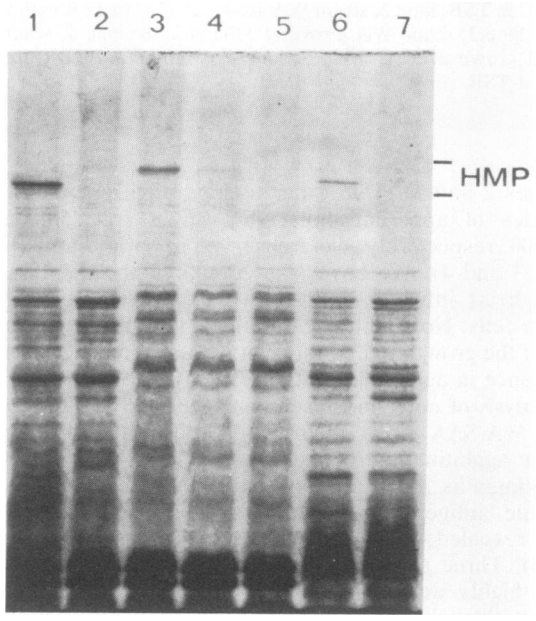

FIG. 4. Detection of virulence-associated polypeptides among virulent and avirulent isogenic, virulence plasmid-cured isolates of *Y. enterocolitica*. Outer membrane polypeptides were analyzed by SDS-PAGE. Avirulent isolates are indicated by MOX after the strain designation. Lane 1, strain IP-383 (serotype O:9); lane 2, strain IP-383-MOX; lane 3, strain C-122-76 (serotype O:3); lane 4, strain E-675 (serotype O:3); lane 5, strain E-675-MOX; lane 6, strain E-654 (serotype O:5,27); lane 7, strain E-654-MOX.

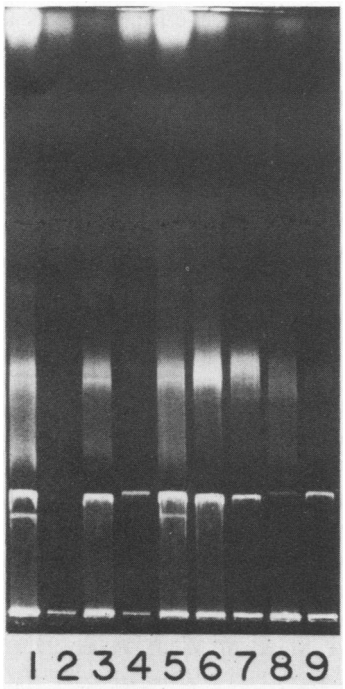

FIG. 5. Detection of 42- and 82-Mdal plasmids among strains of *Y. enterocolitica*. Avirulent isolates are indicated by MOX. Lane 1, strain CDC-A2635 (serotype O:8); lane 2, strain CDC-A2635-MOX; lane 3, strain E-654; lane 4, strain E-701 (serotype O:4,32); lane 5, strain E-736 (serotype O:21); lane 6, strain IP-383; lane 7, strain C-122-76; lane 8, strain WA 20^+ (serotype O:8); lane 9, strain WA.

We have used an antiserum that specifically agglutinates with virulent strains of *Y. enterocolitica* to identify virulence-associated factors. The antiserum, WA-SAA, was prepared from rabbits immunized with live cells of virulent strain WA and then exhaustively adsorbed with both heat-killed and formalinized cells of an avirulent isogenic strain, WA-ETBR. This procedure removes most of the antibodies to common surface antigens and retains antibodies specific to virulent WA cells. As expected, cells of the avirulent strain WA-ETBR did not agglutinate, nor did they stain in immunohistochemical assay with antiserum WA-SAA (8). Three major polypeptides and possibly four (T1, T5, T6, and T7) were identified as virulence-associated polypeptides among the virulent but not the avirulent isolates of *Y. enterocolitica*. Two additional types (based on molecular weight) of virulence-associated polypeptides (HMP) were found in serotypes other than the O:8 and O:21 strains. Strains with this additional HMP generally showed weaker antibody binding in the T1 polypeptide region. It is possible that HMP and T1 are related molecules that determine the degree of virulence. Strains of serotypes O:8 and O:21 with T1 as the major determinant cause both diarrhea and death in the mouse virulence test, whereas strains belonging to other serotypes, i.e., O:3, O:9, and O:5,27, are less virulent and have a modified T1. This possibility can be examined when T1 is purified and specific antibody is available.

Although WA-SAA was prepared by specific adsorption to remove antibodies to common antigens, three common membrane polypeptides for both WA and WA-ETBR strains (T2, T3, and T4 for the TSB-grown cells and E2, E3, and E4 for the E medium-grown cells) were detected by Western blot. We think this is because the antigenic sites of the polypeptides were not exposed in the native membrane but were exposed only after isolation and/or sample denaturation in SDS-PAGE. We have also examined the cytosol of WA and WA-ETBR and found a few common polypeptides having binding specificity (data not shown). This was expected because our antiserum adsorption procedure does not remove cytosol-reacting antibodies. As a control, normal rabbit serum (preimmune serum) was also tested by the Western blot technique. Unlike the reaction with WA-SAA by which discrete polypeptide regions could be resolved as sharp bands, the reaction with preimmune serum gave a weak staining, diffuse pattern suggesting nonspecific binding.

Growing WA under a nutrient-poor condition (i.e., in medium E) resulted in a slight reduction in agglutination titer and a general shift in the spectrum of major polypeptides in SDS-PAGE, with the exception of T1 polypeptide. This mobility shift may have resulted from a general modification of membrane polypeptides, such as glycosylation, phosphorylation, or methylation, all of which are known to modify polypeptide mobility in SDS-PAGE. If this occurred, T5 and E5 are probably related polypeptides, as are the other three common polypeptides. The mobility shift has been noticed also on several occasions with E1 or T1 polypeptide, suggesting that this characteristic is sensitive to ionic conditions. Therefore, we have always compared results of the Western blot with a gel prepared under identical conditions.

T1 polypeptide, being temperature dependent in its production and being specific to strain WA, is probably very similar, if not identical, to the protein 1 of *Y. enterocolitica* reported by Bölin et al. (3). Purified protein 1 of *Yersinia pseudotuberculosis* (a kind gift from H. Wolf-Watz) appeared to have similar but not identical electrophoretic mobility as T1 of *Y. enterocolitica* and did react with WA-SAA in a Western blot (data not shown). This binding activity, however, was not as great as that of T1 of *Y. enterocolitica*. This finding suggests that protein 1 of *Y. pseudotuberculosis* and T1 of *Y. enterocolitica* may be related molecules. The weak binding reaction of protein 1 to WA-SAA could be due to either poor transfer of protein to nitrocellulose paper or a topographic difference in the antibody-binding site of the molecule. To identify the relationship of T1 and protein 1 requires that these proteins be purified and characterized.

We have in this report also demonstrated that these specific membrane molecules could be found among virulent isolates of *Y. enterocolitica* that harbored 42- and/or 82-Mdal plasmids. In the avirulent isolates, the absence of agglutination reaction and specific polypeptides were accompanied by the loss of both plasmids. Since the production of specific polypeptides was not inhibited by calcium concentration, which affects growth at 37°C, we suggest that these two traits, if coded for by the plasmid(s), may be regulated by independent mechanisms. We do not know which of the two (or both) is related to the production of these specific polypeptides, since avirulent isolates lose both plasmids concomitantly.

In conclusion, our study identified specific polypeptides in the outer membrane of virulent strains of *Y. enterocolitica* that are likely expressed in vivo in infected mice (8). These polypeptides, unlike the V and W virulence antigens of yersiniae (5), were unaffected by calcium concentration and were produced under conditions in which V antigen could not be detected (8). We suggest that these polypeptides are novel markers specific to virulent isolates and may be important to the pathogenicity of this organism.

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