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Purification, Location, and Immunological Characterization of the Iron-Regulated High-Molecular-Weight Proteins of the Highly Pathogenic Yersiniae

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We have previously shown that under iron limitation, different *Yersinia* species synthesize new polypeptides. Two of them, the high-molecular-weight proteins (HMWPs), are expressed only by the highly pathogenic strains. In the present study, the HMWPs from *Y. enterocolitica* serovar O:8 were purified by gel filtration, and specific antibodies were obtained. Using these antibodies, we show that the two polypeptides were synthesized de novo during iron starvation and that they were found essentially in the bacterial outer membrane fractions, although the majority of the molecules were not exposed on the cell surface. We also demonstrate that the two proteins had common epitopes and that the HMWPs of the high-virulence-phenotype species *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serovar O:8 (a strain different from the one used to purify the proteins) are antigenically related. The less pathogenic and nonpathogenic strains did not exhibit cross-reacting material, suggesting that these strains do not synthesize even an altered form of the HMWPs.

Since a few years ago, the role of iron in the pathogenicity of many bacteria has become more and more evident. The multiplicity of the mechanisms used by microorganisms to obtain iron prove that this ion is important for their survival. Each bacterium has its own way to react to iron starvation: synthesis of siderophores, expression of novel proteins involved in the binding, transport, and utilization of iron, and production of toxins.

For the genus *Yersinia*, the importance of iron in the degree of pathogenicity has been known for a long time. There have been many clinical reports about the occurrence of septicemia in patients with iron overload due to thalassemia major (5, 6, 19), hemochromatosis (3, 13), cirrhosis (17), long-term iron therapy (10), or accidental oral overdose with iron (11, 12). In 1956, Jackson and Burrows (8) demonstrated that the reduced virulence of the nonpigmented strains of *Y. pestis* can be restored to full virulence if iron is injected along with the bacteria in mice. Nevertheless, nothing was known about the mechanisms developed by the bacteria to obtain iron.

Recently, many new data have been obtained. Robins-Browne and Kaya Prpic (18) showed that the high-virulence phenotype *Y. enterocolitica* serovar O:8 is inherently lethal for mice, whereas the less pathogenic *Y. enterocolitica* serovars O:3 and O:9 are able to kill the animal only if iron or Desferal Mesylate (deferoxamine mesylate; Ciba-Geigy) is added. The synthesis of a siderophore was first demonstrated in *Yersinia* species by Stuart et al. (21). They identified an aerobactinlike siderophore, produced only by the nonpathogenic environmental strains *Y. intermedia*, *Y. kristensenii*, and *Y. fredericksenii*. Later, Heeseman (7) described a novel type of siderophore present in all mouse-lethal *Y. enterocolitica* and *Y. pseudotuberculosis* strains but absent from nonlethal strains. We previously demonstrated that the different *Yersinia* species express novel proteins under iron starvation (4). Among them, two high-molecular-weight proteins (HMWPs) located in the outer membrane were synthesized only by the highly pathogenic strains *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serovar O:8. They were expressed neither by low-pathogenic or nonpathogenic species nor by the live-vaccine strain EV76, a strain known to need exogenous iron to kill mice.

We report in this paper the purification of the HMWPs and the production of specific antibodies. These antibodies were used to investigate the location and the putative forms of the two polypeptides in the different bacterial compartments. We also studied the immunological relationships between the two HMWPs and the existence of common antigenic determinants among the HMWPs of the highly pathogenic species. We show that the two HMWPs have common epitopes. We also demonstrate that the antibodies directed against the HMWPs of *Y. enterocolitica* serovar O:8 reacted with the HMWPs of all the mouse-lethal strains studied, whereas no protein was recognized by the low- and nonpathogenic strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study were *Y. pestis* 6/69M and EV76, *Y. pseudotuberculosis* IP 2637, *Y. enterocolitica* serovar O:8 strains Ye 8081 and WA, serovar O:3 strain IP 864, and serovar O:9 strain IP 383, *Y. intermedia* IP 16835, *Y. fredericksenii* IP 16840, and *Y. kristensenii* IP 16832 (4). Both strain Ye 8081 bearing the 42- to 47-megadalton (MDa) plasmid and its spontaneously plasmid-cured derivative were used. For the other strains, only the plasmid-cured bacteria were studied to avoid expression of the plasmid-encoded protein 1 described by Bolin et al. (1), which has a molecular weight very close to that of the slower HMWP.

After overnight culture at 25°C in peptone broth (Pasteur Diagnostics), the bacteria were collected by centrifugation and washed twice in glass-distilled water. The final suspension was used to inoculate a chemically defined liquid medium described previously (4). This medium was made

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iron poor by the addition of 2,2'-dipyridyl (Sigma Chemical Co.) to a final concentration of 50 μM or iron rich by adding 150 μM $FeCl_3$. Bacteria were allowed to grow for 3 days at room temperature (20 to 23°C) with shaking.

Purification of HMWPs. The plasmid-cured strain Ye 8081 was grown in the iron-poor chemically defined medium, and total membrane proteins were extracted by sonication and centrifugation at 50,000 × *g* for 1 h at 4°C. The pellet contained the membranes and the proteins bound to them. To separate the proteins from the membranes, the membrane fraction was incubated for 1 h in 1% sodium dodecyl sulfate (SDS) at room temperature and subjected again to a 1-h centrifugation. The majority of the proteins were present in the supernatant, while membrane debris remained in the pellet. The supernatant (4 ml) was dialyzed for 3 h against 50 mM Tris–150 mM NaCl–0.1 mM EDTA–0.2% SDS–0.5% 2-mercaptoethanol, pH 7.8 (buffer A). The protein solution was then loaded on a column (1.6 by 100 cm) containing Ultrogel AcA 34 (IBF) and equilibrated in buffer A. Fractions (0.8 ml) were collected over 24 h at a flow rate of 8 ml/h, and samples corresponding to each peak were subjected to electrophoresis as described below.

Preparation of HMWP-specific antibodies. Five female BALB/c mice (Iffa Credo) were immunized with the purified HMWPs. Each mouse received 200 μl of purified protein (ca. 100 μg) subcutaneously on days 1, 30, 52, and 60. The purified proteins (approximately 100 μg) were emulsified in the same volume of Freund incomplete adjuvant (Difco) before injection. On day 54, 0.5 ml of RPMI 1640 medium (Biochrom KG) containing 10^{10} cells of sarcoma cell line 180/TG (20) were injected intraperitoneally into each mouse. The ascitic fluids (ca. 10 ml per mouse) were collected on day 68 and centrifuged at 800 × *g* for 10 min. The supernatants were divided into equal portions and stored at −20°C before use. As a negative control, the ascitic fluid of a nonimmunized mouse was also collected.

SDS-PAGE and protein blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed essentially by the procedure of Laemmli (9), with a 7.5% acrylamide running gel at a constant current of 50 mA. Protein bands were stained with Coomassie blue R or transferred to nitrocellulose. Molecular weight markers (Pharmacia) were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

Immunoblotting was performed by a modification of the method of Burnette (2). Proteins were transferred overnight to the nitrocellulose filter (Hybond C; Amersham Corp.) with a current of 250 mA. Efficiency of transfer was determined by staining the nitrocellulose sheet with 0.2% Ponceau red in 3% trichloroacetic acid for 10 min. Blots were then incubated successively with the HMWP-specific antibodies diluted to 1/200 in phosphate-buffered saline (PBS)–5% powdered milk (Regilait) and the horseradish peroxidase-conjugated anti-mouse immunoglobulin antibodies (Amersham Corp.) diluted to 1/10,000 in the same buffer. Bound antibody was detected by staining with 3-amino-9-ethylcarbazole (Sigma Chemical Co.).

Preparation of protein fractions. To obtain the proteins present in the periplasm, cytoplasm, and inner and outer membrane fractions, 1 liter of the plasmid-cured strain Ye 8081, corresponding to 5 × 10^{10} bacteria, was harvested by centrifugation and washed twice in glass-distilled water. The final pellet was suspended in 6 ml of 20% sucrose–30 mM Tris hydrochloride (pH 8)–1 mM EDTA and subjected to osmotic shock as described by Neu and Heppel (14). The supernatant fluid was called the periplasmic fraction (S1). The pellet was suspended in 100 ml of 5 mM $MgCl_2$–25 mM K_2HPO_4, pH 7, and the cells were disrupted by sonication. Cell debris was removed by low-speed centrifugation, and membranes were pelleted from the supernatant by centrifugation at 50,000 × *g* for 1 h. The supernatant was called the cytoplasmic fraction (S2). The pellet was further differentiated into inner and outer membrane by treatment with Triton X-100 (16). The supernatant was called the inner membrane fraction (S3). The three supernatants were dialyzed against pyrodistilled water and concentrated to 100 μl by rotary evaporation. The pellet (P), called the outer membrane fraction, was suspended in 350 μl of pyrolyzed water. Before electrophoresis, 50 μl of S1 and S3, 5 μl of S2, and 15 μl of P were loaded on a gel.

Most of the time, freshly grown bacteria were only sonicated; cell debris was removed, and the supernatant was centrifuged at 50,000 × *g* for 1 h. The pellet, called the membrane proteins, was suspended in pyrodistilled water and stored at −20°C before use.

Iodination of the outer membrane proteins. Whole bacteria or the membrane fraction was iodinated as described by Sullivan and Williams (22). Briefly, plasmid-cured strain Ye 8081 was grown for 3 days at 25°C in the iron-depleted liquid medium and washed twice in PBS. The cell pellet, corresponding to 10^{10} bacteria, was suspended in 2 ml of PBS and transferred to a glass vial coated with 50 μg of 1,3,4,6-tetrachloro-3,6-diphenylglycouryl (Iodo-Gen; Pierce Chemical Co., Rockford, Ill.). Then, 50 μl of PBS containing 500 μCi of ^{125}INa (Amersham Corp.) was added and allowed to stand at room temperature for 60 s. The bacterial suspension was transferred to a new tube containing 6 ml of 10 mM sodium iodide–PBS and then washed three times with the same buffer and twice with 1 ml of 0.5 M Tris hydrochloride, pH 6.8. The final cell pellet was suspended in the sonication buffer, and total membrane proteins were obtained as described above. When previously isolated membrane fraction instead of whole cells was iodinated, 600 μg of protein was suspended in 1 ml of PBS and transferred to an Iodo-Gen-coated glass vial. The procedure was then similar to that used for whole cells except for the washes, which were carried out at 10,000 × *g* for 30 min. Electrophoresis of radiolabeled samples was accomplished as described above. One part of the gels was stained with Coomassie blue, and the other part was dried before being exposed to a Kodak X-Omat R film (Eastman Kodak Co.).

Trypsinization of whole bacteria and isolated membrane fraction. Plasmid-cured strain Ye 8081 was grown for 3 days in the iron-poor liquid medium and washed twice with PBS; 1 ml of the suspension, containing 10^{10} bacteria, was incubated at 37°C for 30 min in the presence or absence of trypsin (Sigma Chemical Co.) at a final concentration of 40 μg/ml. Soybean trypsin inhibitor (Sigma Chemical Co.) was added at the same final concentration to stop the reaction, and membrane proteins were extracted as described above. To test the sensitivity to trypsin of membrane proteins, 100 μg of the membrane fraction was suspended in 50 μl of PBS and incubated at 37°C for 30 min with or without trypsin as described above. Membrane proteins were kept at −20°C before being solubilized and subjected to electrophoresis.

Elution of antibodies from nitrocellulose filters. The modified method of Olmsted (15) was used to elute the antibodies bound to the nitrocellulose. Briefly, total membrane proteins from Ye 8081 were subjected to SDS-PAGE and transferred electrically to the nitrocellulose. The two HMWP bands were stained with Ponceau red before being individually cut. They were saturated with PBS-milk and incubated with a

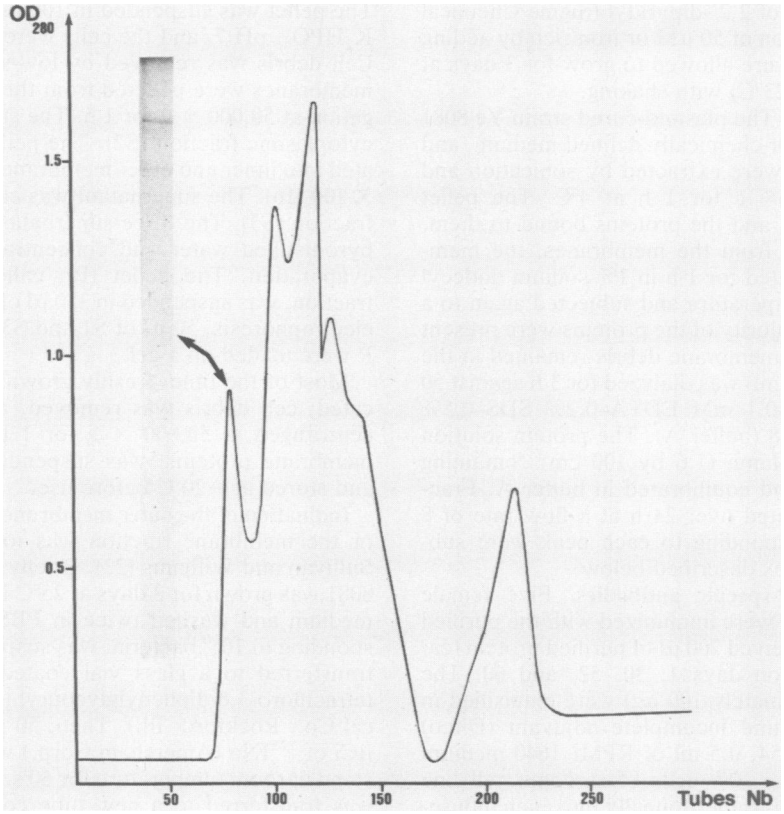

FIG. 1. Purification of HMWPs. Points show the optical density of the different membrane protein fractions from the iron-starved strain Ye 8081 eluted from an AcA 34 gel filtration column. The first peak and its corresponding electrophoresed proteins are indicated by arrows. Nb, Number.

1/100 dilution of the HMWP antibodies. Each strip was then incubated for 10 min at 4°C in 1 ml of 0.2 M glycine hydrochloride (pH 2.2)–1% Tween 20–0.5% powdered milk. The eluted antibody solutions specific for each HMWP were neutralized with 1 M Tris and allowed to react with total membrane proteins previously transferred to nitrocellulose. Immunoblotting was then carried out as described above.

RESULTS

Purification of the two HMWPs by gel filtration. We took advantage of the high molecular weights of the two HMWPs (ca. 190,000 and 240,000) to purify them by filtration. After passage of membrane proteins of *Y. enterocolitica* serovar O:8 (strain Ye 8081) through an AcA 34 column, five peaks were resolved by spectrophotometry at 280 nm and analyzed after electrophoresis. As shown in Fig. 1, the first peak corresponded to the two almost pure HMWPs. Nevertheless, proteins with too high a molecular weight to penetrate the gel could also have been present in the fraction corresponding to the first peak. To try to answer this question, the purified HMWPs were allowed to migrate to the lower part of a 5% acrylamide gel. After staining, nothing except the two HMWPs was visualized on the gel (data not shown).

Production of HMWP-specific antibodies. Purification of the HMWPs was necessary to obtain specific antibodies because the two proteins were poorly antigenic when injected with total membrane proteins and obtained in insufficient amount when eluted from the polyacrylamide gel. The purified HMWP fraction was used to immunize BALB/c mice. After four injections corresponding to a total amount of 400 μg of protein per mouse, the peritoneal exudate induced by neoplastic cells was collected and tested by immunoblotting. The antibodies obtained specifically recognized the two HMWPs, whereas the control ascitic fluid did not react with either protein (data not shown). The 190,000-M_r HMWP, although slightly less stained by Coomassie blue, reacted more strongly with the antibodies than did the 240,000-M_r HMWP. No protein was recognized when bacteria were iron replete.

Effect of disulfide bonds on migration of HMWPs. The two HMWPs were not visible on the polyacrylamide gel if 2-mercaptoethanol was omitted from the denaturing buffer. Immunoblotting with the HMWP antibodies demonstrated that when disulfide bonds were intact, these proteins remained at the top of the running gel (data not shown).

Location of HMWPs in different bacterial compartments. As shown in Fig. 2A, the HMWPs were essentially found in the outer membrane fraction. The polypeptides were not visualized in the inner membrane and were present in minute amounts in the cytoplasmic and periplasmic fractions. These data could indicate the real presence of the HMWPs in the cytoplasm and periplasm or contamination of these fractions by the outer membrane proteins. The presence of the HMWPs in the outer membrane fraction does not mean that the two polypeptides are bound to the outer membrane; they could be only associated with it or may have been extracted with the membrane fraction. The specific antibodies recognized only two bands corresponding to the molecular weights of the HMWPs in the cytoplasm, periplasm, and outer membrane fractions (Fig. 2B). Proteins with a different

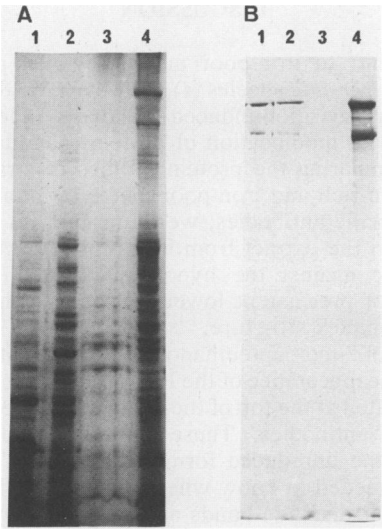

FIG. 2. Subcellular localization of the HMWPs. Shown are SDS-PAGE (A) and immunoblotting with HMWP-specific antibodies (B) of proteins from iron-starved strain Ye 8081. Lanes: 1, periplasmic proteins; 2, cytoplasmic proteins; 3, inner membrane proteins; 4, outer membrane proteins.

size were not detected by the antiserum in the various fractions.

In order to know whether the HMWPs were exposed on the cell surface, two kinds of experiments were carried out. First, iodination of the proteins exposed on the surface of whole iron-starved bacteria was performed. As shown in Fig. 3A, the HMWPs were not iodinated, suggesting that the HMWPs were located on the inner side of the outer membrane. The absence of iodinated HMWPs was not due to a lack of accessible tyrosine and histidine, since it was possible to label them when the membrane fraction was previously extracted (Fig. 3B). Second, we looked at the action of

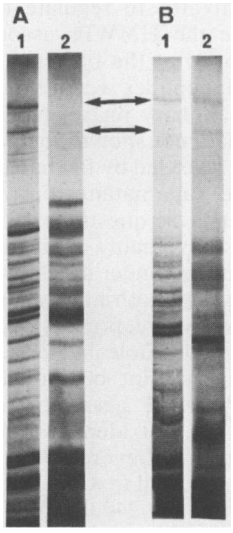

FIG. 3. ^{125}I labeling of membrane proteins from iron-starved strain Ye 8081. SDS-PAGE (lanes 1) and autoradiography (lanes 2) of membrane proteins radioiodinated on whole bacteria (A) and of membrane proteins labeled after being extracted (B). Arrows indicate the HMWPs.

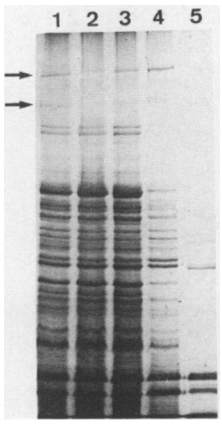

FIG. 4. Trypsin sensitivity of membrane proteins from iron-starved strain Ye 8081, visualized by SDS-PAGE. Lanes: 1, membrane proteins extracted without incubation at 37°C; 2, whole bacteria incubated at 37°C for 30 min before protein extraction; 3, whole bacteria incubated for 30 min at 37°C in the presence of trypsin; 4, membrane proteins incubated for 30 min at 37°C; 5, membrane proteins incubated for 30 min at 37°C in the presence of trypsin. Arrows indicate the HMWPs.

trypsin on the outer membrane proteins associated with either the extracted membrane fraction or whole bacteria. In the isolated membrane fraction, the HMWPs were sensitive to the action of trypsin (Fig. 4, lane 5). This proteolysis was actually due to the enzyme and not to a nonspecific degradation occurring at 37°C, since the same sample incubated under the same conditions without trypsin did not lead to degradation of the HMWPs (Fig. 4, lane 4). In contrast, when associated with whole bacteria, the HMWPs were not trypsin sensitive (Fig. 4, lane 3). These two results strongly support the hypothesis that the majority of the HMWP molecules are not exposed on the cell surface.

Immunological relationships between the two HMWPs. To try to understand whether the HMWPs were unrelated or had some common part, specific antibodies to each polypeptide were eluted from the nitrocellulose and allowed to react with total membrane proteins. As shown in Fig. 5, the anti-240,000-M_r HMWP-specific antibodies recognized with equal intensity the 190,000- and the 240,000-M_r proteins. In contrast, antibodies eluted from the protein band of 190,000 M_r reacted strongly with this protein and only slightly with the 240,000-M_r polypeptide. These results demonstrate the existence of immunological relationships between the two HMWPs.

Comparison of HMWPs from plasmid-bearing and plasmid-cured Ye 8081 by immunoblotting. We have previously shown that both the 42- to 47-MDa-plasmid-bearing strain Ye 8081 and its plasmid-cured derivative express the two HMWPs (4). When the specific antibodies were incubated with total membrane proteins from the two iron-depleted variants, the HMWPs were recognized equally (Fig. 6B, lanes 2 to 5), confirming that the virulence plasmid did not play any role in the expression of these two polypeptides.

Immunological relationships between the HMWPs of various *Yersinia* species. The two HMWPs have been found in all the highly virulent species of *Yersinia* studied (4). These proteins had the same apparent molecular weights on a polyacrylamide gel (Fig. 6A). We used the specific antibodies raised against the *Y. enterocolitica* serovar O:8 HMWPs to compare them further. These antibodies also recognized the HMWPs of the highly pathogenic strains WA (*Y. entero-*

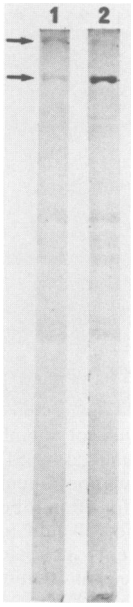

FIG. 5. Immunological cross-reactivity of the two HMWPs. Immunoblotting of membrane proteins from iron-starved strain Ye 8081 was performed with the antibodies eluted from the higher-M_r HMWP (lane 1) or from the lower-M_r HMWP (lane 2). Arrows indicate the HMWPs.

colitica serovar O:8), IP 2637 (*Y. pseudotuberculosis*), and 6/69 M (*Y. pestis*). For these strains, as for strain Ye 8081, no proteins reacted with the antibodies when the bacteria were grown in the presence of excess iron (Fig. 6B, lanes 4 to 11). The nonpathogenic mutant strain EV76, the low-virulence strains IP 864 (Fig. 6B, lanes 12 to 15) and IP 383, and the avirulent environmental strains IP 16835, IP 16840, and IP 16832 (data not shown) did not express any protein recognized by the HMWP antibodies. These results suggest that these strains did not synthesize even a shortened part of either HMWP.

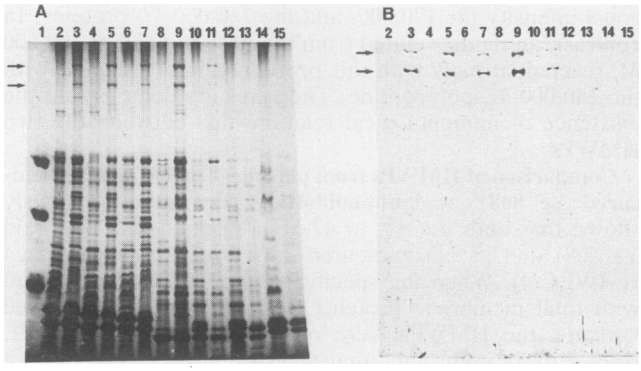

FIG. 6. Immunological cross-reactivities of the HMWPs from various *Yersinia* species. SDS-PAGE (A) and immunoblotting with the HMWP-specific antibodies (B) of membrane proteins from iron-starved (−Fe) or iron-replete (+Fe) strains. Lanes: 2, Ye 8081 +Fe harboring the virulence plasmid; 3, Ye 8081 −Fe harboring the virulence plasmid; 4, plasmid-cured Ye 8081 +Fe; 5, plasmid-cured Ye 8081 −Fe; 6, WA +Fe; 7, WA −Fe; 8, IP 2637 +Fe; 9, IP 2637 −Fe; 10, 6/69M +Fe; 11, 6/69M −Fe; 12, EV76 +Fe; 13, EV76 −Fe; 14, IP 864 +Fe; 15, IP 864 −Fe. Lane 1, Molecular weight standards (see text). *Arrows* indicate the two HMWPs.

DISCUSSION

After a shift to iron-poor medium, novel proteins are expressed in *Yersinia* species (4). However, we did not know whether iron starvation induced de novo synthesis of these polypeptides or modification of proteins already present in the cell. Comparing the protein profiles of strain Ye 8081 grown in iron-rich and iron-poor media by immunoblotting with our specific antibodies, we found that no protein was recognized in the extract from iron-replete bacteria. These results argue against the hypothesis of an iron-induced association of preexistent lower-molecular-weight proteins in a more complex structure.

Omission of 2-mercaptoethanol from the denaturing buffer resulted in disappearance of the HMWPs and the appearance of bands located at the top of the gel that were recognized by the specific antibodies. These proteins probably corresponded to the unreduced forms of the HMWPs. Further studies are needed to know whether the two HMWPs have intramolecular disulfide bonds and whether they are linked together or associated with other molecules.

Iron starvation could have induced the synthesis of the HMWPs in their final form or the synthesis of smaller proteins which would associate to yield a higher-molecular-weight complex. To answer this question, the presence of the two proteins in different bacterial compartments was studied. We showed that the HMWPs are essentially located in the outer membrane fraction and that the specific antibodies recognized only the bands with molecular weights corresponding to those of the HMWPs. The association of iron-induced small polypeptides in the cytoplasm or in the outer membrane to form the HMWPs is therefore improbable. From these results we can hypothesize that iron starvation is responsible for a signal which induces the synthesis of the whole two polypeptides in their final form. The proteins would then cross the inner membrane to reach the periplasmic space, where they would either become anchored on the outer membrane (a great portion of the HMWPs not being exposed on the cell surface) or only associate with the outer membrane. Nevertheless, many questions are still without answers. Does the iron act directly or indirectly on a repressor or an activator to regulate transcription of the HMWP genes? Are the HMWPs associated in the outer membrane? We looked at the bacterial supernatant to see whether the two polypeptides were released during bacterial growth or at the stationary phase. Nothing was recovered from the medium (data not shown), but we cannot state that the HMWPs are not released by the bacteria; they could also be degraded in the supernatant. Further studies will be necessary to answer these questions.

The two HMWPs have many characteristics in common: they are only synthesized under iron starvation by the highly pathogenic species; they both have disulfide links involved in their structure; they are present together in the outer membrane fraction; on whole bacteria, both of them are trypsin resistant and cannot be iodinated; antibodies directed against one HMWP also recognize the other one. Nevertheless, they are not identical, since they have different molecular weights. Several explanations can be proposed. It could be suggested that the 190,000-M_r HMWP is a degradation product of the 240,000-M_r protein created during processing of the samples. This hypothesis is improbable, since the two proteins were also found when whole iron-starved bacteria were directly solubilized in Laemmli buffer and subjected to electrophoresis (data not shown). The 190,000-M_r protein could also be the active form of the

240,000-M_r HMWP obtained after specific enzymatic cleavage. In this case, the antibodies eluted from the 190,000-M_r protein should have recognized the denatured slower HMWP as well as the faster one. The most probable explanation for us is that the two HMWPs are composed in part of a common fragment and in part of another protein segment. Biochemical and genetic experiments are in progress to confirm this hypothesis.

The antibodies directed against the HMWPs of *Y. enterocolitica* serovar O:8 recognized the HMWPs of all the different highly pathogenic species studied. This strongly suggests conservation of the gene coding for these two polypeptides among the high-virulence phenotype *Yersinia* species. The fact that no protein reacted with the specific antibodies in the low- and nonpathogenic strains suggests that these strains did not synthesize even an altered form of the proteins. This could correspond to a mutation in the promoter or the beginning of the gene or to deletion of a great part or all of the gene in the low-virulence species. Alternatively, it could correspond to the insertion of a transposon carrying the sequence coding for the HMWPs in the DNA of the highly pathogenic species. Genetic studies have been carried out to answer this question (Carniel et al., submitted for publication).

The highly pathogenic *Yersinia* species express the HMWPs, whereas the low-virulence and nonvirulent strains do not synthesize these polypeptides. The mutant strain *Y. pestis* EV76 also does not express the HMWPs. Nevertheless, this strain and the low-virulence phenotype strains *Y. enterocolitica* serovars O:3 and O:9 can become lethal to mice if the animal is iron overloaded (8, 18). Our hypothesis is that the HMWPs confer to the bacteria the ability to catch the iron bound to eucaryotic molecules (transferrin, lactoferrin, etc.) in vivo. In contrast, the low-virulence phenotype species and the live-vaccine strain EV76, which do not express the HMWPs, are not pathogenic unless iron is readily available as an exogenous supply. For these reasons it is important to further characterize these proteins. Purification of the HMWPs and production of specific antibodies allowed us to demonstrate the immunological relationships among the two HMWPs and among the highly pathogenic species; to show that these polypeptides are not synthesized even in a truncated form in the low-virulence and nonvirulent strains; and to locate the HMWPs in the outer membrane fraction. Further studies are necessary to understand their role and their regulation.

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