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Expression of Iron-Regulated Proteins in *Yersinia* Species and Their Relation to Virulence

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Under iron-starvation conditions, the different *Yersinia* species expressed various iron-regulated proteins. Among them, two high-molecular-weight outer membrane proteins were synthesized in high-virulence-phenotype *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serovars O:8 and O:Tacoma but were present neither in low-virulence phenotype *Y. enterocolitica* serovars O:3 and O:9 nor in avirulent *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, and *Y. enterocolitica* serovar O:39. Thus, the degree of virulence correlates with the presence of the two high-molecular-weight proteins in *Yersinia* species.

Bacteria need at least minute amounts of iron in their environment or culture medium to grow. In mammalian hosts, proteins such as transferrin in blood and lactoferrin in secretions hold the extracellular iron present in those fluids. Bacteria are unable to grow unless they develop a system to compete with those proteins for iron.

Under iron-starvation conditions, many procaryotes produce iron chelators, called siderophores, used for trapping and transporting iron molecules into the cells (6, 12, 16, 17, 20, 21, 28). Along with the production of siderophores, the synthesis of high-molecular-weight outer membrane proteins is induced in *Escherichia coli* (4, 14), *Salmonella typhimurium* (2), *Vibrio cholerae* (24), *Vibrio anguillarum* (8), *Shigella flexneri* (18), and *Neisseria gonorrhoeae* (15), among others. A role in the transport of the iron-siderophore complex into the cell has been attributed to some of these proteins.

Although Wake et al. (27) showed indirect evidence for the production of siderophores by *Yersinia pestis*, those iron chelators have never been identified in *Yersinia* species. Nevertheless, *Yersinia enterocolitica* can utilize desferrioxamine B, an hydroxamate siderophore from *Streptomyces pilosus*, to grow in vitro (5) and to enhance virulence in mice (23). As do other bacteria, *Yersinia* species need iron molecules to grow. A low-virulence-phenotype *Y. pestis* can be restored to full virulence by concomitant injection of sufficient iron in mice (7). Furthermore, naturally low-virulent *Y. enterocolitica* serovars O:3 and O:9 can become as lethal for mice as highly virulent *Y. enterocolitica* serovar O:8 when iron is available for the bacteria (23). The purpose of the present study was to determine whether *Yersinia* species, like many other bacterial species, synthesize new proteins under iron-starvation conditions and to investigate the possible correlation between the presence of these iron-regulated proteins (IRPs) and the degree of pathogenicity of the strains.

The iron-poor liquid medium used in our experiments was a chemically defined medium derived by Perry and Brubaker (19). It contained 50 mM NaCl, 10 mM NH_4Cl, 0.4 mM K_2HPO_4, 2.5 mM $Na_2S_2O_3$, 40 mM morpholinepropanesulfonic acid, and 10 mM tricin (E. Merck AG). The pH was adjusted to 7 with NaOH, and the medium was heat sterilized at 110°C for 15 min. Fructose (0.1%) and Casamino Acids (0.2%; Difco Laboratories) were filter sterilized before being added to the medium. The final concentration of iron measured by flame atomic absorption spectroscopy was 1.2 μM, a concentration still sufficient to allow the growth of *Yersinia* species. Transferrin (150 μg/ml; Sigma Chemical Co.) or $FeCl_3$ (150 μM) was added to the medium 24 h before use. All glassware used was immersed overnight in 6 N HCl and extensively rinsed with pyrolyzed water.

Strain Ye 8081 of *Y. enterocolitica* serovar O:8 (kindly provided by D. A. Portnoy, Stanford University) was grown overnight in peptone broth (Pasteur Diagnostics) at 25°C, washed twice in pyrolyzed water, suspended in the chemically defined medium, and incubated at 25°C. A 3-ml sample of the culture was harvested each day for 5 days. Bacteria were washed twice in pyrolyzed water, and the pellets were suspended in 30 μl of Laemmli solubilization buffer (13) and boiled for 5 min at 100°C. After centrifugation at 5,000 × *g* for 5 min, 10 μl of the supernatant containing total cell proteins was electrophoresed under denaturating conditions (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) by using a 12.5% running gel and a 3% stacking gel at a constant current of 50 mA. The gels were then stained with Coomassie blue R. SDS-PAGE (Fig. 1) revealed the appearance of at least four new bands (two high-molecular-weight proteins [HMWPs] and two proteins of 81,000 and 79,000 molecular weight) and the amplification of four others (89,000, 70,000, 68,000, and 27,500 molecular weight) during iron limitation. Conversely, some proteins were synthesized in reduced amounts in iron-limited media, confirming that the intracellular iron level induces a switch in protein synthesis. The six IRPs with molecular weights between 27,500 and 89,000 were expressed within 24 h, whereas the two HMWPs appeared only after 3 days and were well visible after 5 days of growth in the iron-poor medium (Fig. 1). This unusually long incubation was necessary because the liquid medium did not allow rapid bacterial growth. Iron-repleted cells reached a plateau after 3 days of culture in this medium, and iron-starved cells reached a plateau only after 5 days (data not shown). Thus, the HMWPs were induced when bacteria were still growing, and maximal expression was obtained at high bacterial concentrations.

To determine the location of these proteins, outer membranes were obtained by sonic disruption of the cells and were separated from inner membranes by treatment with

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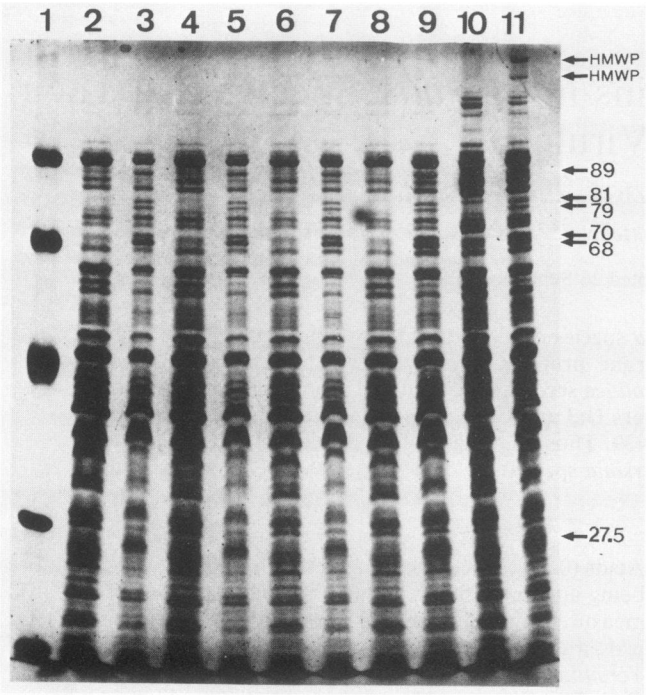

FIG. 1. SDS-PAGE of total cell proteins from Ye 8081 grown for 1 day (lanes 2 and 3), 2 days (lanes 4 and 5), 3 days (lanes 6 and 7), 4 days (lanes 8 and 9), or 5 days (lanes 10 and 11) in an iron-rich (lanes 2, 4, 6, 8, and 10) or iron-poor (lanes 3, 5, 7, 9, and 11) liquid medium. Calibration proteins (Pharmacia; lane 1) are phosphorylase *b* (94 kilodaltons [kDa]), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa). Arrows indicate the IRPs with their molecular weights expressed in thousands.

Triton X-100 (22) before being solubilized in Laemmli buffer. The concentration of the two HMWPs was increased by this treatment (Fig. 2), indicating that they are located mainly on the outer membrane. The six other IRPs, although present, were not major components of the outer membrane.

To ensure that these proteins were not induced by transferrin itself but by iron starvation, bacteria were grown in

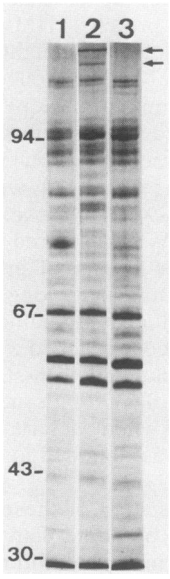

FIG. 2. SDS-PAGE of outer membrane proteins from Ye 8081 grown for 5 days in the presence of 150 μM $FeCl_3$ (lane 1), 150 μg of transferrin per ml (lane 2), or 150 μM iron plus 150 μg of transferrin per ml (lane 3). Tick marks, Standard molecular weights (in thousands). Arrows show the two HMWPs.

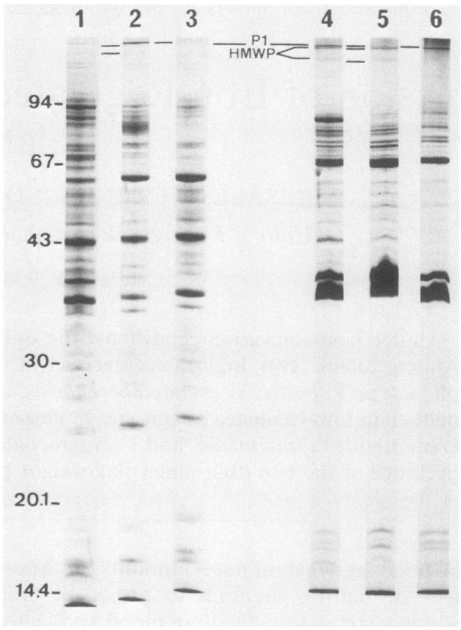

FIG. 3. SDS-PAGE of total cell proteins from Ye 8081 incubated at 25°C in the presence of transferrin (lane 1) or at 37°C in the presence of transferrin (lane 2) or iron (lane 3). Also shown are outer membrane proteins of the same strain incubated at 37°C with transferrin and boiled in Laemmli solubilization buffer for 5 min (lane 4), 10 min (lane 5), or 5 min without 2-mercaptoethanol (lane 6). Tick marks, Standard molecular weights (in thousands); P1, protein 1.

medium containing both transferrin (150 μg/ml) and $FeCl_3$ (150 μM). Iron excess inhibited the synthesis of these proteins, even in the presence of transferrin (Fig. 2, lane 3).

Under calcium-starvation conditions, *Yersinia* species are known to synthesize new proteins at 37°C but not at 25°C (9). To investigate whether the expression of the IRPs was temperature dependent, bacteria were grown at 25 and 37°C in the iron-rich or -poor liquid medium, and total cell proteins were obtained as described above. The IRPs were synthesized at both temperatures, indicating that the genes encoding these proteins are not thermoregulated (Fig. 3). The temperature-dependent protein 1 of Bolin et al. (3) was expressed at 37°C and was very close to the higher HMWP. To differentiate these two polypeptides, samples were boiled for 10 min instead of 5 min in the solubilization buffer. It was previously shown that protein 1 will then dissociate into subunits of 52,500 molecular weight (26). Actually, this treatment considerably decreased the staining of the band corresponding to protein 1 but did not modify the two HMWPs (Fig. 3, lane 5). These results indicate that the HMWPs are not affected by strong reducing conditions. In contrast, when 2-mercaptoethanol was omitted from the solubilization buffer, the two HMWPs disappeared (Fig. 3, lane 6), showing that some disulfide bonds are involved in the structure of these proteins.

Various serovars of *Y. enterocolitica* were then studied for the presence of IRPs after a downshift to an iron-poor

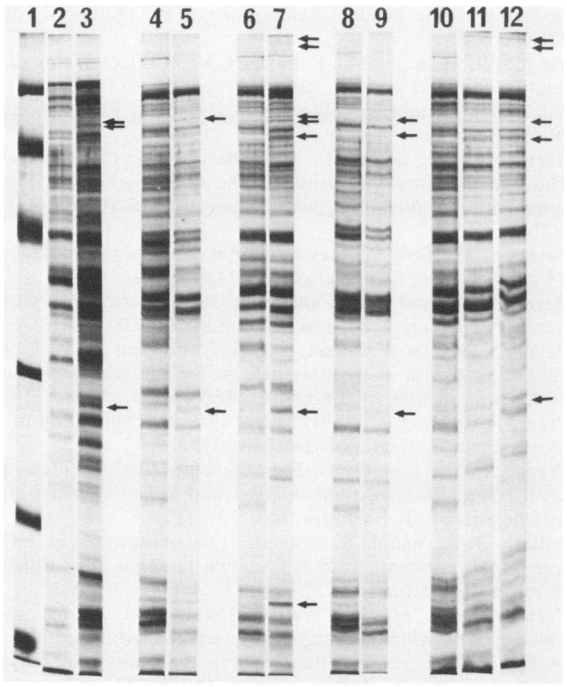

FIG. 4. SDS-PAGE of total cell proteins from various serovars of *Y. enterocolitica* grown under conditions of iron starvation (−Fe) or iron excess (+Fe). Lanes: 1, standard molecular weights as described in the legend to Fig. 1; 2, IP 16498 + Fe; 3, IP 16498 − Fe; 4, IP 383 + Fe; 5, IP 383 − Fe; 6, Ye 9576 + Fe; 7, Ye 9576 − Fe; 8, IP 864 + Fe; 9, IP 864 − Fe; 10, WA + Fe; 11, WA − Fe; 12, WA spontaneously plasmid cured − Fe. Arrows indicate the IRPs.

medium. Strains IP 383 (serovar O:9; Centre National des Yersinia Collection, Institut Pasteur), IP 864 (O:3), IP 16498 (O:39), Ye 9576 (O:Tacoma; from D. A. Portnoy), and WA (O:8; 9) were grown for 5 days in an iron-poor or -rich medium, and total cell proteins were then electrophoresed as described above (Fig. 4). Two IRPs, of 27,500 and 79,000 molecular weight, were present in the various serovars studied, whereas some others were expressed only by specific strains. An 81,000-molecular-weight protein was found only in strains Ye 8081, IP 16498, and Ye 9576; an 18,000-molecular-weight polypeptide was found in strain WA; and a 70,000-molecular-weight protein was found in strains Ye 8081, Ye 9576, IP 864, and WA. Strikingly, the two HMWPs were present in the highly virulent strains of serovars O:8 and O:Tacoma but were expressed neither by the low-virulence-phenotype strains of serovars O:3 and O:9 nor by the avirulent strain of serovar O:39. Because these results strongly suggest that the expression of the two HMWPs is associated with the expression of virulence, we looked at the protein profile of other avirulent or highly virulent *Yersinia* species.

First, the avirulent environment species *Y. frederiksenii* (IP 16840), *Y. intermedia* (IP 16835), and *Y. kristensenii* (IP 16832) were grown in the iron-rich or -poor liquid medium, and total cell proteins were electrophoresed. *Y. intermedia* expressed the 89,000- and 70,000-molecular-weight IRPs, *Y. kristensenii* expressed only the 79,000-molecular-weight protein, and *Y. frederiksenii* expressed the 89,000-, 79,000-, 70,000-, and 27,500-molcular-weight polypeptides, but, as expected, no HMWPs were synthesized by these strains (Fig. 5A).

Second, the protein profiles of the highly virulent *Y. pseudotuberculosis* IP 2637 (25) and *Y. pestis* YP 6/69M (1) and of the avirulent strain *Y. pestis* EV 76 (11; a strain known to need exogenous iron to express its pathogenicity) were studied. The two HMWPs were present in both strains IP 2637 and YP 6/69M but were absent from strain EV 76 (Fig. 5B). In addition, an IRP of 70,000 molecular weight was present in strains YP 6/69M and IP 2637, but no IRP was found in strain EV 76. These results are highly suggestive of an association between the virulence of *Yersinia* species and the presence of the two iron-regulated HMWPs.

As a first approach to determining the genetic basis of the IRPs, virulence-plasmid-bearing strains Ye 8081, IP 383, IP 864, WA, IP 2637, and YP 6/69M and their spontaneously plasmid-cured derivatives were compared. The IRP patterns were identical in strain WA, whatever its plasmid content (Fig. 4, lane 12). The same results were obtained with the other plasmid-harboring strains and their plasmid-cured variants (data not shown). This indicates that the genes encoding the IRPs are not located on the virulence plasmid. Moreover, because no other plasmid is shared by the various pathogenic *Yersinia* species, it is probable that the IRPs are chromosomally encoded. These data strengthen previous results obtained by Heesemann et al. (10) which demonstrated that the presence of the virulence plasmid is not sufficient for the expression of lethality in mice.

The observations reported here demonstrate for the first time the existence of IRPs in *Yersinia* species. Furthermore, they establish a correlation between the presence of the two iron-regulated HMWPs and the virulence of the species. To establish the role of the genes encoding the two HMWPs in the expression of a high-virulence phenotype, genetic complementation experiments are needed.

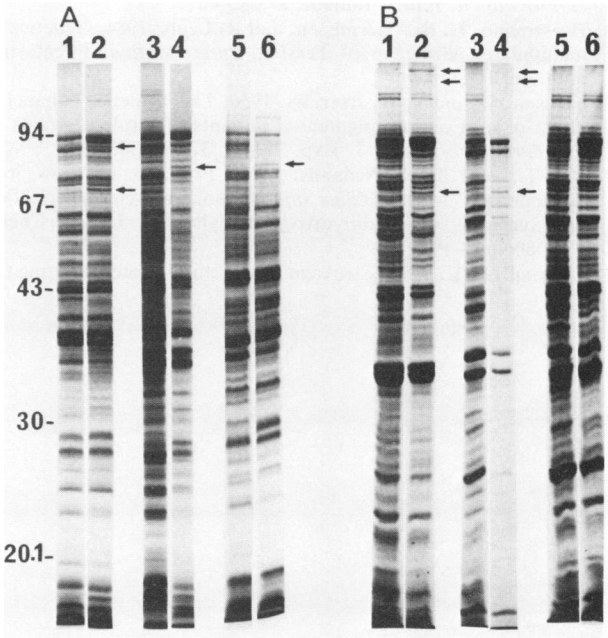

FIG. 5. SDS-PAGE of total cell proteins from various *Yersinia* species grown under conditions of iron starvation (−Fe) or iron excess (+Fe). (A) Environmental species. Lanes: 1, IP 16835 + Fe; 2, IP 16835 − Fe; 3, IP 16832 + Fe; 4, IP 16832 − Fe; 5, IP 16840 + Fe; 6, IP 16840 − Fe. (B) Virulent species. Lanes: 1, IP 2637 + Fe; 2, IP 2637 − Fe; 3, YP 6/69M + Fe; 4, YP 6/69M − Fe; 5, EV 76 (virulent only when exogenous iron is added) + Fe; 6, EV 76 − Fe. Tick marks, Standard molecular weights (in thousands). Arrows indicate the IRPs.

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