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Virulence Plasmid-Associated Autoagglutination in *Yersinia* spp.

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The autoagglutination of *Yersinia enterocolitica* was dependent on the presence of the virulence plasmid and on the active growth of bacteria in tissue culture media at 37°C. Cultures with a high initial concentration of bacteria failed to autoagglutinate, indicating that synthesis of new virulence plasmid-associated surface factors was essential for autoagglutination. The synthesis of a plasmid-encoded polypeptide (molecular weight, 240,000), designated P1, that could be dissociated under strongly reducing conditions into subunits of 52,500 daltons was found to be correlated with autoagglutination. Further, a strain of *Yersinia pseudotuberculosis* [YPIII(PIB102)], which has Tn*5* inserted within the structural gene of P1 that prevents the synthesis of P1, failed to autoagglutinate, in contrast to the wild-type strain, strongly indicating that P1 is involved in this phenomenon. It was also found by immunoblotting that in addition to the common response to temperature, the P1 proteins of *Y. enterocolitica* and *Y. pseudotuberculosis* were immunologically related.

The virulence of the three *Yersinia* species, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, has been associated with a 40- to 50-megadalton plasmid (1, 3, 7–9, 17, 21). This virulence plasmid is associated with several virulence-associated characteristics of yersiniae, i.e., Ca^{2+}-dependent growth at 37°C (8, 17), production of VW antigens (1, 7, 19, 20), and autoagglutination (12, 13, 16, 21). Other virulence plasmid-associated phenomena are production of specific outer membrane proteins (3, 14, 18), serum resistance (14–16), tissue culture cell adherence and cytotoxicity (18, 21), and low 50% lethal dose for test animals (9, 18).

In the present study, one plasmid-associated phenomenon, autoagglutination, was studied. The phenomenon was introduced in 1967 when *Y. pseudotuberculosis* PB1 VW^+, but not its avirulent derivative (VW^-), was observed to autoagglutinate at 37°C in 0.8% Casitone (5). Then, in 1980, autoagglutinability was found to be associated with known virulence characteristics of yersiniae (12); i.e., Ca^{2+}-dependent growth at 37°C and VW antigen production (6). Since then, several articles have been published about virulence plasmids and autoagglutination, but characterization of the autoagglutination phenomenon as such has not been considered. This study was intended to supply some basic information about autoagglutination.

MATERIALS AND METHODS

Bacteria and plasmids. *Y. enterocolitica* serotype O:3 strain 6471/76, serotype O:9 strain Ruokola/71, and *Y. pseudotuberculosis* serotype I strain 2812/79 were isolates from human stool specimens (M. Skurnik, J. Appl. Bacteriol., in press). These strains harbored a virulence plasmid. Plasmid-cured derivatives, strains 6471/76c and Ruokola/71c, were obtained from single colonies growing on magnesium-oxalate agar at 37°C (11). The loss of the virulence plasmid from these strains was verified by direct plasmid isolation (2) and by the autoagglutination test (12). The origin of *Y. pseudotuberculosis* serotype III strains YPIII(PIB1), YPIII, YPIII (PIB101), and YPIII(PIB102) has been described elsewhere (4). Strain YPIII(PIB1) is a wild-type strain that contains the virulence plasmid; strain YPIII is a plasmid-cured derivative of strain YPIII(PIB1); strain YPIII(PIB101) has in its virulence plasmid a Tn*5* insertion that prevents the synthesis of all plasmid-coded outer membrane proteins; and strain YPIII(PIB102) has its virulence plasmid a Tn*5* insertion that prevents the synthesis of polypeptide P1 (4).

Infection of guinea pigs. Strain 6471/76 and its isogenic plasmidless derivative strain 6471/76c were tested by experimental infection of guinea pigs. The strains were grown aerated overnight at RT (room temperature, 22°C) in Luria broth. The bacteria, suspended in 10% $NaHCO_3$, were introduced with a balloon catheter intragastrically into guinea pigs. Three days later, the guinea pigs were killed and their small intestines were removed aseptically. The intestines were homogenized, and the concentrations of *Y. enterocolitica* strains in the homogenates were determined by dilution plating.

Serum resistance test. The serum resistance assay was performed in 100% human serum at 37°C. The bacterial concentration in the beginning was ca. 10^6 per ml. The strains were precultured overnight in meat extract broth at RT or at 37°C. Measurements of living bacteria in serum tubes were done by dilution plating after incubation for 0, 1, and 2 h.

Culture conditions. Bacteria for autoagglutination tests were precultured overnight in Luria broth at RT. BME (basal medium Eagle, Flow Laboratories, Irvine, United Kingdom) was used to perform autoagglutination tests. Quantitations of bacterial concentrations were made either by measuring the absorbance of cultures at 590 nm or by determining the viable counts by the dilution plating method. The bacterial suspensions were pipetted vigorously before dilution plating and absorbance measurements, to break down the bacterial aggregates.

Electron microscope samples of whole cells were prepared on carbon-coated copper grids and negatively stained with 0.2% phosphotungstate. Autoagglutinated cells appeared characteristically as clumps of cells stuck together.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) runs were made in gels of 1 mm thickness. Commercial molecular weight standards were used (Bio-Rad

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TABLE 1. Effect of initial concentration of bacteria in BME on autoagglutination and on growth of *Y. enterocolitica* 6471/76

Initial CFU/ml	Incubation time (h)	Autoagglutination[a]	Final CFU/ml	Multiplication ratio[b]
2.6×10^8	5	−	3.8×10^8	1.5
5.3×10^7	5	+	1.4×10^8	2.6
5.3×10^5	6	+	2.0×10^7	38
5.3×10^3	8.25	−	1.4×10^6	264
5.3×10^1	8.25	−	1.3×10^4	245

[a] The cultures were checked every 30 min for the presence of autoagglutinated bacteria.

[b] Multiplication ratio = $\frac{\text{final CFU/ml}}{\text{initial CFU/ml.}}$

Laboratories, Richmond, Calif. or Pharmacia Fine Chemicals, Uppsala, Sweden). Bacteria for SDS-PAGE were pelleted and suspended in 1 ml of water in 1.5-ml Eppendorf tubes. The suspensions were sonicated for 5 s and centrifuged for 5 min in an Eppendorf 5413 centrifuge. The pellets were washed twice with 1 ml of water, and the supernatants were combined and lyophilized. The lyophilized material was solubilized in water and used as a protein sample in SDS-PAGE. Outer membranes of the bacteria were prepared as described previously (3). The protein samples were incubated for 5 to 10 min in a boiling water bath in a reduction mix containing 0.066 M Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 9% glycerol, and bromophenol blue as a dye marker.

Western blot of outer membrane proteins was done as described previously (4). The specific anti-P1 serum was prepared in rabbits immunized with purified material (4).

RESULTS

Characterization of strains 6471/76 and 6471/76c. Strain 6471/76c was obtained from a large colony growing on a magnesium-oxalate agar plate at 37°C. Plasmid isolation experiments from strain 6471/76c showed the absence of the 48-megadalton plasmid present in strain 6471/76. Strain 6471/76c did not autoagglutinate in tissue culture medium at 37°C as did strain 6471/76. Results of experimental infections of guinea pigs with strains 6471/76 and 6471/76c showed that infection with the plasmid-bearing strain had caused inflammation of the small intestine and a high number of bacteria were recovered from the tissue homogenate, whereas infection with plasmid-cured strain did not cause inflammation and only a few bacteria were recovered from the homogenate. The results of serum resistance tests of strains 6471/76 and 6471/76c showed that serum resistance was virulence plasmid dependent but also temperature dependent, so that plasmid-bearing bacteria grown at 37°C were serum resistant, but not those grown at RT. These results indicated that strain 6471/76 was virulent and strain 6471/76c was avirulent.

Optimization of autoagglutination of *Y. enterocolitica*. A sample of an overnight RT culture of *Y. enterocolitica* O:3 strain 6471/76 in Luria broth was mixed with BME in test tubes, and the growth of bacteria was followed at 37°C. After a lag phase of approximately 2 to 3 h, the bacteria entered the exponential growth phase. Due to the rather high initial concentration of bacteria used in the experiment, the exponential phase lasted only three to four generations. The bacteria autoagglutinated after an incubation period of ca. 5 h, i.e., during exponential growth. This was seen by the naked eye as a formation of small aggregates in the previously homogeneous bacterial suspension.

The results of an experiment in which different initial concentrations of *Y. enterocolitica* 6471/76 bacteria were used in autoagglutination tests are shown in Table 1. The viable counts of the cultures were determined in the beginning and at the time of autoagglutination. No autoagglutination took place in a suspension with a heavy initial bacterial concentration, where bacterial growth was minimal. When the initial concentration was smaller, the time needed to reach the autoagglutination point increased as the initial concentration decreased, suggesting that a minimal bacterial concentration (ca. 10^7 per ml) was obligatory for autoagglutination. Indeed, the bacteria were able to autoagglutinate in the low-concentration cultures "before time" if they were concentrated by centrifugation and suspended to a higher concentration (data not shown). This indicated that the factors responsible for autoagglutination were already present but the bacterial concentration was too low to favor autoagglutination.

In electron microscope preparations, autoagglutinated bacteria appeared as clusters of cells lying side by side. No fimbriae or any other appendages were visible. The surface of the autoagglutinated cells was slightly rougher than the surface of nonautoagglutinated cells.

Plasmid-associated proteins in autoagglutinated *Y. enterocolitica* and *Y. pseudotuberculosis*. When the protein profiles from autoagglutinated bacteria were compared with those from non-autoagglutinated bacteria, it was found that the synthesis of the temperature-inducible outer membrane protein P1 (3, 4) correlated with autoagglutination. Results of a typical experiment are shown in Fig. 1, lanes A to D. Two major plasmid-associated polypeptides were present in autoagglutinated *Y. enterocolitica* O:3 strain 6471/76 with apparent molecular weights of ca. 240,000 and 52,500 (designated P1 and P2, respectively; Fig. 1, lane A). *Y. enterocolitica* O:9 strain Ruokola/71 behaved identically (Fig. 1, lanes I and J). P1 was visible also in protein preparations from

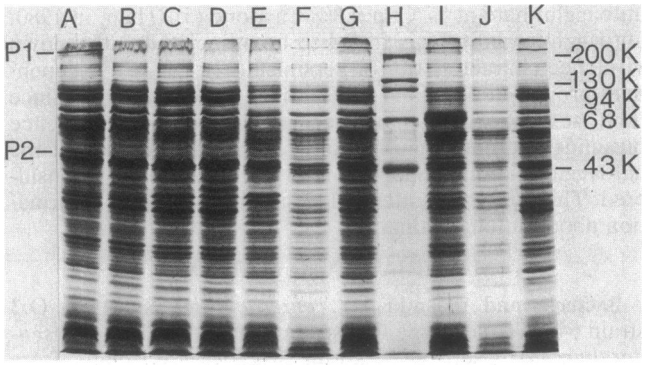

FIG. 1. SDS-PAGE (12% polyacrylamide gel) of water-soluble proteins of *Y. enterocolitica* O:3 and O:9 and *Y. pseudotuberculosis* I grown under different cultural conditions. Lanes: A, strain 6471/76, 24-h culture in BME at 37°C; B, strain 6471/76c, 24-h culture in BME at 37°C; C, strain 6471/76, 24-h culture in BME at RT; D, strain 6471/76c, 24-h culture in BME at RT; E, strain 6471/76, overnight culture in Luria broth at RT; F, strain 6471/76, 1-h culture in BME at 37°C; G, strain 6471/76, 4-h culture in BME at 37°C. Lane H, molecular weight standards, from top to bottom: 200,000 (myosin), 130,000 (beta-galactosidase), 94,000 (phosphorylase b), 68,000 (bovine serum albumin), and 43,000 (ovalbumin). Lane I, strain Ruokola/71, 24-h culture in BME at 37°C; J, strain Ruokola/71c, 24-h culture in BME at 37°C; K, strain 2812/79, 24-h culture in BME at 37°C.

autoagglutinated *Y. pseudotuberculosis* bacteria (Fig. 1, lane K). Protein profiles were prepared from cells of strain 6471/76 grown at RT and after 1 and 4 h of incubation at 37°C (Fig. 1, lanes E, F, and G, respectively). In the 1-h preparation, P1 was already present, whereas P2 was not visible (Fig. 1, lane F). In the 4-h preparation, both P1 and P2 were present (Fig. 1, lane G). In other experiments, it was shown that P1 could be dissociated into 52,500-dalton (= P2) subunits (see below and Fig. 3). In this particular experiment (Fig. 1, lanes E, F, and G), the bacteria autoagglutinated after an incubation period of 2.5 h. These results indicated a possible involvement of P1 in the mechanism of autoagglutination. To try to confirm the correlation of P1 with autoagglutination, mutant strains of *Y. pseudotuberculosis* were tested for autoagglutination, as strains of *Y. pseudotuberculosis* also express P1 (3, 4). Strain YPIII(PIB1) (plasmid bearing, wild type) was positive, whereas strains YPIII (plasmid cured) and YPIII (PIB102) (Tn*5* insertion in the structural gene coding for P1) (4) were negative. As all other plasmid-coded proteins, except for P1, are synthesized by strain YPIII(PIB102) (data not shown), the role of P1 in autoagglutination was suggestive. The similarity of P1 from *Y. enterocolitica* O:3 and from *Y. pseudotuberculosis* was confirmed with specific anti-P1 antiserum in a Western blotting analysis of outer membrane proteins of autoagglutinated bacteria (Fig. 2). The results showed that P1s from both species were immunologically related although their molecular weights seemed to differ slightly. Purified P1 of *Y. pseudotuberculosis* (Fig. 2, lane C) gave rise to two different polypeptide bands with apparent molecular weights of ca. 240,000 and 52,500, respectively, visible in the immune blot, suggesting that P1 actually could be a polymerized structure. This was confirmed as prolonged boiling of a purified P1 sample with an excess of 2-mercaptoethanol or freshly made reduction mix gave only one single band in SDS-PAGE, the 52,500-dalton polypeptide (Fig. 3).

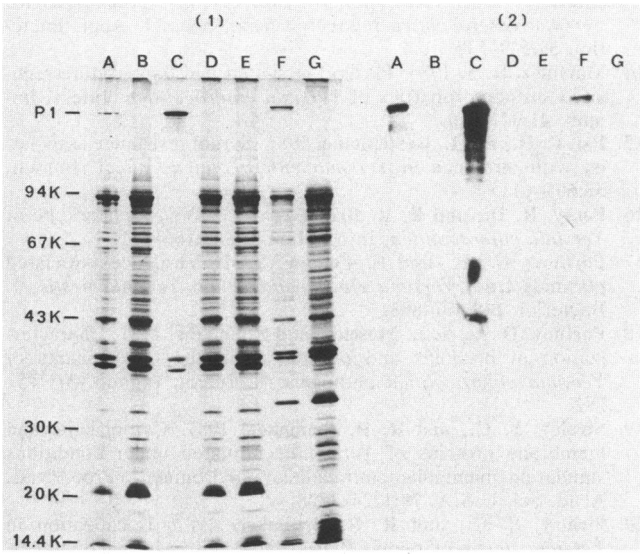

FIG. 2. SDS-PAGE (10 to 17% polyacrylamide gradient gel from top to bottom). (1) Gel stained with Coomassie brilliant blue; (2) autoradiograph of the corresponding Western blot of outer membrane proteins of *Y. enterocolitica* and *Y. pseudotuberculosis* strains cultured in tissue culture medium overnight at 37°C. Specific anti-P1 antiserum was used in Western blot, and the bound antibodies were detected by radioiodinated protein A. Lanes: A, strain YPIII/PIB1; B, strain YPIII; C, purified P1 from *Y. pseudotuberculosis* YPIII (PIB1); D, strain YPIII(PIB101); E, strain YPIII(PIB102); F, strain 6471/76; G, strain 6471/76c.

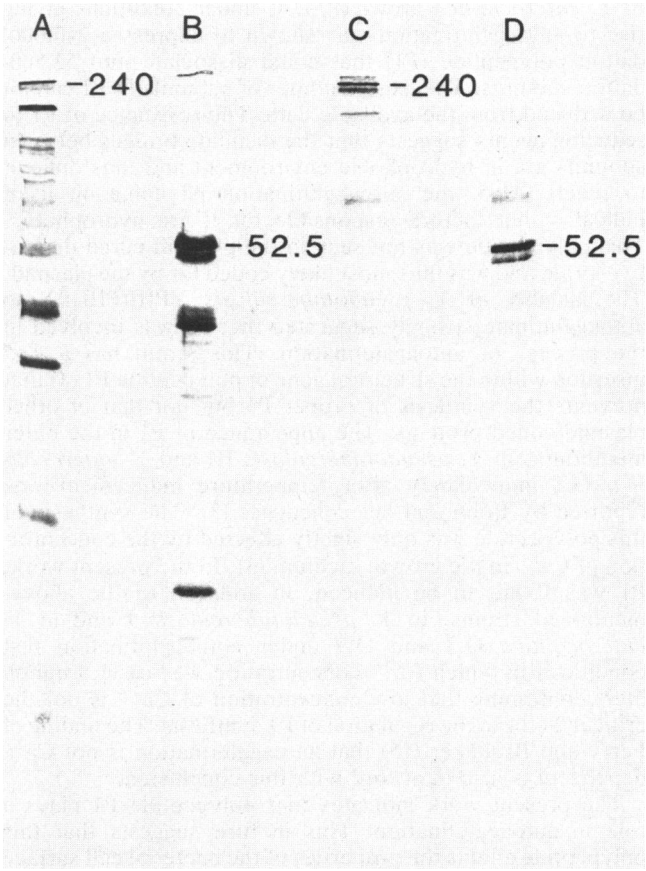

FIG. 3. SDS-PAGE (12% polyacrylamide gel) of protein preparations of *Y. enterocolitica* 6471/76. Lane A: autoagglutinated bacteria were sonicated in distilled water, and the soluble proteins were precipitated by adding 25 mg of $(NH_4)_2SO_4$ per ml at 22°C for 15 min. The precipitate was solubilized into a freshly made reduction mix solution at 22°C. Lane B: same as lane A, except that the sample was boiled for 10 min. Lane C: protein P1 was purified as described previously (4, 10). The sample was solubilized into the reduction mix solution mix at 22°C. Lane D: same as lane C, except that the sample was boiled for 10 min. The numbers indicate molecular weights in kilodaltons.

DISCUSSION

The study of virulence plasmid-associated functions of *Yersinia* spp. requires an isogenic pair of strains, one with and one without the virulence plasmid. In this work, such a pair was used and the association of the plasmid with virulence was confirmed. The results of the guinea pig infections are in good agreement with the results of Pai and DeStephano (15), who studied experimental infections in mice. Also, the results of serum resistance tests supported those of Pai and DeStephano (15) and Perry and Brubaker (16), i.e., plasmid-bearing bacteria were resistant when grown at 37°C but sensitive when grown at RT, whereas plasmid-cured bacteria were sensitive when grown at both temperatures.

This work showed that autoagglutination is a rapid event and depends on the test conditions. A plasmid-bearing strain

of *Y. enterocolitica* grown at 37°C under conditions giving rise to autoagglutination was shown to express a 240,000-dalton polypeptide (P1) that could dissociate into 52,500-dalton subunits. The exact number of subunits in P1 cannot be deduced from the available data. The resistance of P1 to reducing agents suggests that the disulfide bridges between subunits are in hydrophobic environment and thus difficult to reach. Also, the autoagglutination phenomenon itself indicates that factors responsible for it are hydrophobic. This polypeptide was not seen in the plasmid-cured derivative strain and was thus most likely coded for by the plasmid. The inability of *Y. pseudotuberculosis* YPIII(PIB102) to autoagglutinate strongly suggested that P1 was involved in the process of autoagglutination. This strain has a Tn*5* insertion within the structural gene of polypeptide P1 (4) that prevents the synthesis of proper P1 but not that of other plasmid-coded proteins. The appearance of P1 in the outer membranes of *Y. pseudotuberculosis* III and *Y. enterocolitica* O:8 immediately after temperature inducement was reported by Bölin and her colleagues (3). The synthesis of this polypeptide was only sightly affected by the concentration of Ca^{2+} in the growth medium (3). In the present work, P1 was found to be induced, in addition to the above-mentioned strains, in *Y. pseudotuberculosis* I and in *Y. enterocolitica* O:3 and O:9 under autoagglutination test conditions in which Ca^{2+} concentration was ca. 1.8 mmol/liter, confirming that low concentration of Ca^{2+} is not the critical factor in the regulation of P1 synthesis. The finding of Perry and Brubaker (15) that autoagglutination is not Ca^{2+} dependent is also in accord with this conclusion.

The present work indicates that polypeptide P1 plays a role in autoagglutination. This in turn suggests that this polypeptide affects the properties of the bacterial cell surface when grown at 37°C and also in vivo. The recent report of Martinez (14) showed that in the plasmid-bearing *Y. enterocolitica*, the cell surface properties are indeed dramatically altered during growth at 37°C, apparently as a result of synthesis of plasmid-associated outer membrane proteins, including protein P1. Elimination of these proteins with proteolytic enzymes made bacteria serum sensitive and hydrophilic. Thus, P1 could be involved in serum resistance. Furthermore, in two recent reports, the virulence plasmid has been associated with epithelial cell adherence and cytotoxicity of *Y. enterocolitica* (18, 20). In these experiments, bacteria were incubated at 37°C in tissue culture medium, i.e., under conditions comparable to those used in the autoagglutination tests. Thus, protein P1 could be involved as an enhancer element in the process of the bacterial adherence and penetration of epithelial cells. However, the role of protein P1 as a major virulence determinant must be questioned, as the *Y. pseudotuberculosis* YPIII(PIB102) is as virulent as its wild-type strain YPIII(PIB1) when tested in a mouse model system (4). The elucidation of the biological role of protein P1 and other plasmid-encoded proteins must wait for the development of methods to manipulate *Y. enterocolitica* genetically.

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