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Temperature-Inducible Surface Fibrillae Associated with the Virulence Plasmid of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

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When cultivated at 37°C in static broth, human clinical isolates of *Yersinia enterocolitica* (serogroups O:3, O:8, and O:9) and *Yersinia pseudotuberculosis* (serogroup O:III) produced numerous nonflagellar surface appendages, which appeared as a lawn of fine fibrillae, each having a diameter of 1.5 to 2.0 nm and a length of 50 to 70 nm. Cultivation at 22°C resulted in complete disappearance of the fibrillae. The phenotypic expression of these appendages was correlated with the presence of the 40- to 48-megadalton virulence plasmid and was strongly affected by the growth medium. Evidence is presented which suggests that these plasmid-mediated, temperature-inducible surface fibrillae are responsible for autoagglutination and are related to production of one prominent, Sarkosyl-insoluble polypeptide of ca. 180 kilodaltons in the bacterial outer membrane.

Virulent strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, unlike their avirulent mutants, produce a series of ancillary outer membrane proteins (OMPs) (1, 2, 6, 21, 27, 28, 31), which are at least partly externally exposed on the bacterial surface (21). The phenotypic expression of these OMPs is temperature regulated; bacteria cultivated at 22 to 25°C do not produce detectable amounts of the OMPs, whereas large quantities are synthesized at 35 to 37°C (1, 6, 21, 28). The genetic information coding for production of the OMPs is provided by a virulence plasmid of 40 to 48 megadaltons (Mdal) (2, 28), which plays an essential role in the infectious process of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Yersinia pestis* (10, 12, 13, 26, 28). *Y. pestis*, however, apparently fails to express the OMPs in vitro (28). Moreover, the ability to undergo spontaneous autoagglutination when cells are cultivated at 37°C is correlated with presence of the virulence plasmid in *Y. enterocolitica* and *Y. pseudotuberculosis*, whereas *Y. pestis* lacks this property (25). Thus, both ancillary OMPs and autoagglutination are plasmid-mediated, temperature-inducible characteristics of *Y. enterocolitica* and *Y. pseudotuberculosis*. Neither of these two properties is appreciably affected by the addition of Ca^{2+} to the growth medium (6, 25). Accordingly, the possibility exists that autoagglutination and the OMPs reflect the same determinant (25). Recently published data indicate that autoagglutination is associated with one particular high-molecular-weight OMP (30). We have previously shown that autoagglutination in *Y. enterocolitica* is correlated with production of a distinct mannose-resistant hemagglutinin (MRHA) that reacts with guinea pig erythrocytes (17). Hemagglutinins are in many cases associated with fimbriae, fibrillae, or nonfilamentous surface proteins (8, 16, 24). In the present work we report a new type of surface appendage in *Y. enterocolitica* and *Y. pseudotuberculosis*, which is correlated with one particular OMP and with autoagglutination.

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MATERIALS AND METHODS

Bacterial strains. The relevant properties of the eight *Yersinia* spp. strains examined are listed in Table 1. The primary isolates were originally recovered from human cases of gastroenteritis. In the present study, each of these isolates was represented by a pair of isogenic derivatives: (i) a plasmid-bearing variant (P^+) harboring the 40- to 48-Mdal virulence plasmid and (ii) a plasmid-cured mutant (P^-). P^- mutants were derived from P^+ parents by selection for Ca^{2+}-independent growth at 37°C on magnesium oxalate agar (5, 25). Preparation and analysis of plasmid DNA were carried out by the methods of Clewell and Helinski (7) and Meyers et al. (22). All strains were stored at −70°C in heat-inactivated horse serum with 17% glycerol.

Media and growth conditions. Bacteria were grown for 18 to 20 h at 22 or 37°C under aerobic and static conditions in two different liquid media: (i) Eagle basal medium supplemented with 10% fetal calf serum and 0.1% (wt/vol) sodium bicarbonate (EBM-FCS; GIBCO Ltd., Paisley, Scotland) and (ii) brain heart infusion broth (BHI; Oxoid Ltd., Basingstoke, Hampshire, England). One subculture was made in these media before inoculation of the cultures used in the experiments.

Autoagglutination. All strains were tested for spontaneous autoagglutination as described by Laird and Cavanaugh (19). Autoagglutination-positive bacteria formed a flocculate covering the bottom of the tube, leaving a clear supernatant at 37°C, whereas uniform turbid growth was observed at 22°C. Autoagglutination-negative bacteria produced turbid growth at both temperatures.

Hemagglutination. Hemagglutinin production was tested by slide agglutination with erythrocytes from humans (group A Rh^+) and guinea pigs as described previously (17). Hemagglutinins were characterized as mannose resistant (MRHA) if the same degree of agglutination occurred in the presence of 2% mannose.

Preparation of outer membranes. Bacterial outer membranes were prepared by the Sarkosyl (sodium lauryl sar-

TABLE 1. *Yersinia* strains studied

Strain[a]	Serogroup	Biotype[b]	Plasmid size (Mdal)	Country of origin	Source[c]
Y. enterocolitica					
8265 P^+	O:3	4	42.9 ± 0.9	France	H. H. Mollaret
8265 P^-	O:3	4	None		
NY81-71 P^+	O:8	1	40.9 ± 0.9	United States	T. J. Quan
NY81-71 P^-	O:8	1	None		
3315 P^+	O:9	2	42.0 ± 0.8	The Netherlands	J. Oosterom
3315 P^-	O:9	2	None		
Y. pseudotuberculosis					
YPIII P^+	O:III		41.0	Sweden	I. Bölin
YPIII P^-	O:III		None		

[a] P^+, derivatives harboring the virulence plasmid; P^-, mutants that have spontaneously lost plasmid DNA.
[b] According to Wauters (G. Wauters, thèse d'agregation, University of Louvan, Brussels, Vander, Belgium).
[c] Contributors provided the primary isolates that formed the basis for selection of P^+ and P^- isogenic derivatives.

cosinate) solubilization method devised by Filip et al. (11) as detailed by Bölin et al. (1). Briefly, 50-ml stationary phase cultures were harvested into 5 ml of Tris-EDTA buffer (10 mM Tris, 5 mM EDTA, 1 mM β-mercaptoethanol, pH 7.8). Approximately equal amounts of bacteria were used for the preparation of membranes. Bacterial cells were lysed by ultrasonic disintegration, and unbroken cells and cell debris were removed by centrifugation for 15 min at 6,000 × *g*. The total membrane fraction was sedimented by centrifugation of the supernatant at 100,000 × *g* for 1 h. The inner membranes were dissolved by suspending the membrane pellets in 5 ml of Sarkosyl solution (0.5% Sarkosyl, 1 mM β-mercaptoethanol). After incubation at 4°C overnight, the Sarkosyl-insoluble outer membranes were sedimented by centrifugation for 1 h at 100,000 × *g* and suspended in 100 μl of electrophoresis sample buffer (62.5 mM Tris, 1% sodium dodecyl sulfate, 0.5% β-mercaptoethanol, 10% glycerol, pH 6.8).

Analysis of OMPs. Samples of 15 μl of the outer membrane preparations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18) with slab gels containing 10% total acrylamide with 0.26% bisacrylamide. After polyacrylamide gel electrophoresis, the gels were fixed and stained overnight in a solution containing 0.1% Coomassie brilliant blue G, 45% methanol, and 5% acetic acid in distilled water. Destaining was performed by passive diffusion in a mixture of 10% methanol and 10% acetic acid in distilled water. All steps were carried out at room temperature. The resultant Coomassie blue-stained gels were scanned at 600 nm in a Gilford spectrophotometer equipped with a linear gel transport. The densitometric traces thus obtained enabled quantitative comparison of individual OMPs, which were visualized as peaks on the densitograms. The amount of protein was assumed to be proportional to the peak area, calculated as peak height multiplied by peak width at half-maximum height. For evaluation of extremely narrow peaks, gels were rescanned with a ratio of gel transport speed to chart speed equalling 1:20, which resulted in a more bell-shaped presentation. Molecular weights were estimated by comparison with standard proteins obtained from Pharmacia AB (Pharmacia Fine Chemicals, Uppsala, Sweden).

Electron microscopy. Before electron microscopical examination, 2-ml volumes of bacterial cultures were harvested by low-speed centrifugation (2,000 × *g* for 5 min), and the bacteria were washed once in distilled water and suspended in 1 ml of distilled water. Droplets (5 μl) of the suspensions were applied to carbon-filmed grids. The filmed grids had previously been conditioned by glow discharge in air to facilitate spreading of both stain and specimens (23). The grids were blotted almost dry after 1 min and subsequently stained with 0.25% sodium phosphotungstic acid (pH 7.0) for 1 min. Electron microscopy was performed on a JEM 100B microscope operated at 80 keV and with liquid nitrogen traps to prevent contamination. The lowest possible doses were used to reduce radiation damage, and optical diffraction analysis was carried out to select micrographs recorded at identical foci (14).

RESULTS

Cultivation in EBM-FCS. Electron microscopic examination of *Y. enterocolitica* and *Y. pseudotuberculosis* revealed distinct morphological differences between plasmid-bearing (P^+) and plasmid-cured (P^-) derivatives. When cultivated at 37°C in EBM-FCS, all strains harboring the virulence plasmid produced numerous, nonflagellar fibrillae that were evenly distributed on the bacterial surface. These plasmid-associated appendages measured 1.5 to 2.0 nm in diameter and 50 to 70 nm in length (Fig. 1a). Cultivation at 22°C resulted in complete disappearance of fibrillation. The plasmid-cured mutants, however, did not produce surface fibrillae, regardless of incubation temperature (Fig. 1b).

The presence of surface fibrillae was invariably correlated with the expression of three plasmid-mediated characteristics related to the bacterial surface (Table 2); when cultivated at 37°C in EBM-FCS, all plasmid-bearing, fibrillated strains, unlike their plasmid-cured, unfibrillated mutants, (i) underwent autoagglutination, (ii) produced an MRHA which agglutinated guinea pig erythrocytes, and (iii) synthesized one prominent, Sarkosyl-insoluble, high-molecular-weight protein (HMWP) of ca. 180 kilodaltons (kdal) in the bacterial outer membrane. None of these characters was expressed at 22°C.

The HMWP constituted a substantial proportion of the OMPs produced; thus, the total amount of protein applied to each gel lane could not be used as a standard to assess the relative amount of HMWP. For each strain being compared, the relative amount of HMWP was calculated by using one major OMP of ca. 40 kdal as an internal standard (Fig. 2). This polypeptide was a constitutive and dominant component of the OMP profiles of all strains included in the study. The mean values from two individual analyses based on two separate OMP preparations are presented in Table 2. HMWP was shared by all plasmid-bearing strains examined, with little quantitative variation (Table 2). Likewise, only slight variations in the number of surface fibrillae were observed.

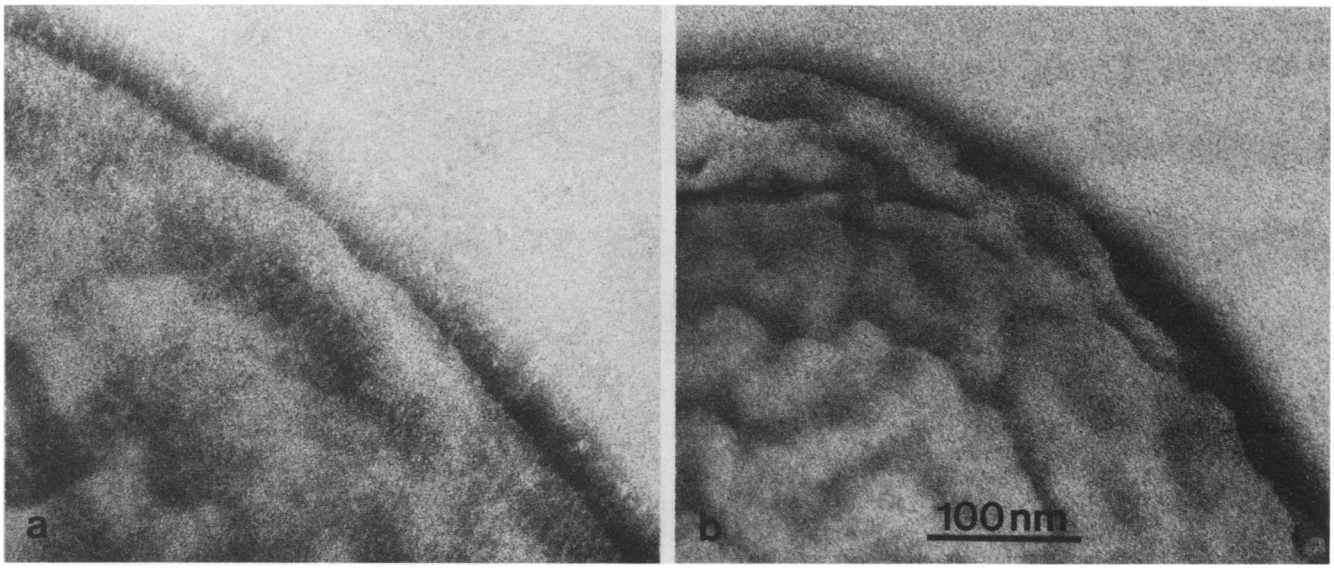

FIG. 1. Electron micrographs showing two isogenic derivatives of *Y. enterocolitica* strain 3315 (serogroup O:9) grown in EBM-FCS at 37°C. The specimens are negatively stained with 0.25% sodium phosphotungstic acid (pH 7.0). (a) A plasmid-bearing derivative (3315 P^+) harboring the virulence plasmid, which exhibits numerous temperature-inducible surface fibrillae, each having a diameter of 1.5 to 2.0 nm and a length of 50 to 70 nm (×200,000). (b) A plasmid-cured mutant (3315 P^-) showing complete disappearance of the fibrillae (×200,000).

In the light of this strongly suggestive evidence of a close relationship between the novel surface fibrillae, autoagglutination, MRHA, and HMWP, we studied the phenotypic expression of these characters after cultivation in BHI. This medium has been reported to diminish the production of plasmid-mediated OMPs in *Y. pseudotuberculosis* (1).

Cultivation in BHI. Only strains harboring the virulence plasmid were examined in this part of the study, and the incubation temperature was invariably 37°C. After cultivation in BHI, all strains possessed a reduced number of fibrillae compared with bacteria grown in EBM-FCS. This repression of the fibrillated state was accompanied by a parallel reduction of HMWP and a qualitative alteration of the autoagglutination phenomenon (Table 2, Fig. 2). However, MRHA was never detected in BHI-grown cultures. Autoagglutination, HMWP production, and surface fibrillae were still detectable in all three *Y. enterocolitica* strains examined, whereas *Y. pseudotuberculosis* had essentially lost these properties. Among the *Y. enterocolitica* strains, almost complete phenotypic repression of fibrillation was observed with strain NY81-71 P^+, which correspondingly exhibited a 90% reduction in the amount of HMWP (Table 2). Considerably less reduction of both HMWP and fibrillation was demonstrated for strains 8265 P^+ and 3315 P^+. Strain 8265 P^+, which produced the greatest amount of HMWP in BHI, showed no apparent alteration of the autoagglutination process when cultivated in this medium. In contrast, substantial alteration in autoagglutination was observed with strains 3315 P^+ and NY81-71 P^+; flocculates of agglutinating bacteria were clearly seen on the bottom of the cultures, although significant amounts of bacteria still remained in suspension, leaving a turbid supernatant.

Unlike the HMWP, which was repressed in BHI-grown cultures, the production of two closely migrating OMPs of approximately 65 kdal was enhanced in this medium (Fig. 2). These two proteins, which were shared by all *Y. enterocolitica* strains examined, were also produced in EBM-FCS, but after cultivation in BHI they appeared as major components of the OMP profiles. Although OMPs of similar size were synthesized by *Y. pseudotuberculosis*, no increase was detected in the BHI profiles of this species. Furthermore, all strains produced polypeptides of approximately 50 kdal in both media. However, strains YPIII P^+ and NY81-71 P^+ produced large quantities when cultivated in BHI, whereas relatively low levels were detected in EBM-FCS (Fig. 2). Strains 8265 P^+ and 3315 P^+, on the contrary, produced low levels of the 50-kdal OMP, regardless of the growth medium.

TABLE 2. Effect of cultivation medium on four temperature-inducible, plasmid-mediated surface properties of *Y. enterocolitica* and *Y. pseudotuberculosis*[a]

Strain[b]	EBM-FCS				BHI			
	AA	MRHA	FIBR	HMWP[c]	AA	MRHA	FIBR	HMWP[c]
8265 P^+	+	+	+++	1.0	+	−	+	0.6
3315 P^+	+	+	++(+)	0.9	(+)	−	+	0.3
NY81-71 P^+	+	+	++	1.2	(+)	−	(+)	0.1
YPIII P^+	+	+	++	0.8	−	−	−	<0.01

[a] Abbreviations: AA, autoagglutination; MRHA, production of a MRHA, which reacts with guinea pig erythrocytes; FIBR, presence of surface fibrillae (the number of fibrillae was scaled from − [negative] to +++); HMWP; synthesis of a ca. 180-kdal OMP.

[b] Strains were cultivated at 37°C.

[c] Relative to the amount of protein produced by 8265 P^+ in EBM-FCS. Mean values from two separate analyses are presented.

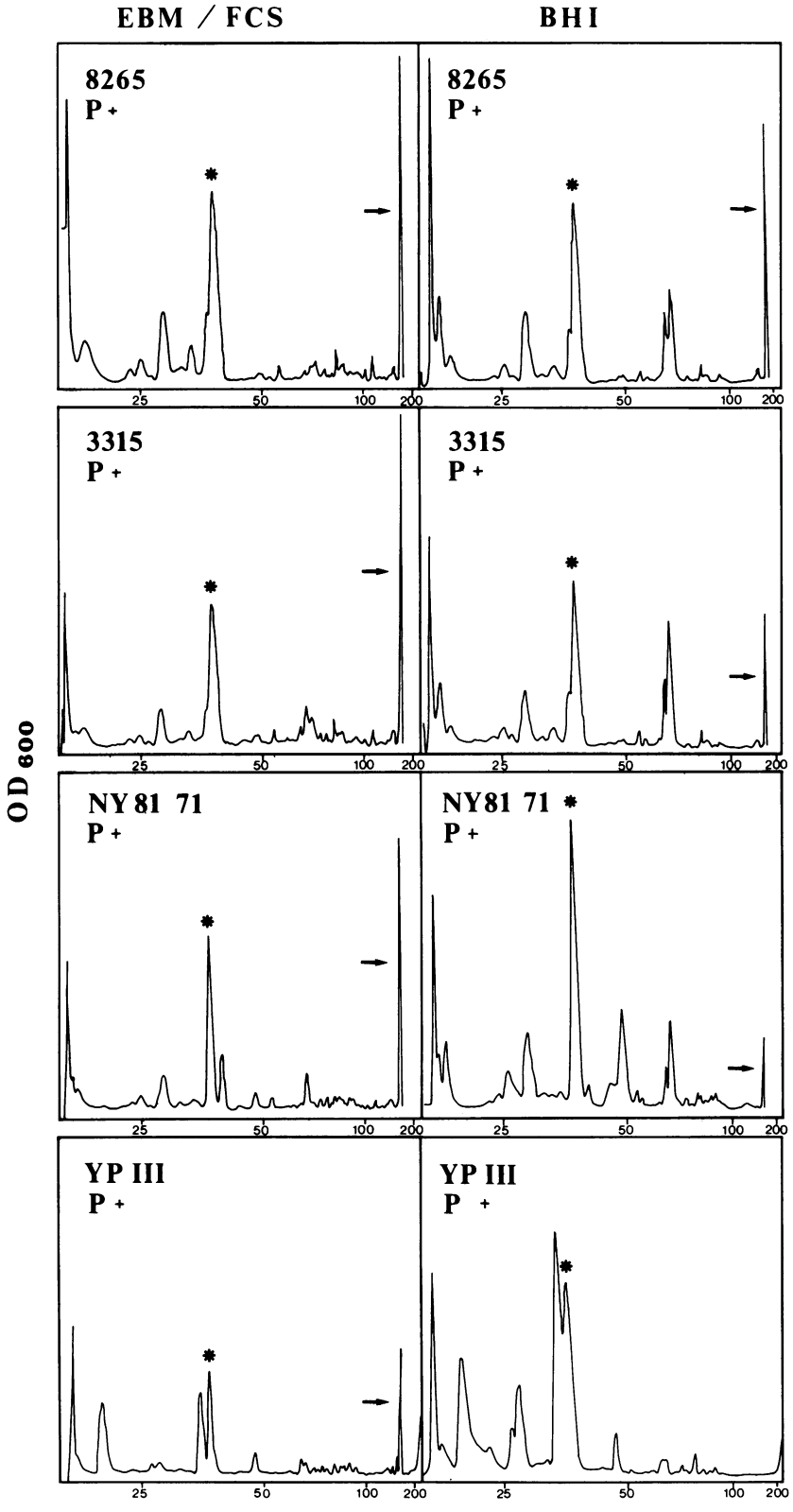

FIG. 2. Effect of cultivation medium on the OMP profiles of *Y. enterocolitica* and *Y. pseudotuberculosis*. Sarkosyl-insoluble OMPs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation (10% acrylamide) followed by spectrophotometric scanning (600 nm) of the resultant Coomassie blue-stained gels. The figure shows densitometric traces representing four different strains, all of which harbor the virulence plasmid (40 to 48 Mdal): (i) 8265 P^+, *Y. enterocolitica* serogroup O:3; (ii) 3315 P^+, *Y. enterocolitica* serogroup O:9; (iii) NY81-71 P^+, *Y. enterocolitica* serogroup O:8; and (iv) YPIII P^+, *Y. pseudotuberculosis* serogroup O:III. Each strain is represented by two OMP profiles, obtained after cultivation at 37°C in two different media: (i) EBM-FCS and (ii) BHI. Arrows indicate one prominent plasmid-mediated, temperature-inducible HMWP. When cultivated in BHI, all strains exhibit a reduced amount of HMWP compared with bacteria grown in EBM-FCS. The asterisks signify one major OMP of ca. 40 kdal, which was used as an internal standard.

DISCUSSION

Many species of *Enterobacteriaceae* produce filamentous, nonflagellar surface appendages (8, 16). The term fimbriae has been applied to appendages that appear regular in form and dimension, with discrete points of origin on the bacterial surface, as opposed to fibrillae, which lack these characteristics (16). The new surface structures of *Y. enterocolitica* and *Y. pseudotuberculosis* described in this report appeared as a lawn of fine filaments, which were distinctly thinner, shorter, and more numerous than all fimbriae so far described (24). Although the dimensions of these filaments are close to the expected resolution limit in biological electron microscopy, they are comparable in size to several other ultrastructural fibers previously observed (e.g., DNA filaments, bacteriophage tail fibers [15]). Glow discharge treatment (23) of the grids allows significant reduction in stain concentration, giving lower contrast (S/N), but prevents minute structures from being immersed in the stain carpet. Since the structure and function of the filaments described in this report are incompletely known, their classification must necessarily be provisional. The question of whether they represent a delicate type of fimbriae, or merely artifacts produced from a nonfilamentous substance, must be regarded as unsettled. Pending further investigations, it seems appropriate to classify such filaments tentatively as fibrillae, as recommended by Jones (16) and Duguid and Old (8).

Two distinct types of fimbriae have previously been described in *Y. enterocolitica*, but both differed in number and dimension from the fibrillae reported here (20). The phenotypic expression of fimbriae as well as fibrillae was found to be temperature inducible (9, 20). However, the fibrillae were expressed only at elevated growth temperatures, i.e., 37°C, whereas the fimbriae prevailed at temperatures below 30°C (20). Furthermore, the fibrillae showed consistent correlation with presence of the virulence plasmid, unlike the fimbriae, which were produced by both virulent and avirulent strains (9, 20, 29). The possibility thus exists that the fibrillae are involved in the infectious process of the bacteria concerned. Many nonflagellar surface appendages are responsible for the adhesive properties of bacteria (8, 16). However, until it is determined whether the fibrillae are expressed in vivo, their potential pathogenic significance remains unsettled.

The presence of fibrillae was consistently correlated with production of one particular OMP (HMWP) and with the capability of autoagglutination, both of which are surface-related properties. These characteristics were lost or gained concomitantly in a plasmid-dependent, temperature-regulated manner. As shown in this study (Table 2) and in earlier works (1, 6, 28), synthesis of the ancillary OMPs is affected by the growth medium. A corresponding growth medium dependence was also exhibited by the fibrillae and the autoagglutination (Table 2). Our results are consistent with the observation of Brubaker (3) that the presence of high concentrations of peptides inhibits autoagglutination in *Y. pseudotuberculosis*. Although not absolutely conclusive, our findings suggest that surface fibrillae, autoagglutination, and HMWP all reflect the same plasmid-dependent determinant and that the fibrillae may well be responsible for autoagglutination.

Bölin et al. (1) detected one particular plasmid-dependent, Sarkosyl-insoluble OMP in *Y. enterocolitica* and *Y. pseudotuberculosis*, designated protein 1, with a molecular weight apparently corresponding to that of the HMWP. It was later demonstrated that proteins 1 from these two bacterial species were immunologically related (30). Since we used the same strain (YPIII) and the same method for preparation of OMPs as did Bölin et al., it is most probable that the HMWP and protein 1 are, indeed, identical. By introducing a transposon into the virulence plasmid of *Y. pseudotuberculosis*, Bölin and Wolf-Watz (2) obtained a mutant [YPIII(PIB102)] that did not produce protein 1 and failed to autoagglutinate (30), strongly indicating that this protein may be involved in the autoagglutination process. This is in accordance with our present suggestion that HMWP and autoagglutination both reflect the possession of distinct surface appendages. The YPIII(PIB102) mutant was still virulent to orally infected mice, suggesting that protein 1-HMWP is not an essential virulence determinant in this bacterial species (2). However, this observation does not exclude the possibility that protein 1-HMWP plays an essential role in the pathogenesis of *Y. enterocolitica* infections. Virulence is a multifactorial character, the mechanism of which is known to vary within the genus *Yersinia* (4). It has been demonstrated that serum from patients convalescing from *Y. enterocolitica* infections contains antibodies that react with the ancillary OMPs, indicating that these polypeptides are synthesized in vivo and therefore may well be involved in the pathogenetic process (21). Martinez (21) correlated the acquisition of ancillary OMPs in *Y. enterocolitica* with resistance to the bactericidal activity of normal human serum. In *Y. pseudotuberculosis*, on the contrary, serum resistance is probably unrelated to these OMPs, as evidenced by Perry and Brubaker (25).

Many nonflagellar surface filaments enable the bacteria to agglutinate different species of erythrocytes in vitro (8, 16). When *Y. enterocolitica* and *Y. pseudotuberculosis* were cultivated in EMB-FCS, we found a close correlation between fibrillation and production of a distinct hemagglutinin (MRHA), whereas no such relationship was detected in BHI-grown cultures of the same bacteria (Table 2). This would suggest that neither fibrillae nor their accompanying HMWP are responsible for the expression of MRHA. It is possible, though, that the failure to detect MRHA in BHI may be a purely quantitative effect due to the substantial reduction of fibrillation obtained in this medium.

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ADDENDUM IN PROOF

After the acceptance of this article, important new information which may support our present results has become available. Lachica et al. (**R. V. Lachica, D. L. Zink, and W. R. Ferris,** Infect. Immun. **46**:272–275, 1984) demonstrated by transmission electron microscopy that one strain of *Y. enterocolitica* serogroup O:3 harboring the virulence plasmid elaborated a hydrophobic matrix of fibrils distal to the outer membrane when cultivated at 37°C but not at 22°C. Plasmid-cured cells did not exhibit any surface matrix, regardless of the growth temperature. The formation of this hydrophobic component was associated with increased cell surface charge, formation of long chains, and spontaneous autoagglutination. Although Lachica et al. did not provide any information on the dimensions of individual fibrils, it is most probable that these components are identical to the plasmid-dependent, temperature-inducible surface fibrillae described in our present work.

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