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Expression of Antigens Encoded by the Virulence Plasmid of *Yersinia enterocolitica* Under Different Growth Conditions

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The expression of polypeptides of the virulence plasmid of *Yersinia enterocolitica* serotype O:3 was studied with the immunoblotting technique and specifically absorbed antisera to *Y. enterocolitica* O:3. At least 16 polypeptides were apparently specified by the virulence plasmid when plasmid-bearing bacteria were grown at 37°C or intraperitoneally in semipermeable capsules. The different growth media used (also with added Ca^{2+}) had quantitatively or qualitatively only a little influence on the expression of these polypeptides, whereas the growth temperature had a stronger influence. The best expression was achieved at 37°C, at 22°C the expression was weak, and at 4°C the plasmid genes were inactive. Two chromosomally encoded polypeptides were expressed only at 37°C, whereas the expression of eight polypeptides expressed at 22°C was repressed at 37°C. The intraperitoneal growth in capsules was used to detect the virulence plasmid-associated polypeptides of *Yersinia pestis*. Four plasmid-associated polypeptides were detected in *Y. pestis* with antiserum to *Y. enterocolitica* virulence plasmid antigens, and three were detected with antiserum to *Y. pestis* EV76. These results suggested that the virulence plasmid of *Y. pestis* was activated in the interstitial environment in vivo, where Ca^{2+} concentration was high, and also that the virulence plasmids of *Y. enterocolitica* and *Y. pestis* have three to four immunologically related polypeptides in common.

Yersinia enterocolitica, *Yersinia pseudotuberculosis*, and *Yersinia pestis* are known human pathogens (8) that show marked temperature dependence, among a variety of characteristics. High growth temperature (35 to 37°C) affects, for example, growth requirements of *Yersinia* species (10), O-specific sugars, the fatty acid composition of the membranes of *Y. enterocolitica* (1, 41), the infectivity of *Y. pseudotuberculosis* and *Y. enterocolitica* strains (11), adherence to tissue culture cells (11, 27), the surface properties of *Y. enterocolitica* (27), and the receptors for bacteriophages (22–24). On the other hand, *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* harbor a 40- to 50-megadalton plasmid associated with the virulence of these species (4, 11, 15–17, 19, 29, 31, 32, 34, 36, 42, 43). The virulence plasmids seem to be associated with virulence determinants that almost uniformly are also temperature dependent, namely, Ca^{2+} dependence for growth at 37°C (14, 15, 29, 38), serum resistance of *Y. enterocolitica* (27, 29, 34, 35), autoagglutination of *Y. enterocolitica* and *Y. pseudotuberculosis* at 37°C (26, 29, 35, 42), and synthesis of V and W antigens (6–8, 29, 30, 38) and other polypeptides (11–13, 27, 32, 33, 35, 38, 39). Thus, many genes in the chromosome and in the plasmid genome seem to be temperature dependent, and most are probably involved in the pathogenesis of these bacteria.

The virulence plasmids were shown to code for at least 20 different polypeptides in minicells (33). The polypeptides of *Y. pestis* and *Y. pseudotuberculosis* plasmids are almost identical, whereas polypeptides of the *Y. enterocolitica* plasmid are different, analogous with the DNA homology data on these plasmids (19, 31–33). Some of the polypeptides have been shown to be expressed in vitro in the outer membrane preparations of *Y. enterocolitica* and *Y. pseudotuberculosis*, but not of *Y. pestis*, grown at 37°C (11, 12, 27, 32, 33, 39). A large outer membrane polypeptide designated protein P1 (240 kilodaltons [kdal], made up of 52.5-kdal subunits; 35) is synthesized in all media tested (11, 33). Protein P1 was shown to be responsible for autoagglutination of *Y. enterocolitica* and *Y. pseudotuberlosis* (35). The expression of other outer membrane polypeptides was reported to require special media, apparently with low Ca^{2+} concentrations (32, 33).

The in vivo role of protein P1 is unknown. Also, the biological significance of the other plasmid-encoded outer membrane polypeptides is obscure, although *Y. enterocolitica* loses serum resistance when the outer membrane polypeptides are proteolytically removed from the cell surface (27). In any case, during *Y. enterocolitica* infections, plasmid-encoded polypeptides are expressed both in animals and humans (14, 27). More information on the gene products of the virulence plasmids, the chromosomal gene products involved in the pathogenesis, and the regulation of them both is needed for the understanding of the plasmid functions.

For the present article, I studied the expression of plasmid-associated polypeptides of *Y. enterocolitica* O:3 grown under different conditions, and I show that the plasmid of *Y. enterocolitica* O:3 apparently specified at least 16 polypeptides, the expression of which was not regulated by calcium but mainly by temperature. Most of the polypeptides were also expressed in vivo in interstitial fluid. I also present results that indicate immunological relatedness between some *Y. pestis* and *Y. enterocolitica* plasmid-specified polypeptides and that the expression of these plasmid-associated polypeptides of *Y. pestis* is not Ca^{2+} dependent in vivo.

MATERIALS AND METHODS

Bacterial strains. *Y. enterocolitica* serotype O:3 strain 6471/76 is a pYV^+ (pYV is a prefix for Yersinia plasmids associated with virulence; 31) stool isolate, and strain 6471/76c is a plasmid-cured (pYV^-) avirulent derivative; both have been described previously (34a, 35). *Y. pestis* EV76 and EV766 are a similar pair of strains (31) and were generously given by Daniel Portnoy.

Immune sera. Immunization of rabbits with strains 6471/76c and 6471/76 was performed as follows. Bacteria were grown overnight at 22°C in Luria broth (per liter: 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl), (LB), washed three times with 0.9% NaCl, and suspensed in the appropriate concentration (measured by absorbance at 590 nm) to a total volume of 0.5 ml. Three young rabbits were immunized with live bacteria by subcutaneous injection of five sites (0.1 ml each) on the back of the rabbit, according to the schedule used with *Y. pestis* (9). Shortly, avirulent strain 6471/76c bacteria were given in preimmunization injections once a week for 3 weeks, after which the schedule continued with the virulent strain (6471/76) injections either once a week or once every 2 weeks, with increasing doses. During the early stages of immunization with the virulent strain, local inflammatory reactions in the injection sites were seen. The bacteria caused the formation of abscesses which subsequently ruptured. During the later stages, the injection sites were almost unaffected. The rabbits were killed and the blood was collected after 5 to 10 months of immunization.

Immunizations of guinea pigs with *Y. pestis* EV76 were done by intraperitoneal injections once a week for 6 weeks with increasing doses (from 10^1 to 10^9 cells) of live organisms.

The rabbit sera were absorbed by three methods with strain 6471/76c organisms (pYV^-) grown with aeration in LB at 37 or 22°C. About 2 g of bacteria for 10 ml of serum was used in each absorption step. Method 1 included sequential absorptions; twice with autoclaved organisms and twice with Formalin-killed organisms grown at 37°C. In method 2, sera were absorbed twice with acetone-dried organisms grown at 37°C and twice with acetone-dried organisms grown at 22°C. In method 3, absorptions of methods 1 and 2 were done sequentially. After the absorptions, the sera were sterilized by membrane filtration and stored frozen in small portions.

Agglutination titers of the rabbit sera were measured in microtiter plates with U-bottomed wells by determining the highest dilution of the sera that still agglutinated the antigens. The bacteria for antigen preparations werer grown overnight at 22 or 37°C in medium E (MedE; 0.1 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid, 10 g of K_2HPO_4, and 3.5 g of $NaNH_4HPO_4 \cdot 4H_2O$ per liter, supplemented with 0.2% glucose, 0.2% Casamino Acids, and 1 mg vitamin B_1 per liter) (11; H. Wolf-Watz, personal communication). The bacteria were washed with phosphate-buffered saline (0.01 M, pH 7.2), incubated for 1 h in 0.3% Formalin, and washed with and suspensed into phosphate-buffered saline.

Intraperitoneal culture of bacteria in capsules. The bacteria for capsule experiments were grown in LB at 22°C overnight, except for the *Y. pestis* strains, which required prolonged incubation for sufficient growth, and suspended in distilled water, so that the bacterial concentration was ca. 10^8 cells per ml. The capsules were prepared from 1-ml syringes that were cut into four pieces (0.25 to 0.3 ml each). The ends of the pieces were smoothed by warming them slightly on a Bunsen burner and by pressing the softened ends against a smooth surface. After that the ends were roughened a little with fine sand paper, and small pieces of nitrocellulose membrane filter (Millipore type GS; pore size, 0.22 μm) were glued (UHU all purpose adhesive no. 13, Lingner & Fischer, 7580 Bühl, Federal Republic of Germany, diluted with ethanol) on the other end of the syringe pieces. Before that the syringe and also the membrane filter pieces were sterilized by incubation in ethanol. The bacterial suspension was pipetted inside the capsule, which was closed by gluing another piece of filter paper over the open end. The excess filter was cut away, and the capsules (up to 20 per guinea pig) were surgically placed into the peritoneal cavity of guinea pigs under ether narcosis. The animals were killed after 1 to 3 days, and the capsules were removed. The liquid was collected from the capsules, and the bacteria were pelleted, washed once with 0.1 ml of phosphate-buffered saline to remove the guinea pig serum proteins (mainly albumin) from the residual liquid on the pellet, and stored frozen.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially by the procedure of Laemmli (25) in 1-mm-thick slab gels with the acrylamide gradient from 10 to 17% and the bisacrylamide gradient from 0.26 to 0.45%. The high- and low-molecular-weight standards (Bio-Rad Laboratories, Richmond, Calif.) were run simultaneously with the samples. The samples were whole bacteria incubated in a boiling water bath for 5 to 10 min in a reduction mix solution (0.066 M Tris-hydrochloride [pH 6.8], 2% SDS, 5% 2-mercaptoethanol, 9% glycerol, and bromophenol blue as a dye marker). The protein bands were stained with Coomassie brilliant blue R250 or transferred to nitrocellulose filter papers as described below.

Protein blotting. The proteins were transferred electrically with a 0.2- to 0.3-A current from the slab gel to the nitrocellulose membrane filter paper (BA 85; Schleicher & Schüll, Inc., Keene, N.H.) (NCP) for 16 to 20 h in the Towbin buffer (40). The lanes containing the molecular weight standards were cut away from the NCP and stained with India ink as described (18). The rest of the NCP was placed in a roller bottle (the neck of a plastic bottle was cut off so that a rim of ca. 5 to 10 mm was left) flat against the wall of the bottle; the transferred proteins were facing inside. The bottle was placed on its side on a test tube roller that gave the bottle ca. 1 to 2 rpm. The volume of reagents needed to flood the NCP in this arrangement could be decreased down to 5 ml for an NCP of 15 by 25 cm, and still a very even distribution of reagents all over the NCP was achieved. All the incubations and washings of the NCP followed the principles of Towbin and others (40) and were performed in the roller bottle at 22°C. The immunoperoxidase method that was used nonspecifically stained background protein bands, the reason for which most probably was the nonspecific binding of the peroxidase-conjugated antibodies, since longer incubation times resulted in stronger background staining. In any case, the bands that were stained specifically were usually more intensively colored and were easily distinguishable.

RESULTS

Characterization of the antiserum to the pYV-associated antigens of *Y. enterocolitica*. The antisera to *Y. enterocolitica* were prepared in three rabbits that were immunized with live organisms of virulent strain 6471/76. The agglutination titers of the pre- and postabsorption sera (absorption method 1) were measured with antigens prepared from both strains grown either at 22 or 37°C (Table 1). The titers of the preabsorbtion sera showed that rabbit B had responded the most strongly to the immunization. The titers varied, depending on which antigen was used. The pYV^- strain (6471/76c) grown at 22°C was poorest antigen, whereas the others were equal. The absorbtions had been sufficient, as no agglutinating antibodies against pYV^- bacteria were detected in the postabsorption sera. Surprisingly, the pYV^+

TABLE 1. Agglutination titers of the antisera prepared in rabbits to *Y. enterocolitica* 6471/76 before and after absorptions with strain 6471/76c bacteria (method 1)[a]

Antiserum	Agglutination titer with antigens from strain:			
	6471/76c		6471/76	
	22°C	37°C	22°C	37°C
Rabbit A				
Unabsorbed	1:1,280	1:2,560	1:2,560	—[b]
Absorbed	0	0	1:640	—
Rabbit B				
Unabsorbed	1:5,120	1:10,240	1:10,240	—
Absorbed	0	0	1:2,560	—
Rabbit C				
Unabsorbed	1:2,560	1:5,120	1:5,120	—
Absorbed	0	0	1:2,560	—

[a] The agglutination antigens were prepared from strains 6471/76 and 6471/76c grown at 22 and 37°C in MedE.

[b] Agglutination titers could not be evaluated because the bacteria were autoagglutinated.

bacteria grown at 22°C gave agglutination titers of 1:640 to 1:2,560, suggesting that in these bacteria some plasmid-associated antigens were expressed at 22°C.

As the serum of rabbit B had the highest specific antibody levels both before and after the absorptions, it was used in the following experiments.

The next step was to characterize the difference between the pre- and postabsorption rabbit sera by the immunoblotting technique. The immunoblot (Fig. 1) showed that a vast number of antigens of the pYV$^+$ and pYV$^-$ bacteria were recognized by antibodies in the preabsorption serum, so that essentially no differences between the strains were distinguishable (Fig. 1, lanes A and B), except for the 240-kdal polypeptide band (Fig. 1, lane B). As expected, only a few protein bands of the pYV$^+$ strain were stained specifically by the postabsorption serum (Fig. 1, lanes D and F), whereas the pYV$^-$ strain seemed to be lacking any specific bands (Fig. 1, lanes C and E). Sera absorbed by methods 1, 2, or 3 gave identical results in immunoblotting (Fig. 1, lanes C through F). These results indicated that the absorbed sera contained only specific antibodies to the virulence plasmid-encoded or -associated antigens and that absorption methods 1 and 2 were equally efficient and specific.

The results of the agglutination tests (Table 1) suggested that the activity of the virulence plasmid of *Y. enterocolitica* serotype O:3 was not very strictly temperature regulated. Thus, with absorbed rabbit serum (method 1) and the immunoblotting technique the expression of the virulence plasmid-associated antigens was studied. The pYV$^+$ *Y. enterocolitica* strain 6471/76 grown under different conditions and the pYV$^-$ strain 6471/76c as a negative control were used in these studies. The expression of the antigens was studied at three different growth temperatures, (4, 22, and 37°C) combined with four growth media: LB, basal medium Eagle (BME; Flow laboratories, Irvine, United Kingdom), MedE, and MedE, supplemented with 5 mM Ca^{2+} (MedECa). At 37°C the bacteria also were grown in the four media anaerobically, i.e., the cultures were overlaid with paraffin oil. Growth in the intraperitoneal capsules was used to simulate the in vivo growth conditions.

Growth at 4°C. The SDS-PAGE and the corresponding immunoblot of the strains grown at 4°C for 4 days in the four media revealed no difference between the protein profiles of the two strains (data not shown), suggesting that the plasmid genome is inactive at this temperature.

Growth at 22°C. The SDS-PAGE and the immunoblot of the strains grown at 22°C are shown in Fig. 2. In the SDS-PAGE, no differences were detectable in the protein profiles between the strains, but some quantitative differences were seen between the growth media. The immunoblot, however, revealed that the virulence plasmid was active at 22°C in media other than LB. The pYV$^+$ strain expressed new polypeptide bands (240, 52.5, 40, and 38 kdal; Fig. 2) not detectable in the pYV$^-$ strain. It has been previously shown that the 52.5-kdal polypeptide is a subunit of the 240-kdal polypeptide (35).

Growth at 37°C. The SDS-PAGE and corresponding immunoblot of the strains grown in the four media at 37°C are shown in Fig. 3. The SDS-PAGE revealed a few differences between the strains. The 240-, 52.5-, and 38-kdal polypeptides, already present at 22°C (see the immunoblot, Fig. 2),

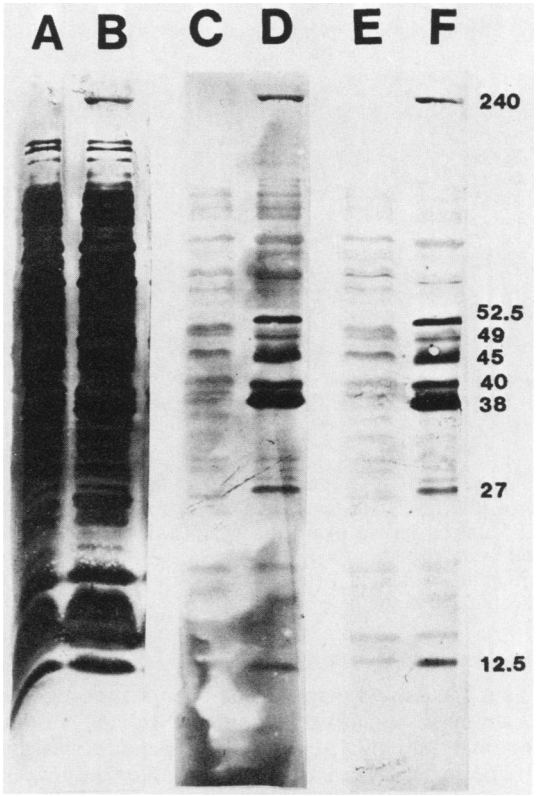

FIG. 1. Immunoblot overlaid with 1:100-diluted unabsorbed (lanes A and B) or 1:50-diluted absorbed (lanes C and D, method 2; lanes E and F, method 1) sera of rabbit B immunized with *Y. enterocolitica* 6471/76. The lanes contained total proteins of *Y. enterocolitica* 6471/76c (lanes A, C, and E) and 6471/76 (lanes B, D, and F) grown overnight at 37°C in MedE and transferred from a SDS-PAGE gel. The samples were electrophoresed from top to bottom. After electrophoresis, the bands were transferred electrically onto an NCP filter, coated with 4% bovine serum albumin, and overlaid with the antisera mentioned above. The peroxidase-conjugated antibodies to rabbit immunoglobulins (DAKO immunoglobulins; P217, lot 112) were used in a 1:200 to 1:400 dilution to detect the bound rabbit antibodies. Incubation periods with the antisera at 22°C ranged from 2 h to 3 days. 3,3-diaminobenzidine tetrahydrochloride was used as the chromogen for peroxidase staining. The molecular masses of the bands in this and other figures are in kdal.

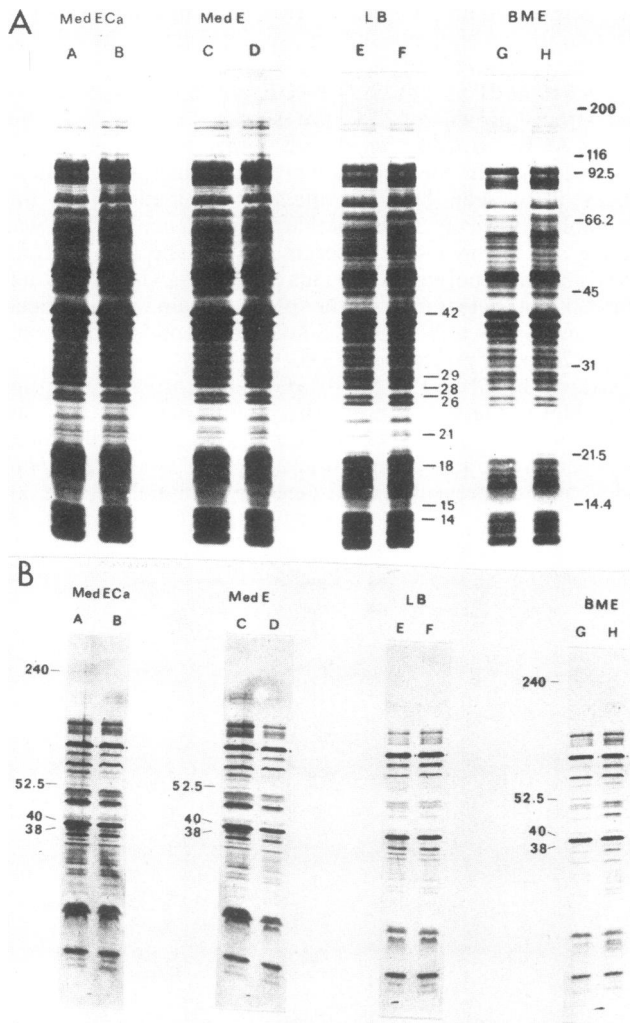

FIG. 2. SDS-PAGE (A) and immunoblot (B) of total proteins of *Y. enterocolitica* 6471/76c (lanes B, D, F, and H) and 6471/76 (lanes A, C, E, and G) grown overnight at 22°C. The immunoblot was prepared as described in the legend to Fig. 1, with absorbed rabbit serum (method 1, diluted 1:200) in the overlay. The growth media are indicated above the lane letters.

and a new 27-kdal polypeptide had been synthesized by the pYV^+ strain in quantities detectable by the Coomassie brilliant blue staining. A 68-kdal polypeptide was synthesized by both strains in LB, but not at all or only weakly in other media used; also, the pYV^- strain grown in MedE and MedECa had a new 22-kdal polypeptide band.

The lanes in SDS-PAGE containing protein profiles of the pYV^- strain 6471/76c grown at 22 and 37°C were tracked with a densitometer, and several temperature-dependent changes in the protein profiles were evident (Fig. 2, lane F, and Fig. 3, lane F). Eight polypeptide bands (42, 29, 28, 26, 21, 18, 15, and 14 kdal) were weaker or absent in bacteria grown at 37°C when compared with bacteria grown at 22°C, and two were stronger (65 and 13 kdal).

In the immunoblot, the presence of more than 16 plasmid-associated polypeptide bands was evident, i.e., 240 kdal, several bands between 200 and 180 kdal, and 165, 145, 132, 115, 108, 52.5, 51.5 (detectable only in anaerobic LB; Fig. 3, lane I), 49, 45, 40, 38, 27, and 12.5 kdal (Fig. 3). The media slightly affected the synthesis of these polypeptides, so that small quantitative and qualitative differences were seen. BME seemed to be the poorest growth medium for the synthesis of these polypeptides; the other media seemed to be equal in this respect. The 51.5-kdal polypeptide was present only in anaerobic LB (Fig. 3, lane I), although the 52.5-kdal polypeptide may have covered it totally in other preparations. In the anaerobic cultures, the 49-kdal polypeptide was absent in BME, MedE, and MedECa, and the 27- and 12.5-kdal polypeptides were absent in BME (data not shown). In LB and anaerobic LB and in BME and anaerobic BME, the 68- and 56-kdal polypeptide bands and the 25- and 21-kdal polypeptide bands, respectively, were present in both strains, but this was not true in other media (Fig. 3).

Growth in intraperitoneal capsules. Figure 4 presents the SDS-PAGE and the immunoblot of *Y. enterocolitica* 6471/76 and 6471/76c and *Y. pestis* EV76 and EV766 grown in capsules. The SDS-PAGE revealed some differences between the pYV^+ and pYV^- strains. In *Y. enterocolitica*, the 240-, 52.5-, 38-, and 12.5-kdal polypeptide bands could be seen, and in *Y. pestis*, the 44-, 40-, and 12-kdal polypeptide bands could be seen. In the corresponding immunoblot overlaid with absorbed rabbit antiserum to *Y. enterocolitica*, all of the more than 16 *Y. enterocolitica* plasmid-associated polypeptide bands were present (see above). One additional plasmid-associated band (35 kdal) was visible in *Y. pestis*. An immunoblot of *Y. pestis* EV76 and EV766 cultured in capsules and overlaid with guinea pig antiserum to strain EV76 is shown in Fig. 5 (lanes C and D). This antiserum detected the pYV-associated 40-, 35-, and 23-kdal polypeptides of *Y. pestis*. Figure 5 shows also the immunoblots where transferred polypeptides of *Y. enterocolitica* pYV^+ and pYV^- strains were overlaid with antisera to *Y. pestis*. In addition to the ca. 20 chromosomally coded polypeptides, the antiserum detected three pYV-specified polypeptides (45, 38, and 27 kdal; Fig. 5, lane B).

DISCUSSION

In this work antibodies to pYV^+ *Y. enterocolitica* were used to characterize the antigens encoded by the virulence plasmid. The antiserum was absorbed exhaustively with pYV^- bacteria prepared in various ways. The immunoblots showed that in absorptions only antibodies to pYV-specified antigens were left in the sera. A similar conclusion can be made from the agglutination experiments, since in absorbed sera only agglutinating antibodies to pYV^+ bacteria were detectable. The ability of the absorbed antiserum to agglutinate pYV^+ bacteria grown at 22°C was explained by the expression of plasmid-associated antigens that occurred also to some extent at low temperatures (see Fig. 2). pYV^- bacteria had a higher titer when grown at a higher temperature, suggesting that in vivo (during the immunizations) chromosomal temperature-regulated genes also are activated and that their products are antigenic. These genes may play the chromosomal part of the evident synergism in pathogenesis between the chromosome and plasmid in *Y. enterocolitica* (20, 30). The comparison of the pYV^- strain grown at 22 and 37°C revealed significant differences between the protein profiles, supporting this hypothesis.

The results of this work, showing that the pYV-associated polypeptides were synthesized weakly at 22°C, do not agree with those of Portnoy and others (33), who detected no pYV-associated polypeptides in the outer membrane preparations of *Y. enterocolitica* O:8 grown at 25°C. However, the

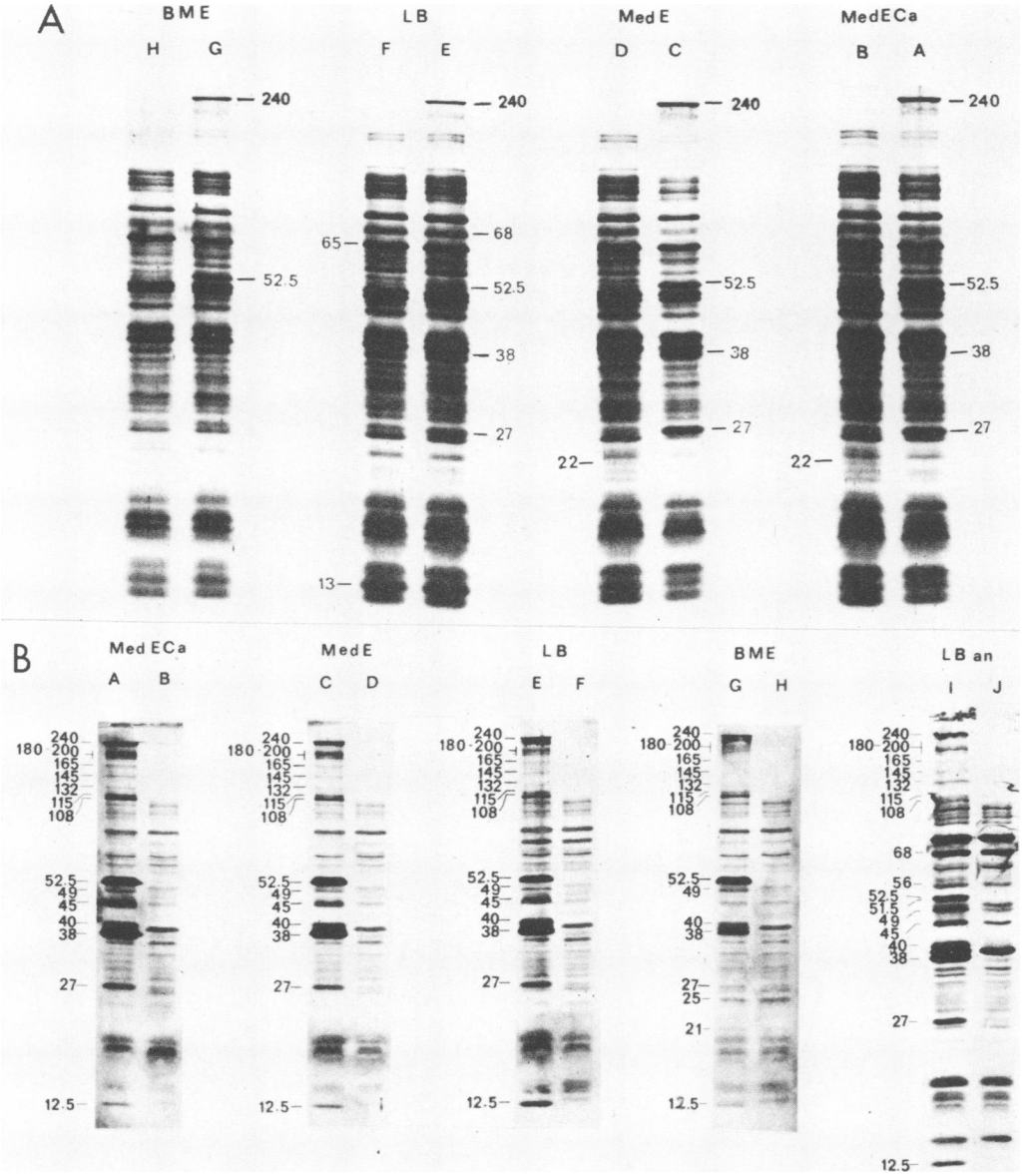

FIG. 3. SDS-PAGE (A) and immunoblot (B) of total proteins of *Y. enterocolitica* 6471/76 (lanes A, C, E, and G) and 6471/76c (lanes B, D, F, H, and I) grown overnight at 37°C. Other conditions were as described in the legend to Fig. 2.

SDS-PAGE stained with Coomassie brilliant blue used by Portnoy and others (33) was in this work too insensitive, as the polypeptide bands were visible only in the immunoblots. These results give also an explanation for the observation of Kapperud and Lassen (21) that some *Y. enterocolitica* strains autoagglutinate weakly at 22°C.

The repression of eight and the induction of two chromosomally coded polypeptides of *Y. enterocolitica* at 37°C may explain the observation that *Y. pseudotuberculosis* and *Y. enterocolitica* grown at 25°C adhered better to epithelial cells than bacteria grown at 37°C (11, 19). Also, it may explain the results of Martinez (27), showing that the surface hydrophobicity of a pYV$^-$ *Y. enterocolitica* O:8 strain was temperature dependent. Bacteria grown at 25°C were hydrophobic, whereas those grown at 37°C were hydrophilic. Similarly, Barber and Eylan (5) reported antigenic differences between bacteria grown at 25 and 37°C. These results demonstrate that the bacterial behavior, at least the protein profiles, changes markedly at different temperatures.

The synthesis of over 16 polypeptides apparently associated with the virulence plasmid is in accordance with the results of Portnoy and others (33), who could detect over 20 polypeptides in minicell analysis of the virulence plasmid. However, the synthesis of these polypeptides in all media tested and also in media with added Ca^{2+}, although marked by a certain variability, is contradictory to the results of others in this respect (11, 12, 32, 33). The pYV plasmids of *Y. enterocolitica* serotype O:3 and O:8 are not homologous (19, 32; unpublished data) and may explain the controversy between the results of this work and the results of others. This work suggests that the activity of the pYV plasmid of *Y. enterocolitica* O:3 increases gradually with increasing tem-

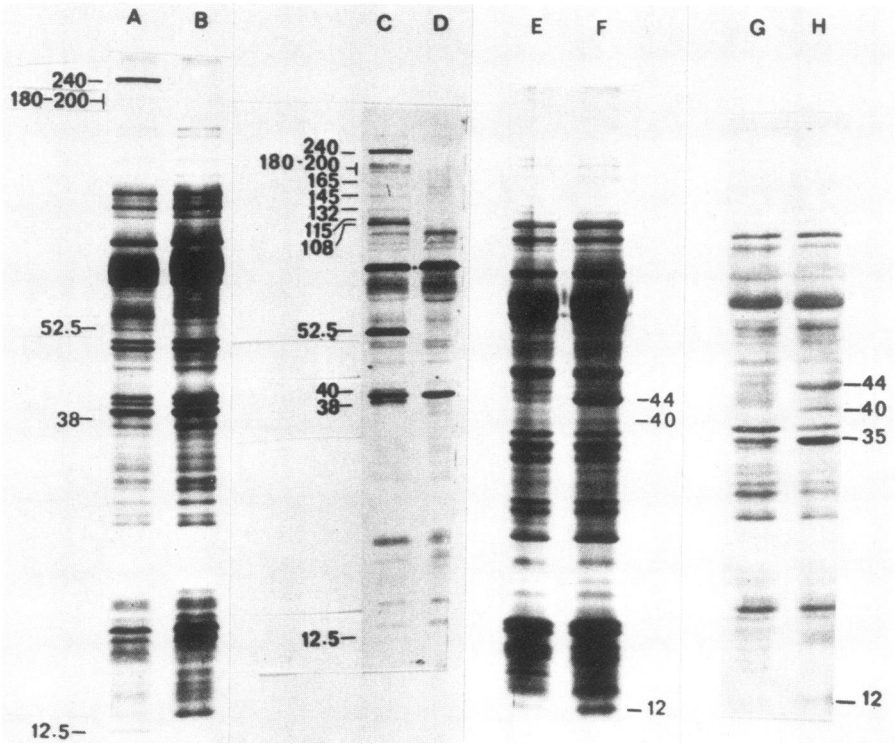

FIG. 4. SDS-PAGE (lanes A, B, E, and F) and immunoblot (lanes C, D, G, and H) of total proteins of *Y. enterocolitica* 6471/76 (lanes A and C) and 6471/76c (lanes B and D) and *Y. pestis* EV76 (lanes F and H) and EV766 (lanes E and G), grown in capsules in the peritoneal cavity of guinea pigs for 3 days. Other conditions were as described in the legend to Fig. 2.

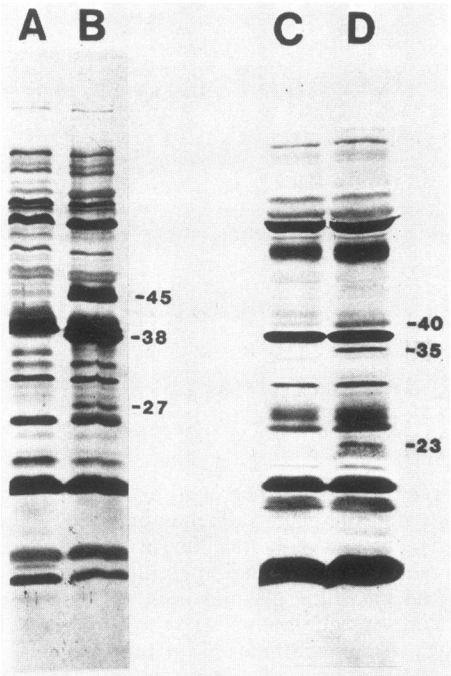

FIG. 5. Immunoblots overlaid with 1:50-diluted serum of a guinea pig immunized with *Y. pestis* EV76. The lanes contained total proteins of *Y. enterocolitica* 6471/76c (lane A) and 6471/76 (lane B) grown overnight at 37°C in MedE and of *Y. pestis* EV766 (lane C) and EV76 (lane D) cultured in intraperitoneal capsules. The immunoblots were prepared as described in the legend to Fig. 1, except that peroxidase-conjuguated antibodies to guinea pig immunoglobulins (DAKO immunoglobulins; P141, lot 053) were used to detect the specifically bound antibodies in the blot.

perature and that other environmental conditions, such as calcium concentration, are not so important.

The in vivo experiments with capsules added new data on the behavior of the virulence plasmid. The intraperitoneal fluid diffusing into the capsules is essentially similar to interstitial fluid, and thus its Ca^{2+} concentration is high. To find out that the virulence plasmid-associated polypeptides of *Y. enterocolitica* 6471/76 were synthesized in the capsules in an environment with a high calcium concentration was not surprising in the light of previous results. However, the evidence of the activation of the *Y. pestis* virulence plasmid contradicts the previous in vitro results that indicate total inactivation of plasmid genes when calcium is present (8, 33, 38, 39), although the synthesis of V and W antigens has been reported in the peritoneal exudate of dying guinea pigs (37). The absorbed antiserum to *Y. enterocolitica* recognized some pYV plasmid-associated polypeptides of *Y. pestis* in the immunoblot, and analogously the antiserum to *Y. pestis* recognized some pYV plasmid-associated polypeptides of *Y. enterocolitica*, supporting the observation of Portnoy and others (33) that some of the pYV plasmid-specified polypeptides of *Y. pestis* and *Y. enterocolitica* are related. These cross-reacting antigens could be partially responsible for the immunological cross-protection that *Y. enterocolitica* showed against *Y. pestis* in mice (2, 3), although these species share also many other related antigens (Fig. 5).

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