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Immunochemical Analysis of Plasmid-Encoded Proteins Released by Enteropathogenic *Yersinia* sp. Grown in Calcium-Deficient Media

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Enteropathogenic *Yersinia* sp. releases plasmid-associated proteins of low molecular mass (26–67 kilodaltons) at 37°C. In this study, the optimum conditions for the release of proteins were assessed and the released proteins (RPs) were analyzed for the manner of release, immunochemical characteristics, and the location of the genes necessary for their synthesis. Protein release was strongly enhanced when growth media were markedly depleted of calcium ions by precipitation with oxalate or chelation with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. RP yields were greatest when *Yersinia* spp. were in the exponential growth phase. The RPs appeared to be released from the *Yersinia* spp. by secretion rather than by pinching off of membrane vesicles, because the RPs did not sediment during high-speed centrifugation nor were they contaminated to any significant degree with lipopolysaccharide. Moreover, immunoblot analysis revealed only traces of protein species related to RPs within the outer membranes of plasmid-positive *Yersinia* spp. grown at 37°C under calcium-restricted conditions. Immunoblot studies also showed that the RPs of *Y. enterocolitica* serotypes O:3, O:8, and O:9 and the RP of *Y. pseudotuberculosis* serotype I are highly cross-reactive. Finally, the immunoprecipitates of the products of minicells which harbor *Yersinia* plasmids were used to demonstrate that at least three proteins immunochemically related to the released fraction were plasmid encoded. These results suggest that at least three of the RPs may be related to or identical with previously described plasmid-encoded *Yersinia* outer membrane proteins.

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are enteric pathogens that invade the gastrointestinal tract (6, 19, 35). By contrast, *Yersinia pestis*, the causative agent of bubonic plague, enters the bloodstream directly after a bite by an infected flea (36). A common feature of these three human pathogens is that they possess closely related plasmids of about 42 to 46 megadaltons that are essential for the full expression of virulence (7–9, 37; for a review, see reference 29). Plasmid-associated virulence factors of *Yersinia* spp. include resistance to the bactericidal effect of serum (13, 26), the ability to adhere to HEp-2 cells (11, 13, 15, 22, 28, 34, 37), cytotoxicity for HeLa cells (28), and lethality for mice and guinea pigs (7–9, 13, 18, 23, 37).

In addition to these plasmid-mediated virulence factors, chromosomal determinants also appear to be relevant to the pathogenicity of *Yersinia* sp. For example, lethality for mice is expressed by plasmid-positive *Y. enterocolitica* serotype O:8 but not by *Y. enterocolitica* serotypes O:3 and O:9 (11, 14). A further example is that serum resistance is under plasmid control in *Y. enterocolitica* (13, 26), whereas this property is plasmid independent for *Y. pseudotuberculosis* and *Y. pestis*. A common feature of all plasmid-harboring human-pathogenic *Yersinia* species is calcium-dependent growth at 37°C and the production of V antigen, a plasmid-encoded 37-kilodalton (kDa) protein (21, 31). Moreover, the virulence plasmids of *Y. enterocolitica* and *Y. pseudotuberculosis* have the coding capacity for about five temperature-inducible outer membrane proteins (YOPs) with molecular masses ranging from 250 to 25 kDa (3, 4, 28, 30). However, not all human-pathogenic *Yersinia* strains, in particular *Y. pestis*, are able to express these YOPs in detectable quantities under in vitro conditions (4, 30). In spite of this, animals and humans suffering from yersiniosis respond to these YOPs with specific antibodies, indicating that YOPs are expressed in vivo (4, 12, 25).

In a recent report, we identified five proteins of different molecular weights that are released into calcium-deficient media by plasmid-bearing strains of *Y. enterocolitica* O:3 and O:9 (11). By molecular weight, these protein species are closely related to YOP2, YOP4, and YOP5 of *Y. enterocolitica* serotype O:8 (4).

The purpose of this investigation was fourfold: (i) to optimize the conditions for release of the proteins of *Yersinia* spp.; (ii) to determine whether these proteins are released by secretion or membrane vesiculation; (iii) to compare by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis the proteins released by *Y. enterocolitica* and *Y. pseudotuberculosis*; and (iv) to assess with the *Escherichia coli* minicell expression system whether the proteins released by *Y. enterocolitica* and *Y. pseudotuberculosis* are plasmid encoded.

We found that protein release was optimal in brain heart infusion (BHI) broth which was depleted of calcium by using 1 mM to 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. At least three proteins of the released fraction were plasmid encoded and antigenically closely related within the group of enteropathogenic *Yersinia* sp. Lipopolysaccharide analysis and high-speed centrifugation results indicated that the released proteins (RPs) were not membrane vesicles.

(Portions of this work appear in the theses of U. Gross and N. Schmidt.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Y. enterocolitica* serotypes O:3 (Y-108 Nal^r, plasmid positive), O:9 (Y-96 Nal^r, plasmid positive), and O:8 (WA-314 Nal^r, plasmid positive, and its plasmid-negative derivative WA-cNal^r)

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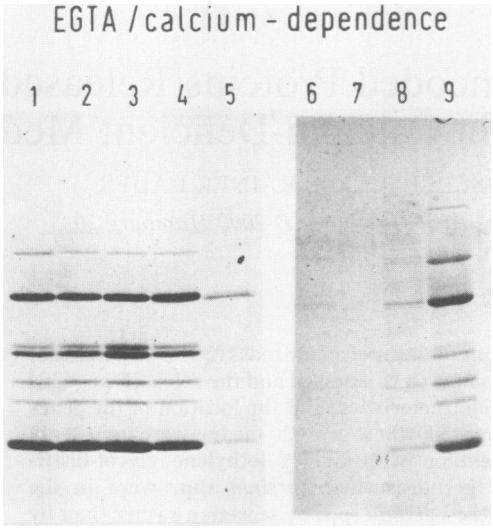

FIG. 1. SDS-PAGE (12% polyacrylamide gel) (Coomassie stain) of ammonium sulfate precipitates from supernatants (5 ml) of *Y. enterocolitica* O:8 strain WA-314 Nal^r cultivated in BHI medium containing different concentrations of EGTA and either magnesium chloride or calcium chloride. Lanes: 1, 20 mM $MgCl_2$, 10 mM EGTA; 2, 10 mM EGTA; 3, 5 mM EGTA; 4, 1 mM EGTA; 5, 0.1 mM EGTA; 6, 20 mM $CaCl_2$, 10 mM EGTA; 7, 10 mM $CaCl_2$, 10 mM EGTA; 8, 5 mM $CaCl_2$, 10 mM EGTA; 9, 1 mM $CaCl_2$, 10 mM EGTA.

were used (11, 14). A *Y. pseudotuberculosis* serotype I strain was the kind gift of S. Aleksić, Hygienisches Institut, Hamburg, Federal Republic of Germany; this Y-pNal^r strain harbors a 42-megadalton plasmid and is lethal for mice and guinea pigs. The minicell-producing *E. coli* DS410 was a gift of J. N. Reeve, Ohio. *Yersinia* species and *E. coli* were subcultured on solid Luria medium (0.5% NaCl, 1% tryptone, 0.5% yeast extract, 1.5% agar).

Isolation of proteins secreted from *Yersinia* sp. and SDS-PAGE. For optimal protein release *Yersinia* sp. was precultured in BHI broth (Difco Laboratories, Detroit, Mich.) at 26°C with shaking (120 rpm) overnight. This preculture was then diluted 1:20 with fresh BHI broth (optical density at 600 nm [OD_{600}], ~0.07 to 0.1; 100 ml of medium in a 250-ml Erlenmeyer flask) and incubated with shaking (120 rpm) at 37°C for 90 min (final OD_{600}, ~0.4; about 6×10^7 cells per ml). Next, the medium was supplemented with filter-sterilized EGTA, and incubation was continued for 90 min at 37°C (final OD_{600}, ~0.9; about 2×10^8 cells per ml). The bacterial cells were then removed by centrifugation (7,000 × *g* at 4°C for 20 min), and the clarified culture supernatant was filter-sterilized. Proteins were precipitated from this culture supernatant by the addition of solid ammonium sulfate (40 g/100 ml of supernatant). RPs were subjected to SDS-PAGE as described recently (11, 20). Variations on the standard procedure for RP isolation are described in Results. Other media used to prepare RPs included Luria broth (LB) and the cell culture medium RPMI 1640. Cell growth in LB was comparable to that in BHI medium, whereas *Yersinia* sp. grew slowly in RPMI 1640 and reached an OD_{600} of ≈0.6 after 3 h of incubation. The calcium- and magnesium-ion concentrations of the three media as measured by a multianalyzer (Astra 8; Beckman Instruments, Inc., Fullerton, Calif.) were as follows: BHI medium, 0.2 mM Mg^{2+}, 0.4 mM Ca^{2+}; LB, 0.02 mM Mg^{2+}, 0.15 mM Ca^{2+}; RPMI 1640, 0.45 mM Mg^{2+}, 0.42 mM Ca^{2+}.

Isolation of bacterial outer membranes. Bacteria cultivated for protein release were sedimented by centrifugation (7,000 × *g* at 4°C for 20 min), washed once in Tris buffer (100 mM Tris/Tris hydrochloride [pH 8.0]), and then disrupted by sonic lysis as described by Achtman et al. (1). Unbroken cells were separated from envelope vesicles by centrifugation (2,000 × *g* at 4°C for 20 min). The envelope vesicles, which consisted of inner and outer membranes, were then harvested by high-speed centrifugation (100,000 × *g* at 4°C for 30 min). The protein contents of the membrane preparations were determined by the method of Markwell et al. (24). About 100 μg of protein per sample (50 μl) was subjected to SDS-PAGE (11, 20). The gel was then bidirectionally blotted onto nitrocellulose (see below) and stained with Coomassie blue. Quantitative determination of 2-keto-3-deoxyoctonate (KDO) as the lipopolysaccharide component was performed by the method of Karkhanis et al. (17).

Immunoblot analysis and antiserum preparation. After SDS-PAGE, the gels were sandwiched between two piles of filter paper. Each pile of filters consisted of 15 dry Whatman 3MM papers (Whatman, Inc., Clifton, N.J.), one Western blot buffer-wetted 3MM paper (33), and one Western blot buffer-soaked nitrocellulose sheet (BA85; Schleicher & Schuell, Inc., Dassel, Federal Republic of Germany).

This sandwich was then placed for 1 h in a vacuum gel dryer (Bio-Rad Laboratories, Munich, Federal Republic of Germany) after initial heating for 10 min to facilitate the transfer of proteins from the gel to the nitrocellulose. The Whatman 3MM paper was then removed, and the nitrocellulose gel sandwich was submerged in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM Na_2HPO_4/NaH_2PO_4 [pH 7.0]) plus 0.5% Tween 20 (PBS-Tween) to dissociate the gel from the nitrocellulose. The gel was stained with Coomassie blue (11). The nitrocellulose filters were shaken in PBS-Tween for 30 min (blocking procedure) and then incubated with rabbit antiserum to the RPs of *Y. enterocolitica* O:9 strain Y-96 Nal^r (anti-O:9RP; dilution, 1:100 with PBS-Tween) overnight. Then the filter was washed three times for 10 min each with PBS-Tween and incubated with swine anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Dakopatt, Copenhagen, Denmark) diluted 1:1,000 with PBS-Tween for 2 h. After three washes as above, the reactive bands were developed by the method of Blake et al. (2). Anti-O:9RP was obtained by intracutaneous immunization with a crude preparation of RPs of strain Y-96 Nal^r (precipitate of a 100-ml culture suspended in 1 ml of PBS) emulsified in Freund incomplete adjuvant by the method of Harboe and Ingild (10).

Immunprecipitation of solubilized minicells. *Yersinia* plasmids were mobilized via cointegration (11, 14) into minicell-producing *E. coli* DS410. Isolation of minicells and labeling (37°C for 1 h) by using [^{35}S]methionine (Amersham Corp., Buchler, Federal Republic of Germany) were performed by the method of Reeve (29). After separation of the labeled minicells by centrifugation (Eppendorf microfuge; 4 min) the pellet of a 1-ml culture (about 60,000 to 120,000 cpm) was solubilized in electrophoresis sample buffer for SDS-PAGE.

For immunprecipitation, the pellet of a 1-ml culture was frozen (−20°C) and warmed up (37°C) three times (to enhance solubilization) and was solubilized (32) in 200 μl of PBS plus 1% Zwittergent 3-14 (solubilization buffer; Calbiochem-Hoechst, Frankfurt, Federal Republic of Germany). After unsolubilized material was removed by centrifugation (Eppendorf microfuge), 4 μl of undiluted anti-O:9RP was added, and the mixture was incubated for 10 h at 20°C. About 50 μl of sedimented protein A-Sepharose CL-4B

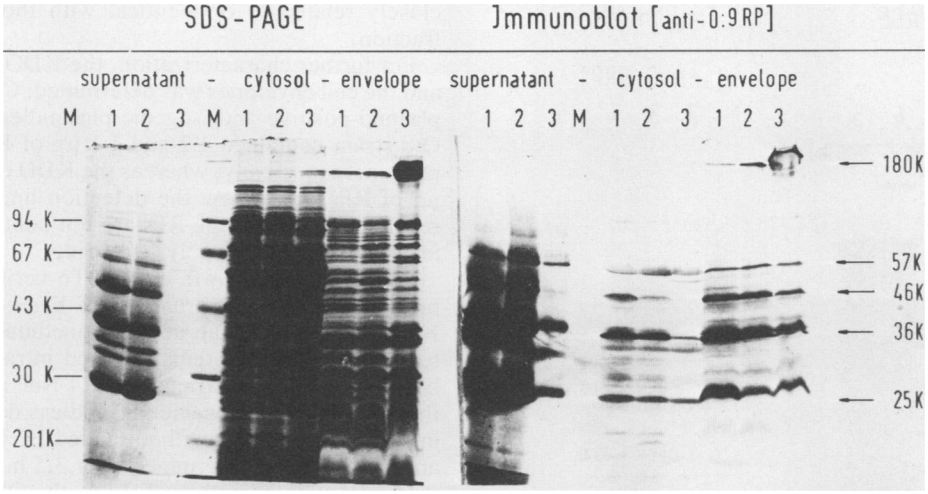

FIG. 2. Left panel: SDS-PAGE (Coomassie-stain) of RPs precipitated from supernatants (5 ml), cytosol proteins, and cell envelopes of *Y. enterocolitica* O:8 strain WA-314Nal^r grown in different calcium-depleted (10 mM EGTA) media. Lanes: 1, BHI medium; 2, LB; 3, RPMI 1640. Right panel: Western blot (obtained from the SDS-PAGE) developed with anti-O:9RP. The molecular masses of the major reactive protein bands are indicated in kilodaltons.

(Pharmacia Fine Chemicals, Uppsala, Sweden) was then added. After 2 h of incubation at 20°C, the sample was washed four times with solubilization buffer and finally boiled with sample buffer for SDS-PAGE (12%).

RESULTS

Culture conditions which affect release of plasmid-encoded proteins from *Yersinia* sp. Protein release by *Y. enterocolitica* O:8 strain WA-314 Nal^r was systematically investigated under various growth conditions. The results are representative also for enteropathogenic *Yersinia* sp. of other serotypes.

(i) Effect of ionized calcium and magnesium. An overnight culture (BHI medium; 26°C; OD_{600}, ~3.0) was diluted 1:20 with fresh BHI medium and grown with shaking at 37°C for 90 min (final OD_{600}, ~0.45). Then EGTA and either calcium chloride or magnesium chloride were added, and incubation was continued for 90 min at 37°C followed by isolation of RPs.

Equal amounts of RPs were obtained in BHI medium supplemented with 10 mM to 1 mM EGTA (Fig. 1, lanes 2 to 4). At an EGTA concentration of 0.1 mM, protein release was no longer supported (lane 5). Repression of protein release occurred under calcium chloride supplementation even in the presence of 10 mM EGTA (lanes 6 to 8). Surprisingly, a large surplus of EGTA over calcium (ratio, 10:1; lane 9) was required for protein release. By contrast, a surplus of magnesium ions in the presence of EGTA had no effect on protein release (lane 1).

(ii) Effect of temperature and growth phase. Overnight cultures diluted (1:40 to 1:1) in BHI medium were incubated for 90 min at a defined temperature. Then 10 mM EGTA was added, and incubation was continued for 90 min. Under these growth conditions, significant protein release was not observed for cultures at exponential or stationary growth phase at 20 or 30°C, indicating that a growth temperature of about 37°C is required for protein release (results not shown). We further investigated the effect of the incubation time under calcium restriction on protein release. Overnight cultures (26°C) diluted 1:40 to 1:1 in BHI medium plus 10 mM EGTA and then incubated at 37°C with shaking for 1 to 10 h did not release significant amounts of plasmid-encoded proteins (not shown).

A similar result was obtained for cultures exponentially grown at 37°C for 90 min in BHI medium followed by a brief incubation (up to 30 min) under calcium restriction (10 mM EGTA). However, extension of the incubation time in calcium-depleted BHI medium to more than 60 min resulted in large amounts of RPs. The optimal growth conditions (about 20 mg of RPs per liter) were as follows. An overnight culture (BHI medium; 26°C) was diluted 1:20 in fresh BHI medium (100 ml/250 ml-Erlenmeyer flask; OD_{600}, ~0.08) and then incubated with shaking (120 rpm) for 90 min at 37°C (OD_{600}, ~0.4). After 10 mM EGTA was added, incubation was continued for 90 min (final OD_{600}, ~0.8 to 0.9). Bacteria were then separated by centrifugation and the RPs were precipitated.

(iii) Effect of various culture media. We found that the highest yields of RPs were obtained with BHI medium, although LB also gave good results (Fig. 2, lanes 1 and 2). However, cell culture media such as RPMI 1640 did not favor protein release (lane 3). The reason for this may be related to the low multiplication rate (one-third of that in BHI medium) of plasmid-positive *Yersinia* spp. in this medium.

Localization of RP species in different cell compartments. Plasmid-positive *Y. enterocolitica* O:8 WA-314 Nal^r was grown in different media (BHI, LB, and RPMI 1640) under conditions which were optimal for protein release (see above). RPs were precipitated from culture supernatants. Cell envelope proteins (inner and outer membrane) and cytosol proteins (soluble fraction) were isolated by differential centrifugation after disruption of the bacteria by sonication. The three fractions obtained from three different cultures (Fig. 2, designated 1, 2, and 3) were analyzed by SDS-PAGE and by immunoblotting by using anti-O:9RP. The cytosol and envelope protein profiles 1, 2, and 3, respectively, are quite similar (Fig. 2). Furthermore, they do not exhibit distinct protein bands which could be related to protein species of the released fraction (Fig. 2, supernatant). The only plasmid-encoded membrane protein which can be clearly seen is the 180-kDa protein designated YOP1 (3, 5).

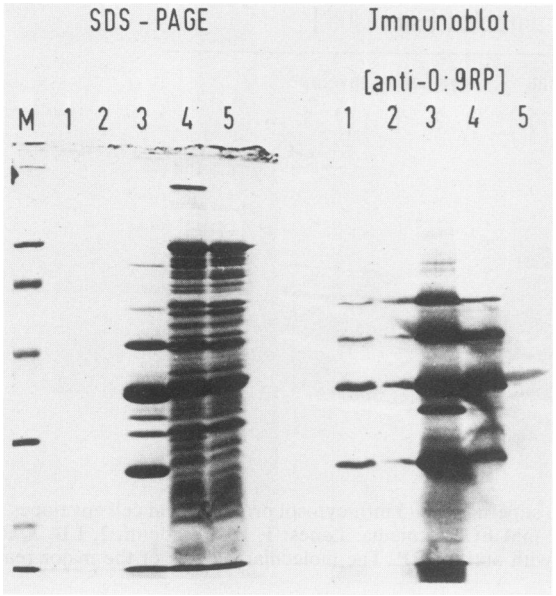

FIG. 3. Left panel: SDS-PAGE (Coomassie-stain) of RPs and cell envelopes of *Y. enterocolitica* O:8 grown under calcium restriction (10 mM EGTA) in BHI medium at 37°C for 1.5 h. Lanes: 1 and 2, protein pellets obtained by centrifugation (135,000 × *g* for 5 h) of 5- and 20-ml culture supernatants, respectively; 3, ammonium sulfate precipitate of the corresponding culture supernatant (5 ml) after high-speed centrifugation; 4 and 5, cell envelopes (120 μg) of the serotype O:8 strains WA-314 Nal^r (plasmid positive) and WA-cNal^r (plasmid negative). Right panel: Western blot (obtained from the SDS-PAGE) developed with anti-O:9RP. M: Marker proteins (see legend to Fig. 2).

The expression of this protein was obviously favored by RPMI 1640 (Fig. 2, overloaded band).

Taking advantage of the high sensitivity of the immunoblot technique, we were able to detect RP species in cytosol, membrane, and culture supernatant fractions (Fig. 2). By this technique we were also able to visualize RPs in RPMI 1640 culture supernatant (lane 3). However, the 46-kDa protein was found in a relatively low concentration; the same was true for the cytosol fraction (lane 3).

To determine whether the RPs originated by membrane vesiculation, the culture supernatant containing the RPs was subjected to high-speed centrifugation (135,000 × *g* at 5°C for 5 h) to separate membrane vesicles from soluble proteins. Only trace levels of material sedimented after centrifugation were solubilized with electrophoresis sample buffer. The remaining supernatant was treated with ammonium sulfate to precipitate RPs.

The two samples obtained were subjected to SDS-PAGE. Only trace levels of RPs were sedimented by high-speed centrifugation (Fig. 3, lanes 1 and 2), whereas the bulk of RPs remained in the supernatant (also shown in the Coomassie stain). For comparison, the cell envelope patterns of the plasmid-positive and the plasmidless derivative of *Y. enterocolitica* O:8 are also shown in Fig. 3. The Coomassie-stained patterns are similar, with the exception of the 180-kDa protein YOP1, which is characteristic for the plasmid-positive strains. Again, immunoblotting revealed the presence of four protein species within the cell envelope of the plasmid-positive strain which are not found in the plasmid-negative strain (Fig. 3, lane 5). These protein bands are closely related to or identical with those of the released fraction.

For further characterization, the KDO content of the RPs and the cell envelopes was determined. Cell envelopes of the plasmid-positive and isogenic plasmidless *Y. enterocolitica* O:8 strain contained 1.2 and 1.1 μg of KDO per 100 μg of protein, respectively, whereas the KDO content of about 100 μg of RPs was below the detection limit of 0.05 μg. This result suggests that the RPs are not contaminated by significant amounts of lipopolysaccharide.

Comparison of growth curves. To verify that the proteins present in culture supernatants of *Y. enterocolitica* WA-314 Nal^r grown in calcium-depleted medium are secreted proteins rather than proteins released intracellularly after cell lysis, the growth of strain WA-314 Nal^r was compared with the growth of the isogenic plasmidless derivative WA-cNal^r in BHI broth with or without added EGTA (10 mM). CFUs and OD_{600}s were determined over 3.5 h.

In BHI with or without EGTA, the OD_{600} of the cultures increased in proportion to the CFUs, indicating a continuous increase in cell mass and cell number without cell lysis. When the calcium chelator EGTA was added to the culture broth, the rate of multiplication of the plasmid-bearing strain was slowed slightly compared with the plasmidless strain (result not shown). These results suggest slight inhibition of multiplication rather than cell lysis during growth under calcium restriction.

Analysis of RPs by SDS-PAGE and immunoblotting. The SDS-PAGE profile of proteins precipitated with ammonium sulfate from culture supernatants (5 ml) of plasmid-positive *Y. enterocolitica* O:3, O:8, and O:9 as well as *Y. pseudotuberculosis* serotype I are shown in Fig. 4. RPs were not detected from any of the plasmidless strains (results not shown; 11). The protein profiles of the different plasmid-bearing strains are quite similar. *Y. enterocolitica* and *Y.*

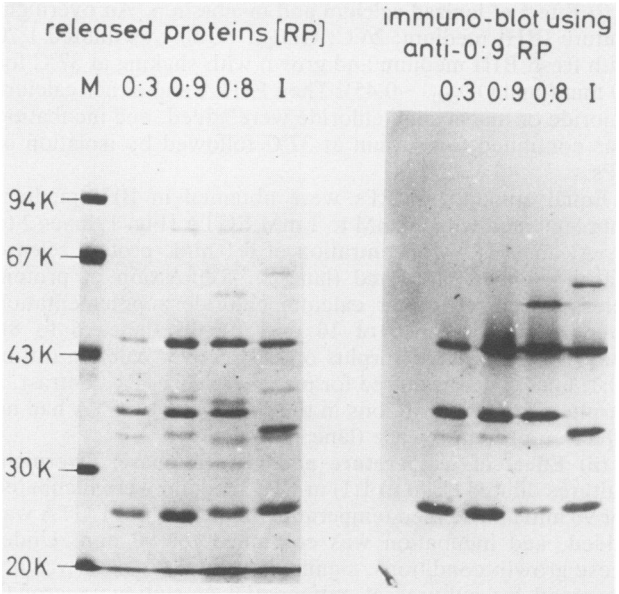

FIG. 4. SDS-PAGE (left panel; Coomassie stain) and immunoblot (right panel; developed with anti-O:9RP) of RPs (5 ml of supernatant) of *Y. enterocolitica* O:3, O:9, and O:8 and *Y. pseudotuberculosis* serotype I. The proteins were bidirectionally transferred from the SDS-PAGE by vacuum blotting. M: Marker proteins are indicated in kilodaltons.

pseudotuberculosis strains all exhibited three major proteins (25, 37, and 47 kDa for *Y. enterocolitica* and 26, 34, and 47 kDa for *Y. pseudotuberculosis*) and three to four minor proteins (30, 34, 38, 57, and 67 kDa).

To analyze the serological relationships among the RPs from different *Yersinia* species, immunoblotting was performed. Anti-O:9RP recognized all major proteins regardless of the serotype. Furthermore, anti-O:9RP recognized the 57-kDa protein of *Y. enterocolitica* O:8 and the 67-kDa protein of *Y. pseudotuberculosis* serotype I, although these protein species are not seen within the protein pattern of *Y. enterocolitica* O:9. When antiserum against the RPs of *Y. pseudotuberculosis* or *Y. enterocolitica* O:8 were tested, similar results were obtained (data not shown). From these experiments, it was concluded that the RPs from different *Yersinia* species share antigenic determinants.

Identification of plasmid-encoded proteins by using *E. coli* minicells. The virulence plasmids of *Y. enterocolitica* and *Y. pseudotuberculosis* were transformed as cointegrates (consisting of the virulence plasmid and the vector pRK290B) (11, 14) into minicell-producing *E. coli* DS410. The labeled minicell products were immunoprecipitated with anti-O:9RP and analyzed by SDS-PAGE. Three major proteins (25, 37, and 47 kDa for *Y. enterocolitica* and 26, 34, and 47 kDa for *Y. pseudotuberculosis*) were precipitated by antibodies. These protein bands are virtually identical in molecular mass to those released by *Y. enterocolitica* and *Y. pseudotuberculosis*.

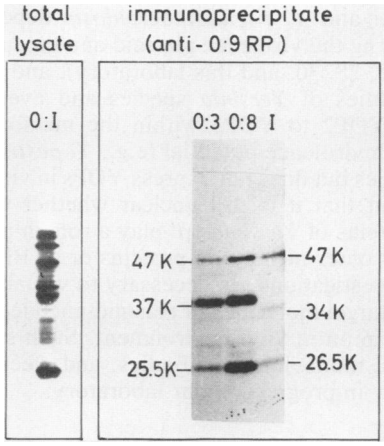

FIG. 5. Detection of protein species of the related fraction in *E. coli* minicells harboring virulence plasmids derived from *Y. enterocolitica* O:3 strains Y-108 Nal^r and O:8 strain WA-314 Nal^r and *Y. pseudotuberculosis* serotype I Y-pNal^r. Left panel: autoradiograph of ^{35}S-labeled products of solubilized minicells harboring a plasmid of *Y. pseudotuberculosis* serotype I. Right panel: immunoprecipitates of different samples obtained after reaction with anti-O:9RP. Molecular masses are given in kilodaltons.

DISCUSSION

Recently we reported several protein species which were isolated from the calcium-deficient growth medium of plasmid-bearing *Y. enterocolitica* O:3 and O:9 strains (11). In the present study we showed that the release of plasmid-encoded proteins is a common phenomenon of enteropathogenic *Yersinia* sp. (*Y. enterocolitica* and *Y. pseudotuberculosis*). By variation of growth conditions, we found that the highest yield of RPs (about 20 mg/liter of culture medium) was obtained from cultures grown at 37°C in rich media (e.g., BHI medium) under calcium-restriction (e.g., calcium chelation by EGTA) during the exponential growth phase.

One fundamental question was whether the RPs were generated by autolysis of cells, by membrane vesiculation, or by secretion. There are four major results which argue against cell lysis or membrane vesiculation. (i) There is no significant indication for cell lysis from growth curves. (ii) SDS-PAGE patterns of RPs substantially differ from those of cell envelopes and cytosol proteins (superposition of both profiles reflect the pattern of lysed cells). (iii) The sedimentation coefficient of RPs significantly differs from that of membrane vesicles. (iv) The RPs are practically free of lipopolysaccharides. From these results, we conclude that plasmid-positive *Y. enterocolitica* and *Y. pseudotuberculosis* are more likely to secrete proteins into the culture medium under calcium-restriction than to release proteins by membrane vesiculation.

To determine the immunochemical relatedness of the RPs of the different strains, we used the immunoblot technique. We were able to demonstrate than an antiserum raised against the RPs of one strain recognized the RPs of different serotypes and species. From this, we suggest that the RPs of different strains share common determinants.

However, one apparent inconsistency of the immunoblot results must be mentioned. Anti-O:9RP also recognized the 57-kDa protein of *Y. enterocolitica* O:8 and the 67-kDa protein of *Y. pseudotuberculosis*, although corresponding bands were not detectable with *Y. enterocolitica* O:9 RPs. The same result was obtained with serum from a convalescent patient (*Y. enterocolitica* O:3 infection [12]). There is evidence from experiments with monoclonal antibodies (12a) which may explain this result; i.e., the 47-kDa protein of the serotype O:3 and O:9 strains consists of two protein species, one of which is immunochemically related to the 57- and the 67-kDa proteins.

By using the *E. coli* minicell system in combination with precipitating antibodies to *Yersinia* RPs, conclusive evidence was obtained that at least the three major proteins of the released fraction of *Y. enterocolitica* (25, 37, and 47 kDa) and *Y. pseudotuberculosis* (26, 34, and 47 kDa) are plasmid encoded. Of course, it is conceivable that there are more than three released major RPs that are plasmid encoded, but they could not be detected because of inefficient expression in *E. coli*.

The question remained whether these three major RPs are related to or identical with the plasmid-encoded outer membrane proteins of *Y. enterocolitica* and *Y. pseudotuberculosis* (designated YOPs [4, 28, 30]). Molecular mass determination and the results of membrane analysis suggest that two of the proteins may be related to YOP5 (26 kDa [4]) and YOP4 (36 kDa for *Y. enterocolitica* and 34 kDa for *Y. pseudotuberculosis* [4]) and the 47-kDa protein may be related to the 46.3-kDa membrane protein YOPI (30). However, the RP of 57 kDa of *Y. enterocolitica* O:8 and that of 67 kDa of *Y. pseudotuberculosis* were not described previously as YOPs. It is still unclear whether the described YOP2 to YOP5 are really inserted into the outer membrane of *Yersinia* sp. under in vivo conditions. There is only evidence from immunological data (infected animals and patients produce antibodies to YOPs) that protein species related to YOPs are expressed in vivo by all pathogenic *Yersinia* sp. (4, 12, 25). Taking into account our results and those which show that (i) *Y. pestis* does not express YOPs in vitro, that (ii) accumulation of YOPs within the outer membrane of *Y.*

enterocolitica and *Y. pseudotuberculosis* depends on the strain harboring the virulence plasmid and on culture conditions (3–5, 25, 28, 30, and this laboratory), and that (iii) the different abilities of *Yersinia* species and even strains to accumulate YOP2 to YOP5 within the membrane do not correlate with virulence potential (e.g., *Y. pestis* is the most virulent species but does not express YOPs in vitro), then we have to admit that it is still unclear whether the plasmid-encoded proteins of *Yersinia* sp. play a role in pathogenesis as cytosol or outer membrane proteins or as RPs.

Further investigations are necessary to visualize the location and the target molecules of plasmid-encoded proteins of *Yersinia* sp. in an in vivo environment. Such studies using genetic tools, monoclonal antibodies, and electron microscopy are now in progress in our laboratory.

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LITERATURE CITED

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