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Genetically Manipulated Virulence of *Yersinia enterocolitica*

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Mobilizable virulence plasmids of *Yersinia enterocolitica* of serotypes O:3 and O:9 were constructed by cointegration of a mobilizable vector into the virulence plasmids. The obtained cointegrates were mobilized into plasmidless *Y. enterocolitica* strains of serotypes O:3, O:5, O:8, and O:9. The transfer experiments revealed the existence of two different subgroups of plasmid-associated traits. (i) Animal virulence functions (mouse lethality and conjuctivitis provocation) were only transferable to plasmid-cured derivatives of virulent parent strains (serotypes O:3, O:8, and O:9), but they were not transferable to *Y. enterocolitica* antigen reference strains (serotypes O:3 and O:8) or to a plasmidless clinical isolate of serotype O:5. A further striking result was that a serotype O:8 strain regained the mouse lethality trait after receipt of a plasmid from a strain not lethal to mice. These results demonstrate that plasmid-mediated animal virulence functions are not uniformly expressed within *Y. enterocolitica*. (ii) The second subgroup of plasmid-mediated traits (calcium dependency, surface agglutinogens, HEp-2 cell adherence, and protein release) were transferable to all *Y. enterocolitica* recipient strains tested (serotypes O:3, O:5, O:8, and O:9 of different origin). For the first time HEp-2 cell adherence and temperature-induced release of five major protein species are described as transferable traits.

Human pathogenic strains of the genus *Yersinia* harbor closely related plasmids of ca. 44 megadaltons (Mdal) (2, 13, 15–17, 23, 31–33, 37, 39). The plasmid-bearing strains of three species (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) are virulent in several animal models, whereas plasmidless derivatives are avirulent (1, 13, 15–17, 23, 26, 32, 37, 39). Recently it was demonstrated that the plague virulence antigen, V (7, 9, 28, 30), and calcium-dependent growth at 37°C (2, 13, 15, 16, 39) are plasmid mediated within virulent *Yersinia* strains. Furthermore, temperature-inducible outer membrane proteins and surface agglutinogens were found to be plasmid mediated by *Y. pseudotuberculosis* and *Y. enterocolitica* (4, 5, 12, 17, 32, 33, 36; J. Heesemann, unpublished data).

Plasmid-associated properties of *Y. enterocolitica* which are evaluated as virulence factors are provocation of guinea pig conjunctivitis, survival in human serum, cytotoxicity for tissue culture cells, lethality for mice, and calcium-dependent growth (1, 12, 13, 15–17, 23, 24, 27, 30, 32, 37–39).

Besides plasmid-controlled functions, chromosomal determinants are evidently relevant for expression of virulence for rodents. This was demonstrated by using mutants of *Y. pestis* (6–8) and by *Y. enterocolitica* strains of different serotypes which had received an identical virulence plasmid (18).

Plasmid transfer experiments are particularly suitable for the analysis of the genetic organization and regulation responsible for virulence expression and to identify plasmid-encoded products, as has been demonstrated with plasmids of three *Yersinia* species (5, 18, 30, 33).

Recently, two different methods have been applied to transfer *Yersinia* plasmids: (i) P1 transduction of Tn*5*-tagged *Y. pestis* plasmids (30, 33) and (ii) construction of *Y. enterocolitica* cointegrates consisting of a mobilizable vector and the virulence plasmid (18). In this paper we present the construction of mobilizable *Y. enterocolitica* cointegrates of serotypes O:3 and O:9, the most common clinical isolates in Europe. The cointegrates were mobilized into plasmidless *Y. enterocolitica* recipients of serotypes O:3, O:5, O:8, and O:9. The obtained transconjugants were tested for transferable traits which may be relevant for the pathogenesis of yersiniosis. A plasmidless serotype O:5 strain was chosen as the test strain for investigating plasmid-mediated cell interaction because of its inability to contact HEp-2 cells.

Furthermore, we attempted to isolate lower-molecular-weight, plasmid-associated outer membrane proteins characteristic for virulent *Y. enterocolitica* (31, 33, 36). These experiments were unsuccessful with our *Yersinia* strains. Instead, we were able to isolate about five protein species which were released into the medium by plasmid-containing strains of serotypes O:3, O:8, O:9, and O:5.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used are listed in Table 1. Growth conditions and media were the same as those described in reference 18. For protein isolation, *Y. enterocolitica* was grown in brain heart infusion (BHI) medium supplemented with 20 mM magnesium chloride, 20 mM sodium oxalate, and 0.2% glucose (BHI-MOX) at 37°C (before use, precipitated calcium oxalate was removed by centrifugation).

Enzymes and reagents. Restriction endonucleases *Eco*RI, *Bam*HI, and *Xba*I and the *Bam*HI molecular linker were purchased from Bethesda Research Laboratories, Neu-Isenheim, Federal Republic of Germany. T4 DNA ligase, DNA polymerase I (Klenow enzyme), T4 polynucleotide kinase, calf intestine alkaline phosphatase, and monodeoxyribonucleotide-5′-triphosphates (thymidine 5′-triphosphate and dATP) were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Recombiant DNA techniques. Plasmid screening was performed as described by Birnboim and Doly (3). A modification of this procedure was used for preparative plasmid isolation (17). Agarose gel electrophoresis of DNA was performed as previously described (25).

Cloning of *Bam*HI-generated *Yersinia* plasmid DNA fragments into the *Bam*HI site of pRK290B was performed as

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TABLE 1. Bacterial strains and plasmids

Organism and strain designation	Relevant characteristics[a]	Plasmids	Source
E. coli			
HB101	*hsdR hdsM recA* Str^r		J. Collins, Braunschweig, Federal Republic of Germany
HB101	*hsdR hsdM recA* Str^r Kan^r	pRK2013	D. Helinski; helper plasmid
HB101	*hsdR hsdM recA* Str^r Tet^r	pRK290B	derivative of pRK290; *Bam*HI linker cloned into the *Eco*RI site (18)
HB101	*hsdR hsdM recA* Str^r Tet^r	pRK290B3-5	This study; hybrid molecule carrying a 2.9-Mdal *Bam*HI fragment of pO:3
HB101	*hsdR hsdM recA* Str^r Tet^r	pRK290B3-5::pO:3	This study; cointegrate (62 Mdal)
HB101	*hsdR hsdM recA* Str^r Tet^r	pRK290B9-5	This study; hybrid molecule carrying a 2.9-Mdal *Bam*HI fragment of pO:9
HB101	*hsdR hsdM recA* Str^r Tet^r	pRK290B9-5::pO:9	This study; cointegrate (60 Mdal)
Y. enterocolitica			
Y-108Nal^r (serotype O:3)	Ml^- Con^+ Mox^+ $Ag3^+$ Nal^r	pO:3	This study; one-step Nal^r mutant of the clinical isolate Y-108, serotype O:3 harboring the 46-Mdal plasmid pO:3
Y-108-cNal^r	Ml^- Con^- Mox^- $Ag3^-$ Nal^r		Spontaneous plasmidless mutant of Y-108Nal^r
Y-75S-Nal^r (serotype O:3)	Ml^- Con^- Mox^- $Ag3^-$ Nal^r		One-step Nal^r mutant of 75-S from W. Knapp, Erlangen, Federal Republic of Germany
Y-96Nal^r (serotype O:9)	Ml^- Con^+ Mox^+ $Ag9^+$ Nal^r	pO:9	This study; one-step Nal^r mutant of the clinical isolate Y-96, serotype O:9, harboring the 44-Mdal plasmid pO:9
Y-96-cNal^r	Ml^- Con^- Mox^- $Ag9^-$ Nal^r		Spontaneous plasmidless mutant of Y-96Nal^r
WA-319Nal^r (serotype O:8)	Ml^+ Con^+ Mox^+ $Ag8^+$ Nal^r	pO:8	One-step Nal^r mutant of the clinical isolate WA-314, serotype O:8 (18)
WA-cNal^r	Ml^- Con^- Mox^- $Ag8^-$ Nal^r		Spontaneous plasmidless mutant of WA-314Nal^r (18)
Y-161M-Nal^r (serotype O:8)	Ml^- Con^- Mox^- $Ag8^-$ Nal^r		One-step Nal^r mutant of 161M from H. Mollaret, Paris, France, through S. Aleksic, Hamburg, Federal Republic of Germany
Y-NF-Nal^r (serotype O:5)	Ml^- Con^- Mox^- Adh^- Nal^r		One-step Nal^r mutant of B-24151-80 (18)

[a] Symbols: *hsdR hsdM recA*, deficient in DNA restriction, modification, and recombination, respectively; Tet^r, Kan^r, Nal^r, and Str^r, resistance to tetracycline, kanamycin, nalidixic acid, and streptomycin, respectively; Ml, mouse lethality; Con, conjunctivitis provocation in the guinea pig eye; Mox, growth inhibition on magnesium oxalate agar at 37°C; Ag3, Ag8, and Ag9, surface agglutinogens with plasmids pO:3, pO:8, and pO:9, respectively.

previously described (11, 18). *Escherichia coli* HB101 was transformed to tetracycline resistance according to the method of Dagert and Ehrlich (10). Triparental patch matings (*E. coli* donor × *E. coli* helper × *Y. enterocolitica* recipient) determination of conjugal transfer frequencies and of cointegrate stability were done as described previously (18).

Virulence assays. The ability to evoke conjunctivitis in the guinea pig eye (phenotype Con) was assessed by the Serény test (17, 35). The lethal response in white mice of strain NMRI (phenotype Ml) was determined by intraperitoneal injection of 1 ml of 10^7 bacteria in 0.9% NaCl.

Calcium requirement test. The calcium requirement test was done as previously described (19). Nutritional dependence on calcium ions at 37°C was determined by plating an appropriate suspension of bacteria onto duplicate BHI-MOX agar. One plate was incubated at 37°C and the other at 27°C. After 20 h of incubation, the colonies (diameter of at least 1 mm) were counted. When the number of colonies on the plates incubated at 37°C was less than 5% of that on the plates incubated at 27°C, the strain was considered to be calcium dependent (phenotype Mox).

Determination of plasmid-associated agglutinogens. Heat-killed, plasmid-bearing strains Y-108, serotype O:3, and Y-96, serotype O:9, previously grown in BHI-MOX at 37°C, were used to immunize rabbits (17). The obtained antiserum was sufficiently adsorbed with the homologous plasmidless heat-killed strain. The resulting antisera were designated pO:3 and pO:9 antiserum, respectively. Plasmid-associated surface agglutinogens (phenotypes Ag3, Ag8, and Ag9, mediated by the plasmids pO:3, pO:8, and pO:9, respectively) were detected by simple slide agglutination (antiserum dilution, 1:50) by using colonies previously incubated at 37°C for 1 to 2 h.

Infection of HEp-2 cells. For the cell infection test we used the procedure described previously (17, 22). Briefly, nonconfluent monolayers of HEp-2 cells on cover slips grown overnight in antibiotic-free medium were inoculated with 10^8 bacteria per ml, which were previously grown for 120 min at 37°C in BHI medium (final optical density at 600 nm, ≈0.3). After an interaction time of 30 min at 37°C, the cover slips were washed twice with phosphate-buffered saline and fixed with methanol. Giemsa-stained cells were examined microscopically for cell-associated bacteria (phenotype HEp) and photographed.

Isolation of excreted proteins and SDS-polyacrylamide gel electrophoresis. An overnight culture of *Y. enterocolitica* (BHI medium, 26°C) was diluted 1:40 with fresh BHI broth and then incubated at 37°C for 90 min (final optical density at 600 nm, ≈0.3). The bacteria were harvested by centrifugation, and the pellet was resuspended in BHI-MOX medium.

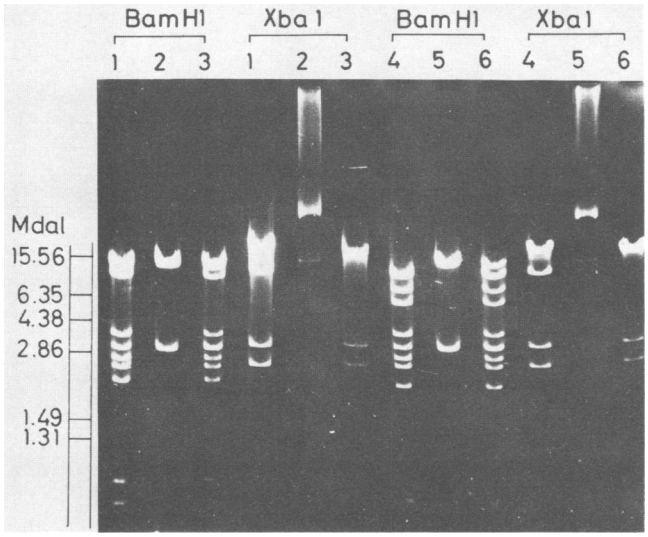

FIG. 1. *Bam*HI and *Xba*I restriction endonuclease digestion patterns obtained after electrophoresis in 0.7% agarose: Lanes 1, Virulence plasmid pO:3 from strain Y-108; lanes 2, hybrid plasmid pRK290B3-5 from strain HB101; lanes 3, cointegrate pRK290B3-5::pO:3 from strain HB101; lanes 4, virulence plasmid pO:9 from strain Y-96, lanes 5, hybrid plasmid pRK290B9-5 from strain HB101; lanes 6, cointegrate pRK290B9-5::pO:9 from strain HB101. After *Xba*I treatment the hybrid plasmid DNS of pRK290B3-5 (lanes 2) and pRK290B9-5 (lanes 5) remained as covalently closed circular molecules. Mdal, *Hin*dIII-digested lambda DNA with molecular weight markers in mdal.

Incubation was continued for 90 min at 37°C, and then the bacteria were separated by centrifugation. The supernatants of the BHI culture and the BHI-MOX culture were filter sterilized and treated with solid ammonium sulfate (final concentration, 3M) (21). After overnight incubation at 4°C, the brown-yellow precipitates were collected by centrifugation (4,000 × *g*, 15 min). Precipitates resulting from 300-ml cultures were resuspended in 1 ml TE buffer (10 mM Tris-hydrochloride [pH 8], 0.1 mM EDTA). The suspensions were centrifuged, and the obtained pellets were dissolved in 1.5 ml of sample buffer (62.5 mM Tris-hydrochloride [pH 6.8] containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue). Samples (30 μl) were electrophoresed at a constant current of 30 mA for 3 h using a 0.15-cm-thick slab gel of 12% acrylamide as the running gel and 3.5% acrylamide as the stacking gel in a discontinuous buffer system as described by Laemmli (20). The gel was stained with 0.25% (wt/vol) Coomassie brilliant blue R-250 in 50% methanol–10% acetic acid and destained at once in 50% methanol–10% acetic acid, followed by 5% methanol–7% acetic acid. Protein concentrations were determined according to the method of Peterson (25).

RESULTS

Cointegrate formation. The 2.8-Mdal *Bam*HI fragments of pO:3 and pO:9 were cloned into the *Bam*HI site of pRK290B by using *E. coli* HB101 as the host strain. The resulting hybrid plasmids, designated as pRK290B3-5 and pRK290B9-5, were used to construct the cointegrates pRK290B3-5::pO:3 and pRK290B9-5::pO:9, respectively, via homologous recombination (for details see reference 18). Comparison of the restriction patterns (using the enzymes *Bam*HI and *Xba*I) of the component plasmids involved in the recombination event and the cointegrates (Fig. 1) shows that the cointegrates are composed of the recombining component plasmids, by which the vector component is flanked by the recombining *Bam*HI fragments (2.9 Mdal) (18). The cointegrates were conjugally transferred from *E. coli* HB101 to plasmidless *Y. enterocolitica* recipients of different serotypes by using the helper plasmid pRK2013. The transfer frequencies were ca. 10^{-4}.

Expression of cointegrate-associated phenotypes. The transconjugants were tested for expression of the following phenotypes: mouse lethality (Ml), conjunctivitis provocation (Con), calcium requirement for growth (Mox), surface agglutinogens (Ag3 and Ag9), and interaction with HEp-2 cells (HEp). The results are shown in Table 2.

After receipt of the cointegrates the cured strains of serotypes O:3, O:9, and O:8 regained the phenotypic profile of the parental strains. A striking result was that the recipient WA-cNal^r, serotype O:8, regained the ability to kill mice, although the cointegrates were derived from strains not lethal to mice. However, transconjugants of serotype O:3 and O:8 which were derivatives of antigen reference strains were avirulent for animals. The same holds for the clinical isolate of serotype O:5.

The ability of *Y. enterocolitica* to interact with HEp-2 cell monolayers was examined by light microscopy. *Yersinia* strains of serotypes O:3, O:8, and O:9 were found to interact strongly with HEp-2 cells. By optical sectioning through the cells, plasmid-containing strains appeared to be extracellularly located, whereas plasmidless strains appeared to be predominantly intracellularly located, which is in accordance with the results of Vesikari et al. (37, 38). The serotype O:5 strain, Y-NF-Nal^r, does not interact with HEp-2 cell (Fig. 2A), as is typical for biotype 1 strains (22). After

TABLE 2. Comparison of phenotypic characteristics of isogenic strains

Origin and strain	Serotype	Plasmid	Phenotype				
			Ml	Con	Mox	Ag[b]	HEp[c]
Clinical isolates							
Y-108		46	−	+	+	+	+
Y-108-cNal^r	O:3		−	−	−	−	+
Transconjugants[a]		62/60	−	+	+	+	+
Y-96		44	−	+	+	+	+
Y-96-cNal^r	O:9		−	−	−	−	+
Transconjugants		62/60	−	+	+	+	+
WA-314		42	+	+	+	+	+
WA-cNal^r	O:8		−	−	−	−	+
Transconjugants		62/60	+	+	+	+	+
Y-NF-Nal^r	O:5		−	−	−	−	−
Transconjugants		62/60	−	−	+	+	+
Reference antigens							
Y-75S Nal^r	O:3		−	−	−	−	+
Transconjugants		62/60	−	−	+	+	+
Y-161M-Nal^r	O:8		−	−	−	−	+
Transconjugants		62/60	−	−	+	+	+

[a] The transconjugants tested harbored the cointegrates pRK290B3-5::pO:3(62 Mdal) and pRK290B9-5::pO:9 (60 Mdal).
[b] Ag denotes plasmid-associated surface antigens Ag3, Ag8, or Ag9.
[c] HEp denotes interaction with HEp-2 cells without discrimination between intracellular and extracellular bacteria.

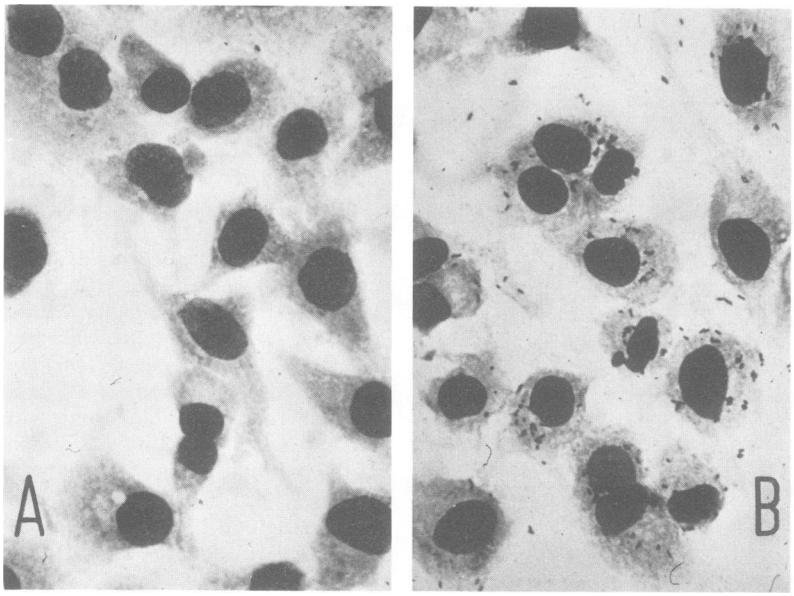

FIG. 2. HEp-2 cell monolayers exposed to *Y. enterocolitica* of serotype O:5, biotype 1, for 30 min and then washed two times and stained by the Giemsa method. ×900. (A) Plasmidless strain Y-NF-Nal^r, no interaction; (B) transconjugant harboring the cointegrate pRK290B3-4::pO:3, abundantly attached bacteria.

receipt of the cointegrates, however, the obtained transconjugant was found to interact strongly with HEp-2 cells (Fig. 2B) and appeared to be extracellularly located. This experiment directly demonstrated that the ability to contact epithelial cells is plasmid mediated.

Plasmid-mediated excretion of proteins. Plasmid-bearing clinical isolates of serotypes O:3 and O:9, their cured derivatives, and transconjugants of serotypes O:3, O:8, O:9, and O:5 harboring the cointegrates or the vector pRK290B (listed in Table 1) were cultivated as described above. Proteins obtained from the culture supernatants were analyzed in SDS-polyacrylamide gel. Supernatants obtained from plasmidless strains or strains harboring the vector pRK290B contained no Coomassie-blue-stainable material (Fig. 3; only representative strains are shown). However, strains harboring the virulence plasmids or the cointegrates apparently release about 10 proteins of discrete molecular weights. Protein release strongly increased after changing the medium from BHI to BHI-MOX. Five major proteins could be identified, with molecular masses of ca. 24, 32, 35, 46, and 49 kilodaltons (kdal).

DISCUSSION

The recently described two-step procedure to generate mobilizable cointegrates of predicted structure (18) was

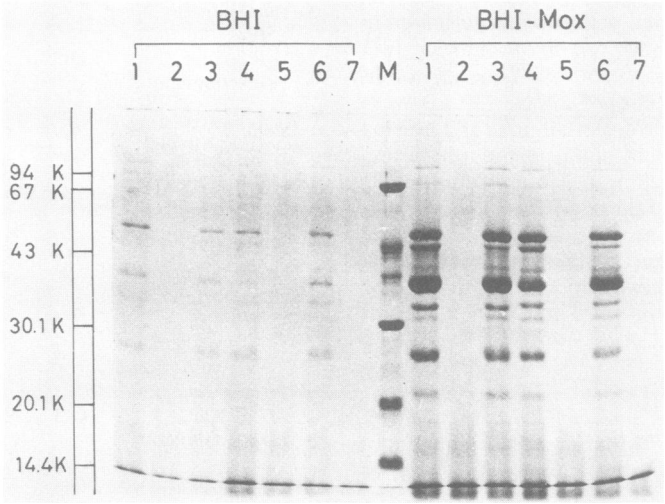

FIG. 3. SDS-polyacrylamide gel electrophoresis of ammonium sulfate-precipitated medium material released by plasmid-positive and plasmid-negative *Yersinia* strains. BHI, Precipitates obtained from 5 ml of BHI of supernatants; BHI-MOX, precipitates obtained from 5 ml of BHI-MOX supernatants. Lanes 1, Strain Y-108Nal^r; lanes 2, strain Y-108-cNal^r; lanes 3, transconjugant of serotype O:5 (pRK290B3-5::pO:3); lanes 4, strain Y-96Nal^r; lanes 5, strain Y-96-cNal^r; lanes 6, transconjugant of serotype O:5 (pRK290B9-5::pO:9); lanes 7, transconjugant of serotype O:5 (pRK290B, vector). Lane M, Reference proteins with indicated molecular weights in kdal.

successfully applied to *Y. enterocolitica* plasmids of serotypes O:3 and O:9 (pO:3 and pO:9).

The cointegrates could be mobilized into *Yersinia* strains of different serotypes. In assessing the obtained transconjugants for suggested plasmid-transferable traits, it became evident that expression of animal virulence (mouse lethality and conjunctivitis) was restricted to strains which were plasmid-cured derivatives of virulent strains. From this we conclude that animal virulence is multideterminant, involving chromosomal and extrachromosomal loci. This conclusion was supported by introducing the mouse lethality trait into the plasmidless WA strain by using a cointegrate derived from a strain not lethal to mice. Transconjugants of serotypes O:3 and O:8 derived from antigen reference strains were avirulent for animals and therefore are suggested to have chromosomal mutations within an unknown virulence locus. These results are similar to the complex phenomenon of animal virulence found with *Y. pestis* and *Shigella flexneri*: (i) plasmid-bearing *Y. pestis* strains may change to avirulence after chromosomal pigmentation mutation (7); and (ii) the conjunctivitis provocation of *S. flexneri* was found to be multideterminant, with contributions of chromosomal and extrachromosomal genes (34).

Besides the multideterminant phenotypes Ml and Con, we were able to show that surface antigens (Ag3, Ag9), calcium dependency (Mox), interaction with HEp-2 cells (HEp), and excretion of proteins were transferable traits within *Yersinia* species. For the first time it was demonstrated directly that HEp-2 cell interaction functions are mediated by *Yersinia* virulence plasmids by comparing an isogenic pair consisting of a plasmidless strain of serotype O:5 (HEp negative) and the cointegrate-bearing derivative (HEp positive).

A further striking phenomenon is the plasmid-mediated release of proteins by *Y. enterocolitica* during cultivation in calcium-deficient media at 37°C. Comparison of the SDS-polyacrylamide gel electrophoresis profiles of these proteins with those of the outer membrane fractions of a virulent *Y. enterocolitica* strain of serotype O:8 and *Y. pseudotuberculosis*, as described by Straley et al. (36) and Portnoy et al. (32, 33), suggests that the released proteins may be outer membrane material. Similar observations were made with an enterotoxigenic *E. coli* strain which released enterotoxin-rich outer membrane fragments (14). Results of minicell analysis of *Y. enterocolitica* plasmid-encoded proteins by Portnoy et al. (33) suggest that the released proteins of 24 and 35 kdal are probably plasmid-encoded and may be closely related to the 38-kdal virulence antigen, V. Experiments are underway to substantiate these suggestions with *E. coli* minicells and immunoblot techniques.

From immunoblot experiments we already know that the 24-, 35-, and 46-kdal proteins react with antibodies of sera from patients who have recovered from yersiniosis (manuscript in preparation).

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LITERATURE CITED

1. **Aulisio, C. C. G., W. E. Hill, J. T. Stanfield, and R. L. Sellers, Jr.** 1983. Evaluation of virulence factor testing and characteristics of pathogenicity in *Yersinia enterocolitica*. Infect. Immun. **40**:330–335.
2. **Ben-Gurion, R., and A. Shafferman.** 1981. Essential virulence determinants of different *Yersinia* species are carried on a common plasmid. Plasmid **5**:183–187.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bölin, J., L. Norlander, and H. Wolf-Watz.** 1982. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. Infect. Immun. **37**:506–512.
5. **Bölin, J., and H. Wolf-Watz.** 1984. Molecular cloning of the temperature-inducible outer membrane protein 1 of *Yersinia pseudotuberculosis*. Infect. Immun. **43**:72–78.
6. **Brubaker, R. R.** 1972. The genus *Yersinia*: biochemistry and genetics of virulence. Curr. Top. Microbiol. **57**:111–158.
7. **Brubaker, R. R.** 1983. The Vwa^+ virulence factor of *Yersiniae*: the molecular basis of the attendant nutritional requirement for Ca^{++}. Rev. Infect. Dis. **5**:748–758.
8. **Burrows, T. W.** 1963. Virulence of *Pasteurella pestis* and immunity to plague. Ergeb. Mikrobiol. Immunitätsforsch. **37**:59–113.
9. **Carter, P. B., R. J. Zahorchak, and R. R. Brubaker.** 1980. Plague virulence antigens from *Yersinia enterocolitica*. Infect. Immun. **28**:638–640.
10. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
11. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **77**:7347–7351.
12. **Doyle, M. P., M. B. Hugdahl, M. T. Chang, and J. T. Beery.** 1982. Serological relatedness of mouse-virulent *Yersinia enterocolitica*. Infect. Immun. **37**:1234–1240.
13. **Ferber, D. M., and R. R. Brubaker.** 1981. Plasmids in *Yersinia pestis*. Infect. Immun. **31**:839–841.
14. **Gankema, H., J. Wensink, P. A. M. Guinée, W. H. Jansen, and B. Witholt.** 1980. Some characteristics of the outer membrane material released by growing enterotoxigenic *Escherichia coli*. Infect. Immun. **29**:704–713.
15. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27**:682–685.
16. **Gemski, P., J. R. Lazere, T. Casey, and J. A. Wollhieter.** 1980. Presence of a virulence-associated plasmid in *Yersinia pseudotuberculosis*. Infect. Immun. **28**:1044–1047.
17. **Heesemann, J., C. Keller, R. Morawa, N. Schmidt, H. J. Siemens, and R. Laufs.** 1983. Plasmids of human strains of *Yersinia enterocolitica*: molecular relatedness and possible importance for pathogenesis. J. Infect.Dis. **147**:107–115.
18. **Heesemann, J., and R. Laufs.** 1983. Construction of a mobilizable *Yersinia enterocolitica* virulence plasmid. J. Bacteriol. **155**:761–767.
19. **Higuchi, K., and J. L. Smith.** 1961. Studies on the nutrition and physiology of *Pasteurella pestis*. VI. A differential plating medium for the estimation of the mutation rate to avirulence. J. Bacteriol. **81**:605–608.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lawton, W. D., R. L. Erdman, and M. J. Surgalla.** 1963. Biosynthesis and purification of V and W antigen in *Pasteurella pestis*. J. Immunol. **91**:179–184.
22. **Lee, W. H., P. P. McGrath, P. H. Carter, and E. L. Eide.** 1977. The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Can. J. Microbiol. **23**:9–12.
23. **Lee, W. H., R. E. Smith, J. M. Damaré, M. E. Harris, and R. W. Johnston.** 1981. Evaluation of virulence test procedure for *Yersinia enterocolitica* recovered from food. J. Appl. Bacteriol. **50**:529–539.
24. **Martinez, R. J.** 1983. Plasmid-mediated and temperature-regu-

lated surface properties of *Yersinia enterocolitica*. Infect. Immun. **41**:921–930.
25. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
26. **Mors, V., and C. H. Pai.** 1980. Pathogenic properties of *Yersinia enterocolitica*. Infect. Immun. **28**:292–294.
27. **Pai, C. H., and L. deStephano.** 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. Infect. Immun. **35**:605–611.
28. **Perry, R. D., and R. R. Brubaker.** 1983. Vwa$^+$ phenotype of *Yersinia enterocolitica*. Infect. Immun. **40**:166–171.
29. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**:346–356.
30. **Portnoy, D. A., H. F. Blank, D. T. Kingsbury, and S. Falkow.** 1983. Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. J. Infect. Dis. **148**:297–304.
31. **Portnoy, D. A., and S. Falkow.** 1981. Virulence-associated plasmids from *Yersinia enterocolitica* and *Yersinia pestis*. J. Bacteriol. **148**:877–883.
32. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
33. **Portnoy, D. A., H. Wolf-Watz, J. Bölin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.
34. **Sansonetti, P. J., T. L. Hale, G. J. Dammin, C. Kapfer, H. H. Collins, Jr., and S. B. Formal.** 1983. Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. Infect. Immun. **39**:1392–1402.
35. **Sereny, B.** 1955. Experimental *Shigella* keratoconjunctivitis: a preliminary report. Acta Microbiol. Acad. Sci. Hung. **2**:293–296.
36. **Straley, S. C., and R. R. Brubaker.** 1981. Cytoplasmic and membrane proteins of *Yersinia* cultivated under conditions simulating mammalian intracellular environment. Proc. Natl. Acad. Sci. U.S.A. **78**:1224–1228.
37. **Vesikari, T. T. Nurmi, M. Mäki, M. Skurnik, C. Sundqvist, K. Granfors, and G. P. Grönroos.** 1981. Plasmids in *Yersinia enterocolitica* serotypes O:3 and O:9: correlation with epithelial cell adherence in vitro. Infect. Immun. **33**:870–876.
38. **Vesikari, T., C. Sundqvist, and M. Mäki.** 1983. Adherence and toxicity of *Yersinia enterocolitica* O:3 and O:9 containing virulence-associated plasmids for various cultured cells. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **91**:121–127.
39. **Zink, D. L., J. C. Feeley, J. G. Wells, C. Vanderzant, J. C. Vickery, W. D. Roof, and A. O. O'Donovan.** 1980. Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*. Nature (London) **283**:224–226.