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Plasmids in *Yersinia enterocolitica* Serotypes O:3 and O:9: Correlation with Epithelial Cell Adherence In Vitro

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Human isolates of *Yersinia enterocolitica* serotypes O:3 (biotype 4) and O:9 (biotype 3) harbored plasmids sized approximately 47 and 44 megadaltons, respectively. No such plasmids were found in "apathogenic" strains of *Y. enterocolitica* belonging to biotype 1. There was a positive correlation among the presence of plasmid, autoagglutination, and adherence to and toxicity for HEp-2 cell cultures; all of these properties were lost by culturing at 37°C in the absence of calcium. Strains of *Y. enterocolitica* O:3 and O:9 cured of the plasmids showed increased invasiveness in the HEp-2 cell culture model, but no invasiveness in guinea pig eye. It is suggested that the plasmids of *Y. enterocolitica* primarily determine epithelial cell adherence, but may also be associated with other pathogenic properties.

Zink et al. recently demonstrated that strains of *Yersinia enterocolitica* O:8 which evoke a positive Serény test in guinea pig eye harbor a plasmid of about 41 megadaltons (Mdal) in size (27). Similar plasmids have also been described in virulent strains of *Y. pseudotuberculosis* (8) and *Y. pestis* (6). Therefore these plasmids appear to be common virulence determinants among yersiniae, but their exact role in the pathogenic process still remains largely open. Gemski et al. showed that plasmid (42.2 Mdal)-positive strains of *Y. enterocolitica* O:8 were pathogenic for mice and calcium dependent for growth at 37°C (7). The latter is a well-established characteristic of virulent strains of *Y. pestis* and *Y. pseudotuberculosis*, associated with the production of V and W virulence antigens (3, 9, 13). More recently, the V and W antigens were also demonstrated in *Y. enterocolitica* O:8 (4), leading to the assumption that plasmid-controlled properties have a role in the intracellular survival of *Y. enterocolitica*. Plasmid association has also been suggested for the intracellular invasiveness (27), which is a frequently reported property of human pathogenic *Y. enterocolitica* (11, 14, 17, 18, 20, 22, 25). Another association could be control of "heat-stable-like" (ST-like) enterotoxin production (10, 18, 21). Finally, Portnoy et al. demonstrated that plasmid positivity in *Y. enterocolitica* is associated with adherence to and detachment of human epithelial cells in tissue culture (23).

We studied *Y. enterocolitica* serotypes O:3 and O:9, which are most commonly detected in human yersiniosis in Finland (1), and found that clinical isolates of these serotypes often contain plasmid deoxyribonucleic acid (DNA) that is similar, but not identical, to the plasmids of *Y. enterocolitica* O:8 or *Y. pseudotuberculosis*. In the search for possible correlations with human pathogenicity, we studied plasmid-positive and -negative strains of *Y. enterocolitica* for invasiveness, toxigenicity, and autoagglutination (12). The last mentioned is another pathogenic determinant associated with calcium-dependent growth at 37°C (12) and appeared to be related to the epithelial cell adherence and toxicity observed during this work.

We now further report some studies on the correlation among the plasmids, the requirement of calcium for growth at 37°C, autoagglutination, and adherence to and invasiveness in HEp-2 cells of *Y. enterocolitica* O:3 and O:9.

MATERIALS AND METHODS

Strains of *Y. enterocolitica*. All strains of *Y. enterocolitica* O:3 and O:9 were human fecal isolates from patients with various clinical symptoms, including diarrhea, mesenterial lymphadenitis, and arthritis. The other strains (biotype 1) were also human stool isolates, found in most cases on routine examinations of symptomless subjects. The isolates were collected from different laboratories: the Department of Medical Microbiology of the University of Turku; the Regional Public Health Laboratory, Turku; the Department of Medical Microbiology, University of Oulu; and the Department of Microbiology, Tampere Cen-

tral Hospital, Tampere, Finland. In the laboratories of Turku and Oulu, the primary isolation was carried out on lactose-MacConkey agar plates at room temperature (RT) or by enrichment at 4°C. All subcultures thereafter were done at RT. In the Tampere laboratory the primary isolation was also carried out at RT, either using Wauters buffer as an enrichment medium or using direct plating on salmonella-shigella-deoxycholate agar. The subsequent biochemical identification and pure cultures for storage were done at 37°C, mainly on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) plates. As a result of the different laboratory procedures, we had a heterogeneous collection of human isolates of *Y. enterocolitica* available for the present study: 45 strains grown only at RT or below and 21 strains subcultured at 37°C.

Two strains of *Y. enterocolitica* O:8 used as reference were kindly provided by Chik H. Pai, McGill University and Montreal Children's Hospital Research Institute, Montreal, Quebec, Canada. Strain MCH314 was identified as plasmid positive (42 Mdal), and strain MCH395 as plasmid negative. Another reference strain (WA) of *Y. enterocolitica* O:8 was a generous gift from P. Gemski, Walter Reed Army Institute of Research, Washington D.C. This strain contained a previously identified plasmid, sized 42.2 Mdal (7).

Serotyping of *Y. enterocolitica* isolates was done for types O:3 and O:9 in the laboratories in Turku, Oulu, and Tampere with rabbit antisera. The other serotypes were kindly determined by P. Ahvonen, Municipal Bacteriology Laboratory, Aurora Hospital, Helsinki, Finland.

Biotyping of *Y. enterocolitica* was carried out as summarized by Winblad (26).

Tests for invasiveness. The in vitro procedure with HEp-2 cell cultures has been described previously (17). The incubation time in the test was 7 h.

In vivo invasiveness was studied by the inoculation of bacterial suspension onto guinea pig cornea (24). The guinea pigs were observed for 5 to 7 days for the development of keratoconjunctivitis. Only obvious inflammation was taken as a positive reaction; mild transient irritation was occasionally seen but was not recorded as a positive result.

Tests for toxin production. Bacteria-free culture supernatants were produced at RT (21). The filtrates were assayed for the presence of ST-like enterotoxin in infant mice (5).

Autoagglutination. The procedure was done essentially as described by Laird and Cavanaugh (12). We used medium 199 (Orion Diagnostica, Espoo, Finland) or RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% newborn calf serum; both media were equally satisfactory for the purpose.

Isolation of plasmid DNA. DNA was isolated from *Y. enterocolitica* strains by the crude lysate method of Meyers et al. with minor modifications (19). Since yersiniae were found to be relatively resistant to lysis with sodium dodecyl sulfate, the latter was used at a concentration of 2%, and a heat-shock treatment was added to the procedure. A bacterial preparation in 2% sodium dodecyl sulfate was transferred to a water bath at 55°C for 5 min. The agarose gel electrophoresis was run according to Meyers et al. (19). After electrophoresis the gels were stained with ethidium bromide.

The size determination of plasmid DNA in *Y. enterocolitica* was based on velocity in agar gel electrophoresis. The principal references were *Escherichia coli* strain V517, containing a 35.7-Mdal plasmid (16), and strain 1100, containing a 56.3-Mdal plasmid (obtained from Kurt Nordström, Department of Molecular Biology, Odense University, Odense, Denmark, through Ilkka Palva, Central Public Health Laboratory, Helsinki, Finland). The above-mentioned plasmid of *Y. enterocolitica* O:8 strain WA, sized 42.2 Mdal, was taken as an additional reference.

RESULTS

Plasmid DNA in *Y. enterocolitica*. About one half of the strains of *Y. enterocolitica* serotype O:3 and most strains of serotype O:9 contained plasmid DNA sized approximately 47 and 44 Mdal, respectively (Table 1). The majority of those strains lacking the plasmid came from the laboratory where the isolates had been subcultured several times at 37°C. In each plasmid-positive strain of *Y. enterocolitica* O:3 and O:9, the size of plasmid DNA appeared identical. Figure 1 shows the agarose gel electrophoresis of plasmid DNA from these serotypes of *Y. enterocolitica*.

Strains of *Y. enterocolitica* belonging to biotype 1 and including several serotypes (5, 6, 10, and 7/13) were found not to contain plasmid DNA of similar size as serotypes O:3, O:9, or O:8. Two strains apparently had some plasmid DNA, sized in one case less than 35 Mdal and in another over 70 Mdal.

Correlation of plasmids in *Y. enterocolitica* to some virulence properties. In summary from the above, human pathogenic strains of *Y. enterocolitica* serotypes O:3, O:9, and O:8 often contained plasmids within the size range 42 to 47 Mdal characteristic of each serotype (and biotype), whereas human apathogenic serotypes (biotype 1) did not. We next studied some possible virulence-associated properties of these strains of *Y. enterocolitica* in search of correlation with the presence of plasmids. These tests included invasiveness in guinea pig eye and

TABLE 1. *Presence of plasmid DNA in various clinical isolates of Y. enterocolitica in relation to biotype and serotype*

Biotype	Serotype(s)	Plasmid DNA present in:	Approx size of plasmid (Mdal)
1	O:5, -6, -7/13, -10	2/12	<35, >70
2	O:8	2/3	42
3	O:9	17/19	44
4	O:3	16/34	47

HEp-2 cells, ST-like enterotoxin, and autoagglutination (Table 2).

None of the strains of *Y. enterocolitica* O:3 and O:9 produced keratoconjunctivitis in the guinea pig eye (occasionally transient irritation was produced), and therefore the test was unsuitable for studies of the pathogenic significance of the plasmids in these bacteria. The plasmid-positive strains of *Y. enterocolitica* O:8 caused conjunctivitis in the guinea pig eye, as described previously (7, 20).

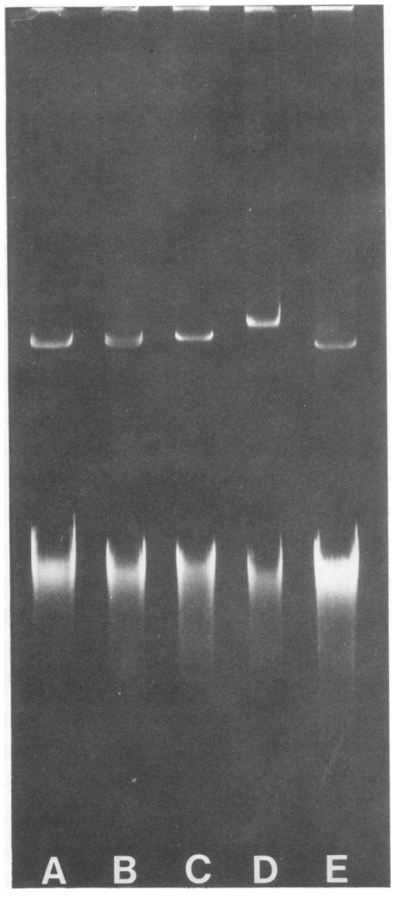

FIG. 1. *Agarose gel electrophoresis of plasmid DNA from various strains of Y. enterocolitica. (A) 44-Mdal plasmid from Y. enterocolitica O:9, (B) 44-Mdal plasmid from Y. enterocolitica O:9 and 47-Mdal plasmid from Y. enterocolitica O:3 run together, (C) 47-Mdal plasmid from Y. enterocolitica O:3, (D) reference E. coli plasmids of 56.3 Mdal and 35.7 Mdal (the latter only weakly visible) run together, and (E) 42-Mdal plasmid from Y. enterocolitica O:8 strain MCH314.*

All of the strains of *Y. enterocolitica* O:3 and O:9, whether plasmid positive or negative, were invasive for HEp-2 cells in vitro. All three strains of *Y. enterocolitica* O:8 were also found invasive in HEp-2 cells, although only two contained plasmid DNA. The invasive properties of *Y. enterocolitica* O:3, O:9, and O:8, biotypes 4, 3, and 2, respectively, were in contrast to biotype 1 strains, of serotypes 5, 6, 7/13, and 10, which were uniformly noninvasive in HEp-2 cells.

In the infant mouse assay, ST-like enterotoxin activity was found in 6 of 34 strains of *Y. enterocolitica* serotype O:3; there was no apparent correlation with the presence or absence of plasmid (Table 2). Among the strains of *Y. enterocolitica* O:9, the infant mouse assay for enterotoxin was positive in 3 out of 18 cases. All three positive strains contained the plasmid. This may, however, be a chance association since there were only two plasmid-negative strains available for testing (Table 2).

Most of the plasmid-containing strains of *Y. enterocolitica* serotype O:3 and all those of serotype O:9 autoagglutinated when grown in tissue culture medium at 37°C, whereas none of the plasmid-negative strains of these serotypes autoagglutinated in those culture conditions (Table 2). In the three instances with negative autoagglutination of a plasmid-positive strain of *Y. enterocolitica* O:3, the amount of plasmid DNA demonstrable by agarose gel electrophoresis was unusually small, suggesting the presence of a heterogeneous (plasmid-positive and -negative) bacterial population.

Adherence onto and toxic effect for HEp-2 cells. During the tests for possible virulence-associated properties in our collection of clinical isolates of *Y. enterocolitica*, we discovered that the interpretation of HEp-2 cell invasiveness was often difficult because of the toxic appearance of the cells. It was observed that fresh isolates of *Y. enterocolitica* O:3 and O:9 grown at RT exerted such a toxic effect on the monolayers (Fig. 2). Large areas of the cells were rounded, and bacteria forming long chains were

TABLE 2. *Correlation between the presence of 44 to 47-Mdal plasmids and three properties in various human isolates of Y. enterocolitica O:3 and O:9*

Serotype of *Y. enterocolitica*	HEp-2 cell invasiveness[a]		ST-like enterotoxin[a]		Autoagglutination[a]	
	Plasmid +	Plasmid −	Plasmid +	Plasmid −	Plasmid +	Plasmid −
O:3	16/16	16/16	2/16	4/18	12/15	0/16
O:9	17/17	2/2	3/16	0/2	17/17	0/2

[a] Data indicate property/number tested.

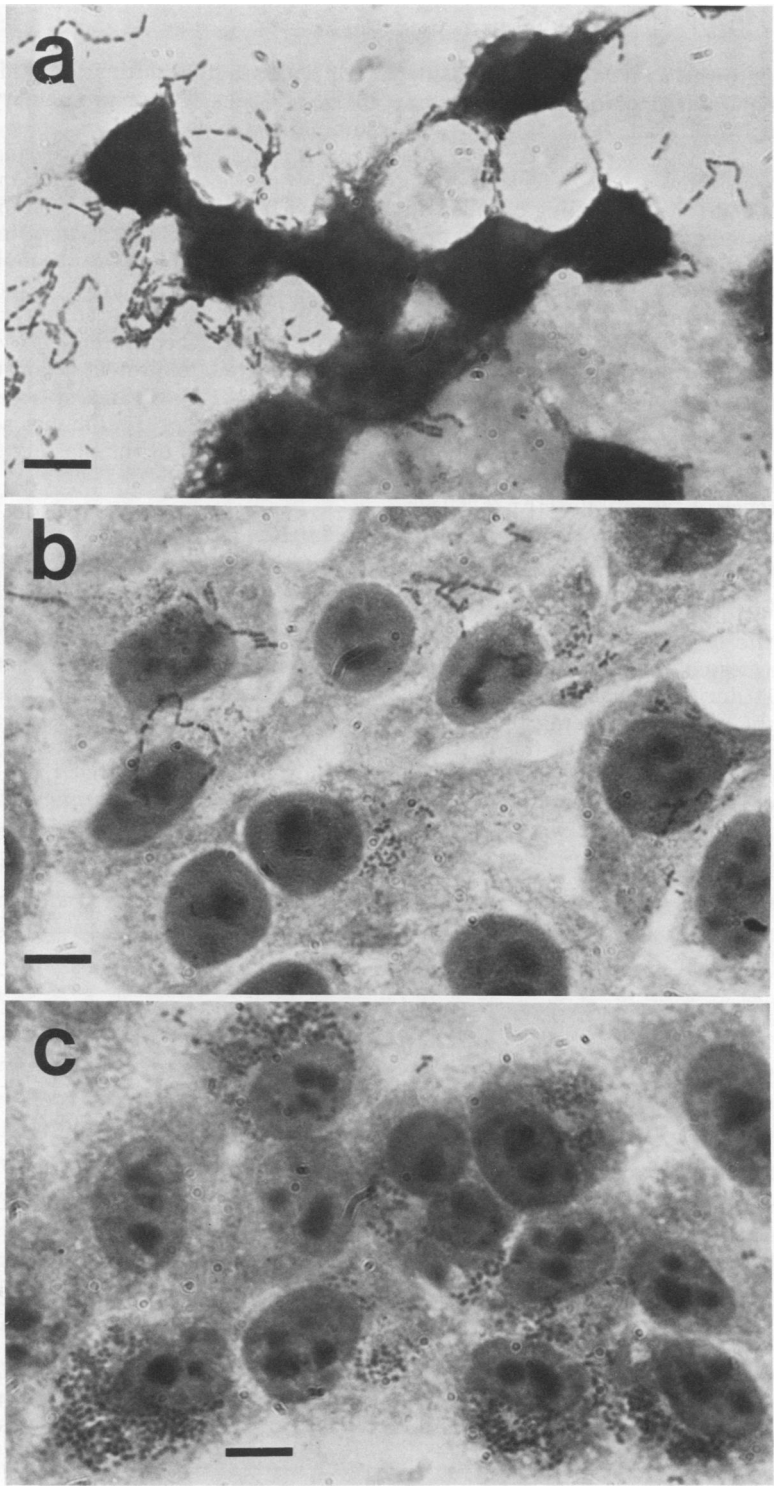

FIG. 2. *Appearance of HEp-2 cell culture after exposure to Y. enterocolitica O:9 for 7 h. (a) Fresh isolate grown at RT. The bacteria formed chains and adhered to the cells, which appear rounded and partly detached from the surface. (b) Bacteria grown at 37°C in regular TSA. Intracellularly located bacteria can be seen, with some chain forms on the cells. (c) Bacteria grown at 37°C in the absence of Ca^{2+} but in the presence of Mg^{2+}. Most cells were invaded by Y. enterocolitica, and there was no toxicity or bacterial chain formation. Bar, 10 μm.*

found adhering to the monolayer. At the same time, occasional intact-looking cells could be found invaded by bacteria.

Old isolates of *Y. enterocolitica* O:3 and O:9 seemed to behave differently in the invasiveness test: there was little or no rounding of cells and no chain formation of bacteria, but a large proportion of cells were invaded by bacteria. The toxic effect and adherence on HEp-2 cells correlated well with the autoagglutination properties of *Y. enterocolitica* O:3 and O:9. Those strains that showed no adherence but were strongly invasive did not autoagglutinate at 37°C, whereas many of the autoagglutination-positive strains were toxic for HEp-2 cells. None of the biotype 1 strains of *Y. enterocolitica* showed adherence or toxicity for HEp-2 cells or autoagglutination at 37°C.

Dependency of plasmid DNA and associated properties on temperature and Ca^{2+}. The absence of plasmid DNA in many of the strains of *Y. enterocolitica* O:3 and O:9 from one laboratory suggested that the plasmid might have been lost during subculture at 37°C. We next studied the possible temperature and Ca^{2+} dependency of the expression of the plasmids and those properties that appeared to be of some pathogenic significance, namely, toxicity for HEp-2 cells, autoagglutination, and invasiveness in HEp-2 cells. These preliminary results are summarized in Table 3.

At RT the plasmids and the plasmid-associated properties, autoagglutination and HEp-2 cell toxicity, were retained even in the absence of calcium. At 37°C in Ca^{2+}-deficient but Mg^{2+}-supplemented medium, the plasmids were cured, and the autoagglutination, as well as the epithelial cell toxicity, disappeared, but invasiveness in the HEp-2 cells became much greater. Excess Ca^{2+} appeared to protect against the loss of plasmids at 37°C. Strains of *Y. enterocolitica* O:3 used in these experiments seemed to be more easily cured of the plasmid than those of serotype O:9.

DISCUSSION

Y. enterocolitica may be divided into two groups regarding the 42- to 47-Mdal plasmids: human apathogenic biotype 1 bacteria, which do not contain such plasmids and human pathogenic biotypes 2, 3, and 4, which may harbor the plasmid. It remains to be established whether these plasmids are characteristic and unique for each serotype or biotype. The recent results of Portnoy et al. suggest that there is among *Y. enterocolitica* a group of related plasmids with

TABLE 3. *Effect of growth temperature and Ca^{2+} depletion on expression of some possible virulence-associated properties in Y. enterocolitica O:3 and O:9*

Property	Serotype	Strain	Expression of property at:				
			RT × 3[a]	RT × 3 (Mg^{2})[b]	37°C × 3	37°C × 3 (Mg^{2+})[b]	37°C × 3 (Ca^{2+})[c]
Presence of plasmid	O:3	6779	+	+	(+)[d]	−	+
		7228	+	+	(+)	−	+
	O:9	05	+	+	+	−	+
		036	+	+	+	−	+
Autoagglutination	0:3	6779	+	+	−	−	+
		7228	+	+	±	−	+
	O:9	05	+	+	+	−	+
		036	+	+	+	−	+
Toxicity for HEp-2 cells	O:3	6779	+	+	−	−	+
		7228	+	+	−	−	+
	O:9	05	+	+	+	−	+
		036	+	+	+	−	+
Invasiveness in HEp-2 cells	O:3	6779	(+)[e]	+	++[f]	+++[f]	+
		7228	(+)	+	++	+++	+
	O:9	05	(+)	(+)	++	+++	++
		036	(+)	+	++	+++	++

[a] TSA containing 0.9 mM Ca^{2+}.
[b] TSA containing 20 mM $MgCl_2$ and 20 mM sodium oxalate.
[c] TSA containing 5 mM $CaCl_2$.
[d] Trace amount of plasmid DNA demonstrable in agarose gel electrophoresis. The plasmid disappeared after three further passages.
[e] Invasiveness difficult to interpret because of the toxic effect for cells.
[f] ++, More than 5% of the cells contained three or more ingested bacteria; +++, more than 30% contained three or more ingested bacteria.

many homologous DNA sequences (23).

The virulence determinants that are plasmid mediated are not known, and therefore the role of the plasmids in the pathogenic process of *Y. enterocolitica* infection remains a matter of speculation. Our results, supported by those of Portnoy et al. (23), suggest that the presence of the plasmid is not necessary in the internalization of bacteria in epithelial cells, since strains cured of the plasmids by growth at 37°C in the absence of calcium were clearly more invasive in HEp-2 cells than the parent strains harboring the plasmid. However, plasmid-coded products may be involved in the process, since the plasmid-negative biotype 1 bacteria never showed any invasiveness in the HEp-2 cells.

The adherence and toxicity for epithelial cells add another possible link to the pathogenic sequence of *Y. enterocolitica* infection. Conceivably, the primary step in intestinal *Y. enterocolitica* infection could be the adherence of plasmid-bearing bacteria onto the gut epithelium. It is uncertain whether the toxic effect has its equivalent in vivo, but there is a possibility that diarrhea could be produced by this mechanism. A soluble toxin is not necessarily involved (23). In our hands the production of ST-like enterotoxin showed no correlation with the presence of plasmids.

The adherence of *Y. enterocolitica* to epithelial cells may be related to autoagglutination (12) or hemagglutination (15) of these bacteria. It has been shown that *Y. enterocolitica* produce fimbriae or pili (2), and these are likely to be responsible for the mannose-resistant hemagglutination shown by many strains of *Y. enterocolitica* (15). Furthermore, the production of the broad-spectrum hemagglutinin appears temperature dependent and is lost by culturing at 37°C (15). This is analogous to the observed loss of autoagglutination and HEp-2 cell adherence at 37°C in the present study.

After adherence, colonization and multiplication of *Y. enterocolitica* in the intestines follow. As this takes place at body temperature, the circumstances may favor the development of invasive bacteria, depending however on the concentrations of bivalent cations. After intracellular invasion, the course of *Y. enterocolitica* infection is apparently determined by the fate of the bacteria in the epithelial cells and in leukocytes. The intracellular survival of *Y. pestis* and *Y. pseudotuberculosis* correlates with the production of the V and W virulence antigens, the synthesis of which is optimal at 37°C in the absence of Ca^{2+} but in the presence of Mg^{2+}, i.e., in conditions mimicking intracellular environment (3). These antigens are also produced by *Y. enterocolitica* O:8, and they are associated with mouse virulence (7). *Y. enterocolitica* serotypes O:3 and O:9 were found to be less pathogenic than O:8 by the Serény test. Whether this is due to differences in the production of virulence antigens and hence intracellular survival remains to be established.

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