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LACK OF CORRELATION BETWEEN PLASMID-ASSOCIATED PHENOTYPES OF *YERSINIA ENTEROCOLITICA* AND PATHOGENICITY IN THE MOUSE¹

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ABSTRACT

Various phenotypic characteristics have been correlated with the pathogenicity of plasmid-bearing virulent strains of Yersinia enterocolitica. Two transposon Tn801-insertion derivatives (JD193 and JD217) of the virulence plasmid from serotype O:3 were used to determine the correlation between pathogenicity and plasmid-associated properties of this organism. Both Tn801-inserted derivatives expressed five plasmid-associated phenotypic characteristics at 37C: (1) colony morphology, (2) calcium-dependent growth or low-calcium response, (3) crystal violet binding, (4) autoagglutination, and (5) hydrophobicity. However, for mouse pathogenicity only JD193 was positive whereas, JD217 was avirulent for mice. Thus, it is possible to have a lack of correlation between plasmid-mediated traits and the actual pathogenicity of the organism in the mouse; however, these plasmid-mediated phenotypic characteristics provide simple and efficient techniques to evaluate the virulence potential of wild-type strains isolated from food poisoning outbreaks and clinical cases.

INTRODUCTION

Yersinia enterocolitica is recognized as an important cause of bacterial gastroenteritis in humans (Doyle and Cliver 1990; Kapperud 1991; Portroy and Martinez 1985). Strains of all serotypes implicated in human disease harbor a 40 to 45 MDa plasmid (Weagant *et al.* 1992). Evidence for the direct involvement of this resident plasmid in the virulence of this bacterium was described (Doyle and Cliver 1990; Kapperud 1991; Kwaga and Iversen 1991). Plasmid-associated

¹ Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

phenotypic characteristics expressed at 37C include colony morphology (appearance as small colony of 1.13 mm in diameter due to slower growth rate), calcium-dependency (also known as low-calcium response: Lcr; appearance of pinpoint colony diameter of 0.36 mm in low calcium medium), crystal violet (CV) binding, autoagglutination (AA), hydrophobicity (HP), serum resistance, and detachment of cells in culture. These characteristics have been used to distinguish between plasmid-bearing virulent and plasmidless avirulent strains and also have been correlated with the pathogenicity of the organism *viz* the mouse virulence test (Bhaduri 1990a; Bhaduri *et al.* 1987; Doyle and Cliver 1990; Kapperud 1991; Kwaga and Iversen 1991; Lazere and Gemski 1983; Mazigh *et al.* 1983; Portnoy and Martinez 1985; Robins-Browne *et al.* 1989); though the mechanisms involved in virulence remain unclear. Studies by several investigators (Bissett *et al.* 1990; Kwaga and Iversen 1991; Mazigh *et al.* 1985; Morris *et al.* 1991; Noble *et al.* 1987; Prpic *et al.* 1985) suggest that the presence of the virulence plasmid and its expression in *Y. enterocolitica* may not unequivocally indicate pathogenicity. Hence, it is important to further evaluate these correlations for the elucidation of the pathogenicity of *Y. enterocolitica*. The present study was initiated to examine the correlation of the plasmid-associated properties with actual pathogenicity by the mouse virulence assay (diarrhea in mice) by using two transposon *Tn801*-insertion derivatives of the virulence plasmid from serotype O:3.

MATERIALS AND METHODS

Bacteria and Growth Conditions

Plasmid-bearing virulent strain GER (serotype O:3) and two ampicillin-resistant *Tn801*-inserted transconjugants of the virulence plasmid from GER (serotype O:3) *Y. enterocolitica* were used in this study as a primary model (Dubel 1983). They were provided by R.V. Lachica (U.S. Army Natick Research, Development and Engineering Center, Natick, MA). The relevant characteristics of the transposon mutants of the original wild type virulent strain GER (Serotype O:3) and their plasmids are presented in Table 1. The transconjugants were constructed as follows: plasmid DNA from the wild-type virulent strain was subjected to transposon-mediated mutagenesis *via* conjugation of the strain with the *Escherichia coli* strain harboring the plasmid pMR5 that carries *Tn801*. The transconjugants JD193 and JD217 constructed by this technique (Dubel 1983) were used in this study. The *Tn801* was inserted onto two different Hind III sites of the virulence plasmid to give rise to transconjugants JD193 and JD217 respectively as determined by Dubel (1983) via southern blot with nick translated pMR5 DNA of the restriction gel. The hybridization pattern confirmed that these two transconjugants were independent strains since they produced two

different fragments containing Tn801 from JD193 and JD217, respectively. The chromosomally isogenic avirulent plasmidless derivatives (P⁻) were obtained from large, flat colonies which emerged spontaneously from corresponding plasmid-bearing virulent strains (P⁺) cultures growing at 37C on calcium-deficient brain heart infusion agarose as described by Bhaduri *et al.* (1990). These cells did not display any of the appropriate plasmid-associated phenotypic properties and showed no presence of plasmid by DNA analysis and hybridization (Bhaduri *et al.* 1990; Dubel 1983).

TABLE 1.
CHARACTERISTICS OF WILD TYPE AND TN801 INSERTED DERIVATIVES OF
Y. ENTEROCOLITICA (STRAINS AND PLASMIDS)

Strain	Serotype	Genotype/Description
GER (P ⁺)	O:3	Clinical isolate containing 48 MDa virulence plasmid
GER-C (P ⁻)	O:3	Isogenic derivative of strain GER that had been cured of the 48 MDa virulence plasmid
JD193 (P ⁺)	O:3	Transpositional (Tn801: 3.2 MDa; Ampicillin resistance) mutant of serotype O:3 (strain GER) at the Hind III site (fragment C) of virulence plasmid (51 MDa)
JD193-C (P ⁻)	O:3	Isogenic derivative of strain JD193 that had been cured of the 51 MDa virulence plasmid
JD217 (P ⁺)	O:3	Transpositional (Tn801: 3.2 MDa; Ampicillin resistance) mutant of serotype O:3 (strain GER) at the Hind III site (fragment A) of virulence plasmid (51 MDa)
JD217-C (P ⁻)	O:3	Isogenic derivative of strain JD193 that had been cured of the 51 MDa virulence plasmid

Preparation of Media

Brain heart infusion (BHI) agarose (BHO) was prepared by adding agarose type V (Sigma Chemical Co., St. Louis, MO) as the gelling agent to a final concentration 1.2% to BHI broth (BHI: Difco Laboratories, Detroit, MI) supplemented with 0.1% magnesium chloride (Bhaduri *et al.* 1990). Brain heart infusion agar (BHA: Difco) was prepared as recommended by the supplier. Since BHI is the common ingredient in these media, the amounts of calcium present in agar and agarose determine the concentrations of calcium in BHA and BHO, respectively. The concentrations of calcium in BHI broth, BHA and BHO were measured by atomic absorption analysis (Bhaduri *et al.* 1990). The calcium content was comparatively high in BHA (1,500 μ M), but low in BHI broth (245 μ M) and BHO (238 μ M). Thus, the addition of agar and agarose as gelling agent to BHI broth allowed to attain high and low level of calcium in these media. Since

the concentration of calcium is high in BHA and low in BHO they were designated and used as calcium-adequate and calcium-deficient (low-calcium) media respectively (Bhaduri *et al.* 1990).

Assays of Plasmid-Associated Phenotypic Characteristics

The colony morphology and Lcr of bacteria was tested by growth on calcium-adequate BHA and calcium-deficient BHO, respectively (Bhaduri *et al.* 1990). The CV binding assay was done on calcium-adequate BHA. Detailed descriptions of these assay conditions are given elsewhere (Bhaduri *et al.* 1987; Bhaduri *et al.* 1990). AA was determined as previously described (Bhaduri *et al.* 1987) with Eagle's minimal essential medium supplemented with 10% fetal bovine serum. HP was examined by latex particle agglutination (LPA) test (Bhaduri *et al.* 1987). The ability of strains of *Y. enterocolitica* to express these plasmid-associated phenotypic characteristics were tested as follows: P⁺ and P⁻ strains were grown separately in BHI broth with shaking for 18 h at 25C to a population density of approximately 1×10^9 cfu/mL. The cells were diluted to 1×10^3 cells/mL using BHI broth and surface-plated onto dye-binding plates. Plates were incubated at 37C for 24 h. Plates were then counted and the presence of the virulence plasmid in the cells was detected by colonial morphology, CV binding, Lcr, AA, and HP. The presence of plasmid DNA in *Y. enterocolitica* and its derivatives was determined by plasmid DNA isolation and agarose gel electrophoresis as described previously (Bhaduri 1990b; Bhaduri *et al.* 1980). The experimental procedures were replicated three times.

Mouse Virulence Assay

The virulence of *Y. enterocolitica* was evaluated by the development of diarrhea in mice as described by Bhaduri *et al.* (1987). Swiss Webster albino male mice (15 to 20 g each) were used. The mice first were pretreated for two days successively with iron salts as described below. The mice were injected intraperitoneally (i.p.) with 5 mg of iron-dextran in 0.5 mL volume (Imferon: Merrell Dow Research Institute, Cincinnati, Ohio). In mice iron-dextran reduced the median lethal dose of administered *Y. enterocolitica*. On the following day mice were given 0.5 mL of normal saline solution by i.p. containing 5 mg/mL of Desferal (iron chelator desferroxamine B mesylate: CIBA-GEIGY Corp., Suffern, New York). Desferal markedly increased the susceptibility of animals to yersinosis. Thus, the administration of both drugs increased the level of available iron in mice to promote the growth of *Y. enterocolitica*. The control mice were injected i.p. with 0.5 mL of normal saline. Bacterial strains were inoculated into BHI broth and incubated at 25C for 24 h. The resultant cultures were diluted with 50 mL of sterile water to a final concentration of 1×10^9 cell/mL. Groups of six mice in one individual cage were orally infected with the

bacterial suspension from both P⁺ and P⁻ cells. This was accomplished by depriving the mice of water for 24 h following the second day after iron injection and then allowing each group to drink from the bacterial suspension *ad libitum* for 24 h. The mice were then given food and water *ad libitum* and each infected mouse from each group were examined daily for diarrhea for 8 d by placing the infected mice twice daily in cages lined with clean, white paper. In the control experiment groups of six mice were also deprived of drinking water for 24 h and then allowed to drink water without the addition of bacterial suspension. These mice were also examined daily for diarrhea as described above.

The protocol for mouse virulence assay was approved by the Institutional Animal Care & Use Committee. The mice were killed by euthanasia with CO₂ at the conclusion of the experiment.

RESULTS AND DISCUSSION

Several studies have shown an unclear correlation between established virulence markers and diarrheal illness in *Y. enterocolitica* (Bissett *et al.* 1990; Kwaga and Iversen 1991; Mazigh *et al.* 1985; Morris *et al.* 1991; Noble *et al.* 1987; Prpic *et al.* 1985). Here the relationship between the presence of virulence plasmid in *Y. enterocolitica*, its phenotypic properties and pathogenicity was investigated using *Tn801*-inserted derivatives of the virulence plasmid.

Initially, the plasmid DNA of transconjugants was analyzed by agarose gel electrophoresis to confirm the insertion of the transposon. The results of this analysis are illustrated in Fig. 1. The wild type virulent strain GER (Serotype O:3) was found to contain a 48 MDa plasmid (Fig. 1, lane C). The *Tn801* derivatives contained a plasmid with a slightly increased molecular weight of 51 MDa (Fig. 1 lanes B and D); this was the anticipated size of a plasmid of the derivative since the 48 MDa virulence plasmid contained 3.2 MDa *Tn801* as reported by southern blot hybridization of the restricted *Tn801*-inserted derivatives of the virulence plasmid (Dubel 1983). This data verified that these two transconjugants are indeed the product of transpositional insertion.

The wild and two transconjugant strains were examined for the expression of mouse virulence and plasmid-associated phenotypic characteristics (Table 2). The isogenic avirulent P⁻ derivatives of these strains were used as negative controls in this study. On calcium adequate BHA the colony size at 37C (1.13 mm) and morphology of JD193 and JD217 were similar to that of GER as reported previously (Bhaduri *et al.* 1990). Both JD193 and JD217 also displayed other plasmid-associated phenotypes at 37C including Lcr, CV binding, AA and HP as observed with GER (Bhaduri *et al.* 1987; Bhaduri *et al.* 1990) (Table 2). Mouse virulence assays were positive for GER and JD193 but not for JD217 as shown by oral infection which led to diarrhea from 5 to 7 days of post infection

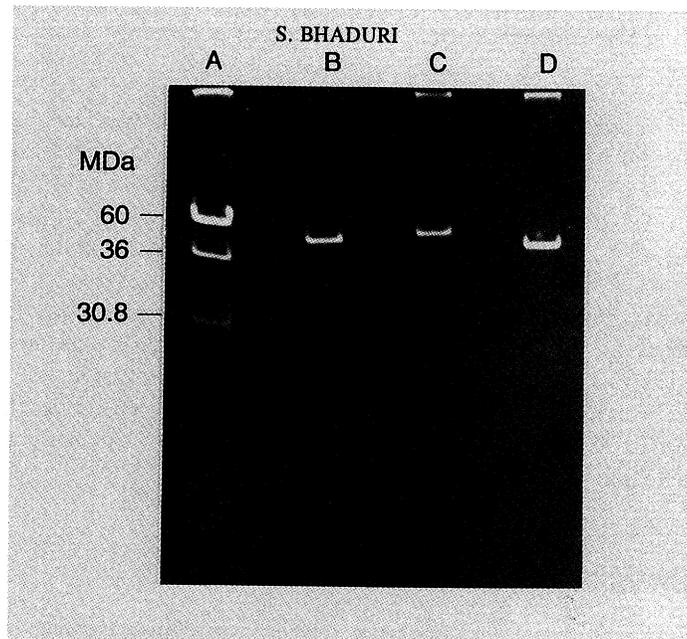


FIG 1. AGAROSE GEL ELECTROPHORESIS OF PLASMID DNA

Electrophoresis was carried out at 80 V for 5 h in 0.7% agarose gel. (A) Molecular mass standards (plasmid from *Salmonella typhimurium* LT2, 60 MDa; plasmid from *Escherichia coli* J53/RP4, 36 MDa; Lambda DNA 30.8 MDa); (B) JD193 plasmid DNA from Tn801 transposon derivative of *Y. enterocolitica* (GER O:3); (C) wild-type virulent strain of *Y. enterocolitica* GER O:3; (D) JD217 plasmid DNA from Tn801 transposon derivative of *Y. enterocolitica* (GER O:3).

in iron-overloaded mice (Table 2). In contrast, none of the isogenic avirulent P-derivatives of these strains contained plasmid as shown by plasmid DNA analysis and did not show plasmid-associated phenotypic characteristics and mouse virulence (Table 2). This indicates transconjugant JD193 expressed both *in vitro* phenotypes and mouse pathogenicity, whereas transconjugant JD217 remained avirulent for mice but expressed the phenotypic properties. The insertion of Tn801 in JD217 might alter the genetic sequence in such way that did not allow the expression of the actual mouse virulence. Any changes in the genetic sequence of a virulence plasmid may occur naturally to prevent the expression of mouse virulence. This demonstrates that it is possible to have a lack of correlation between the presence of *in vitro* virulence traits and biological virulence in the mouse. It is probable that mouse virulence is determined by several complex factors.

In conclusion, a relationship between pathogenicity, the presence of plasmid and phenotypic properties of *Y. enterocolitica* was established by using Tn801-inserted derivatives. It has been found that the presence of the plasmid and its phenotypic expression in *Y. enterocolitica* may have no function in pathogenicity

and may not unequivocally indicate pathogenicity. Further investigations are required to determine the extent to which these plasmid-associated characteristics could be correlated to mouse virulence using other plasmid-bearing virulent strains of *Y. enterocolitica*. However, these tests provide good prediction of the virulence potential in wild type strains isolated from food poisoning outbreaks and clinical cases as reported with other virulent serotypes. There might be other observable plasmid-encoded traits that are more relevant to virulence and it is these that should be sought.

TABLE 2.
EVALUATION OF PLASMID-ASSOCIATED CHARACTERISTICS AND
MOUSE VIRULENCE IN *TN801*-INSERTED DERIVATIVES OF
Y. ENTEROCOLITICA

Strain	Colony Morphology ^a	Lcr ^b	CV binding ^c	AA ^d	HP ^e	Plasmid	Diarrhea in mice ^f
GER	Small	+	+	+	+	+	+
GER-C	Large	-	-	-	-	-	-
JD193	Small	+	+	+	+	+	+
JD193-C	Large	-	-	-	-	-	-
JD217	Small	+	+	+	+	+	-
JD217-C	Large	-	-	-	-	-	-

^a In calcium-adequate agar medium (BHA) P⁺ cells appeared as small colony (diameter, 1.13 mm) compared to the large colony of P⁻ cells (diameter, 2.4 mm) (Bhaduri *et al.* 1990).

^b Lcr: Low Calcium Response (Calcium dependent growth at 37C; P⁺ cells appeared as pin point colony of diameter of 0.36 mm compared to the large colony of P⁻ cells of diameter, 1.37 mm on low-calcium medium: BHO) (Bhaduri *et al.* 1990)

^c CV binding: Crystal Violet Binding

^d AA: Autoagglutination

^e HP: Hydrophobicity

^f Fecal material consistency was liquid; diarrhea was observed on days 5, 6, and 7 postinfection.

REFERENCES

- BHADURI, S. 1990a. Evaluation of different techniques for detection of virulence in *Yersinia enterocolitica*. *J. Clin. Microbiol.* 28, 828-829.
- BHADURI, S. 1990b. Calcium-responsive expression of plasmid-mediated outer membrane proteins from *Yersinia enterocolitica* grown on solid media. *J. Ind. Microbiol.* 5, 207-214.
- BHADURI, S., CONWAY, L.K. and LACHICA, R.V. 1987. Assay of crystal violet binding for rapid identification of virulent plasmid-bearing clones of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 25, 1039-1042.

- BHADURI, S., KASAI, T., SCHLESSINGER, D. and RASKAS, H.J. 1980. pMB9 plasmids bearing the *Salmonella typhimurium* *his* operon and *gnd* gene. *Gene* 8, 239-253.
- BHADURI, S., TURNER-JONES, C., TAYLOR, M.M. and LACHICA, R.V. 1990. Simple assay of calcium dependency for virulent plasmid-bearing clones of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 28, 798-800.
- BISSETT, M.L., POWERS, C., ABBOTT, S.L. and JANDA, J.M. 1990. Epidemic of *Yersinia enterocolitica* and related species: sources, frequency, and serogroup distribution. *J. Clin. Microbiol.* 28, 910-912.
- DOYLE, M.P. and CLIVER, D.O. 1990. *Yersinia enterocolitica*. In *Foodborne Diseases*, (D.O. Cliver, ed.) pp. 223-228, Academic Press, San Diego, CA.
- DUBEL, J.R. 1983. Molecular and genetic characterization of the virulence plasmid of *Yersinia enterocolitica*. Ph.D. Thesis, University of Arizona, Graduate College.
- KAPPERUD, G. 1991. *Yersinia enterocolitica* in food hygiene. *Int. J. Food Microbiol.* 12, 53-66.
- KWAGA, J.K.P. and IVERSEN, J.O. 1991. Laboratory investigation of virulence among strains of *Yersinia enterocolitica* and related species from pigs and pork products. *Can. J. Microbiol.* 38, 92-97.
- LAZERE, J.R. and GEMSKI, P. 1983. Association of colony morphology with virulence of *Yersinia enterocolitica*. *FEMS Microbiol. Letters* 17, 121-126.
- MAZIGH, D., ALONSO, J.M. and MOLLARET, H.H. 1983. Simple method for demonstration of differential colony morphology of plasmid-associated virulent clones of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 17, 555-557.
- MAZIGH, D., CHALVIGNAC, M.A., QUILICI, M.L. and MOLLARET, H.H. 1985. Lack of correlation between plasmid-encoded outer membrane proteins and virulence of *Yersinia enterocolitica*. *Ann. Microbiol. (Inst. Pasteur)* 136(B), 39-47.
- MORRIS, JR., J. *et al.* 1991. *Yersinia enterocolitica* isolated from two cohorts of young children in Santiago, Chile: incidence of and lack of correlation between illness and proposed virulence factors. *J. Clin. Microbiol.* 29, 2784-2788.
- NOBLE, M.A., BARTELUK, R.L., FREEMAN, H.J., SUBRAMANIAM, R. and HUDSON, J.B. 1987. Clinical significance of virulence-related assay of *Yersinia* species. *J. Clin. Microbiol.* 25, 802-807.
- PORTNOY, D.A. and MARTINEZ, R.J. 1985. Role of a plasmid in the pathogenicity of *Yersinia* species. In *Genetic approaches to microbial pathogenicity*, (W. Goebel, ed.) pp. 29-51, Springer-Verlag, New York.
- PRPIC, J.K., ROBINS-BROWNE, R.M. and DAVEY, R.B. 1985. In vitro assessment of virulence in *Yersinia enterocolitica* and related species. *J. Clin. Microbiol.* 22, 105-110.

- ROBINS-BROWNE, R.M., MILIOTIS, M.D., CIANCIOSI, S., MILLER, V.L., FALKOW, S. and MORRIS, J.G. JR. 1989. Evaluation of DNA colony hybridization and other techniques for detection of virulence in *Yersinia* species. *J. Clin. Microbiol.* 27, 644-650.
- WEAGANT, S.D., FENG, P. and STANFIELD, J.T. 1992. *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. In *Food and Drug Administration Bacteriological Analytical Manual*, 7th Ed. (L. Tomlinson, ed.) pp. 95-109, AOAC International, Arlington, VA.