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PRESENCE OF VIRULENCE PLASMID IN *YERSINIA ENTEROCOLITICA* AFTER ITS EXPRESSION AT 37C¹

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ABSTRACT

The phenotypic characteristics associated with the virulence plasmid in Yersinia enterocolitica are expressed only at 37C, a temperature which facilitates the loss of the plasmid. This report investigated the stability of the virulence plasmid in Y. enterocolitica after its phenotypic expression at 37C. Polymerase chain reaction analysis of plasmid DNA showed that Y. enterocolitica did not lose the virulence plasmid during phenotypic expression as assayed by crystal violet binding, Congo red uptake, low calcium response, hydrophobicity and autoagglutination at 37C for 24 h. Prolonged incubation of at least 48 h at 37C is needed to trigger the loss of the plasmid.

INTRODUCTION

Yersinia enterocolitica is recognized as a significant human enteric pathogen (Bhaduri 1994; Brubaker 1991; Cornelis *et al.* 1989; Kapperud 1991). There is considerable confusion in the literature because not all *Y. enterocolitica* strains can cause intestinal infections. Unlike intrinsic pathogens such as *Salmonella*, strain-to-strain pathogenicity variation has been observed in *Y. enterocolitica* (Brubaker 1991; Cornelis *et al.* 1989; Doyle and Cliver 1990; Kapperud 1991). The pathogenicity of *Y. enterocolitica* is directly correlated with the presence of a 40 to 45 megadalton plasmid (Bhaduri 1994; Brubaker 1991; Cornelis *et al.* 1989; Doyle and Cliver 1990; Kapperud 1991). The unique quality of the plasmid is that it is stable in cells maintained at 0-28C but

¹ Reference 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.

expresses virulence genes at 37C. A number of phenotypic characteristics associated with the virulence plasmid are expressed only at 37C and include crystal violet (CV) binding, (Bhaduri *et al.* 1987), low calcium response (Lcr) (Bhaduri *et al.* 1990; Bhaduri *et al.* 1991), Congo red (CR) uptake (Bhaduri *et al.* 1991), hydrophobicity (HP) by latex particle agglutination (LPA) test (Lachica and Zink 1984), and autoagglutination (AA) (Laird and Cavanaugh 1980). At the same time, the expression of plasmid genes at 37C fosters plasmid loss and the concomitant disappearance of the associated phenotypic characteristics. These plasmid-mediated and temperature dependent phenotypic characteristics have been used to detect plasmid-bearing virulent (P⁺) strains of *Y. enterocolitica*. Previously, we showed that elevated temperatures (Bhaduri *et al.* 1988), elevated sodium chloride concentrations (Bhaduri and Mertz 1989), acid pH (Bhaduri and Mertz 1989), anaerobic atmospheric conditions (Bhaduri and Turner-Jones 1993) and standard enrichment procedures (Bhaduri 1995) did not trigger the loss of the virulence plasmid in *Y. enterocolitica*. No systematic and detailed study has yet been performed on the stability of the resident plasmid in *Y. enterocolitica* after its expression at 37C. Moreover, due to the instability of the virulence plasmid at 37C, it is difficult to isolate P⁺ strains after their initial detection. The present study was initiated therefore to determine if the expression of plasmid-associated virulence characteristics is followed by the loss of the virulence plasmid as determined by the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacteria

Recent reports indicate the emergence of Serotype O:3 of *Y. enterocolitica* as the major cause of Yersiniosis in the United States (Lee *et al.* 1990; Lee *et al.* 1991; Metchock *et al.* 1991). Therefore, *Y. enterocolitica* P⁺ GER strain (Serotype O:3) was chosen for this study. A detailed description of the strains, source and preparation of inocula, and incubation conditions are given elsewhere (Bhaduri *et al.* 1987; Bhaduri *et al.* 1991).

Assays of Expression of Plasmid-Associated Virulence Characteristics

To assay the expression of virulence plasmid in *Y. enterocolitica*, P⁺ cells were grown to stationary phase in 5 mL of brain heart infusion (BHI) (Difco Laboratories, Detroit, MI) broth as a starter culture by aerobic incubation for 18 h at 28C with shaking. A portion of the cell culture was diluted to 10³ cells per mL using BHI broth and surface plated onto BHI agar (BHA: Difco Laboratories) and Congo red BHI agarose (CR-BHO). Agarose was purchased from Sigma Chemical Co. (St. Louis, MO). Detailed descriptions of preparation

of these media and assay conditions are given elsewhere (Bhaduri *et al.* 1987; Bhaduri *et al.* 1990; Bhaduri *et al.* 1991). Plates were incubated at 37C for 24 h. The expression of the virulence plasmid in the cells was visualized by CV binding, Lcr, CR uptake and hydrophobicity by the LPA test. The expression of autoagglutination was determined as previously described (Bhaduri *et al.* 1987) with Eagle minimal essential medium supplemented with 10% fetal bovine serum (Bhaduri *et al.* 1987).

Determination of Presence of Virulence Plasmid after its Expression at 37C by PCR Amplification

To demonstrate the presence of the virulence plasmid in cells after its expression *viz* CV binding, Lcr, CR uptake and HP at 37C, colonies of each phenotype and clumped cells from AA test were analyzed by PCR using a key regulatory gene, *virF*, present on the virulence plasmid which encodes a transcriptional activator for the expression of plasmid-encoded outer membrane protein *yop51* (Bhaduri and Pickard 1996; Cornelis *et al.* 1989). DNA samples were prepared as described by Bhaduri and Pickard (1996). The primers (5'-ACATGGCAGAACAGCAGTCAG-3' and 5'-ACTCATCTTACCATTAAGAAG-3') used for detection of the *virF* gene (430- to 1020-nucleotide region) amplified a 591 base pair (bp) product from the virulence plasmid (Bhaduri and Pickard 1996). The oligonucleotide primers utilized in this study were synthesized by the Appligene Company (Pleasanton, CA). PCR was performed as previously reported (Bhaduri and Pickard 1996). The GeneAmp PCR reagent kit with AmpliTaq DNA polymerase was purchased from Perkin-Elmer Cetus Corporation (Norwalk, CT). The standard reaction mixtures (50 μ L) contained 5 μ L template DNA sample, 50 mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM MgCl₂, 0.001% (wt/vol.) gelatin, 100 μ M of each deoxynucleoside triphosphate, 0.2 μ M *virF* primers and 0.5 U AmpliTaq DNA polymerase. The samples were amplified by PCR with a programmable heating block incubator (GeneAmp PCR system 9600, Perkin-Elmer Corporation). It was programmed as follows: predenaturation at 94C for 1 min, 30 cycles of denaturation at 94C for 0.5 min, primer annealing at 55C for 1 min and extension at 70C for 2 min, followed by further extension at 70C for 5 min. P⁺ cells grown at 28C (Bhaduri and Pickard 1996) and purified plasmid DNA from P⁺ strain (Bhaduri 1990) were used as positive controls. A negative control with all of the reaction components except template DNA was included with each test run. After PCR amplification, 5 μ L of stop solution (50% glycerol-0.02% bromo phenol blue-60 mM EDTA, pH 8.0) was added and 20 μ L of each PCR product was analyzed by electrophoresis on a 2.0% agarose gel at a constant voltage of 80 V in TAE buffer (40 mM Tris-acetate-1 mM EDTA, pH 8.0) for 75 min (Bhaduri *et al.* 1980). The amplified DNA fragments were visualized with ethidium bromide (0.5 μ g per

mL) and UV transillumination at 302 nm (Bhaduri *et al.* 1980). Photographs of the strained gel under UV light were made for permanent records.

RESULTS AND DISCUSSION

There have now been several studies to show that CV binding, Lcr, CR uptake, HP, and AA are adequate virulence markers to demonstrate the expression of the plasmid and directly correlate to the virulence of the organism (Bhaduri 1994; Koeppel *et al.* 1993; Kwaga and Iversen 1991). Table 1 showed the expression of plasmid-associated virulence characteristics at 37C after a 24 h incubation. However, when P⁺ cells were grown on BHA and CR-BHO at 37C for 48 h, colonies appeared large flat and white large flat respectively. The cells were examined for the expression of plasmid-associated phenotypic characteristics. They did not display any of the appropriate virulence-associated properties (Table 1) and were designated as isogenic plasmidless strains (P⁻) (Bhaduri 1994).

TABLE 1:
EXPRESSION OF PLASMID-ASSOCIATED VIRULENCE CHARACTERISTICS AT
37C IN *Y. ENTEROCOLITICA*

| Strain | CV Binding ^a | Lcr ^b | CR Uptake ^c | HP ^d | AA ^e |
|----------------|-------------------------|------------------|------------------------|-----------------|-----------------|
| P ⁺ | + | + | + | + | + |
| P ⁻ | - | - | - | - | - |

^a CV binding: Crystal Violet Binding on BHA: P⁺ cells appeared as dark violet colony of diameter of 1.13 mm compared to the white large colony of P⁻ cells of diameter, 2.40 mm, (Bhaduri *et al.* 1987).

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

^c CR uptake: Congo Red Uptake on low-calcium CR-BHO: P⁺ cells appeared as red pin point colonies 0.36 mm in diameter compared to the white large colonies of P⁻ cells, 1.37 mm in diameter (Bhaduri *et al.* 1991).

^d HP: Hydrophobicity by Latex Partice Agglutination Test (Bhaduri *et al.* 1987).

^e AA: Autoagglutination (Bhaduri *et al.* 1987).

To study the presence of the virulence plasmid in *Y. enterocolitica* after the expression of plasmid-associated virulence characteristics including CV binding, Lcr, CR uptake, HP by LPA test, and AA at 37C, the cells from each

of these tests were subjected to PCR analysis to investigate whether the expression of these properties at 37C can cause the loss of the virulence plasmid. PCR results from samples of each phenotypic characteristic are shown in Fig. 1 (lanes 2-6). The primer pair for *VirF* gene was confirmed to amplify a 591 bp product showing the presence of virulence plasmid in each phenotypic characteristic. This was exclusively and consistently obtained from colonies after their respective expression at 37C in the tests previously mentioned. PCR analysis showed that *Y. enterocolitica* did not lose the virulence plasmid during its phenotypic expression at 37C for 24h. To demonstrate the absence of virulence plasmid in P⁻ strain, it was also subjected to PCR analysis using the *virF* regulatory gene as described above. PCR results from this analysis are shown in Fig. 2. The primer pair for *virF* gene failed to amplify a 591 product

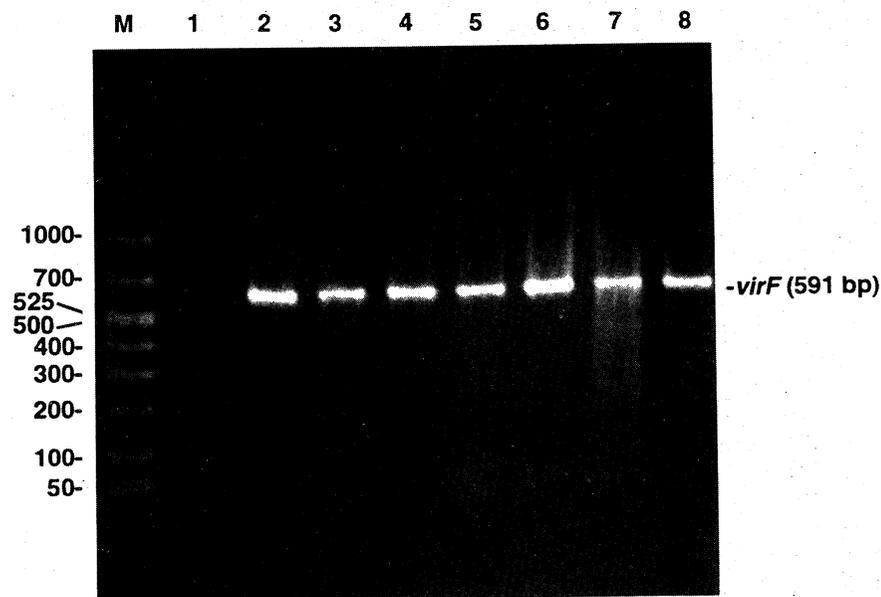


FIG. 1. DETECTION OF PLASMID DNA AFTER ITS EXPRESSION BY SPECIFIC AMPLIFICATION OF 591 BP PRODUCT BY PCR FROM VIRULENCE PLASMID WITH *virF* PRIMERS

Lanes; 1, negative control with no template; 2-6, 591 bp product from virulence plasmid with *virF* primers after expression of CV binding, Lcr, CR uptake, HP and AA respectively; 7, 591 bp product from virulence plasmid with *virF* primers from P⁺ cells grown at 28C; 8, 591 bp product from virulence plasmid with *virF* primers from purified plasmid DNA; M, 50 to 1000 bp ladder marker.

demonstrating the absence of the virulence plasmid in colonies taken from both BHA (Fig. 2, lane 2) and CR-BHO (Fig. 2, lane 4). These observations indicate that prolonged incubation at 37C is needed to trigger the loss of the virulence plasmid.

Of greater significance is the observation that *Y. enterocolitica* retained the virulence plasmid at 37C for 24 h and is therefore more stable than previously thought. Hence, any plasmid-bearing *Y. enterocolitica* recovered after its growth at 37C should still contain the virulence plasmid, and thus allow detection of contaminated samples by PCR.

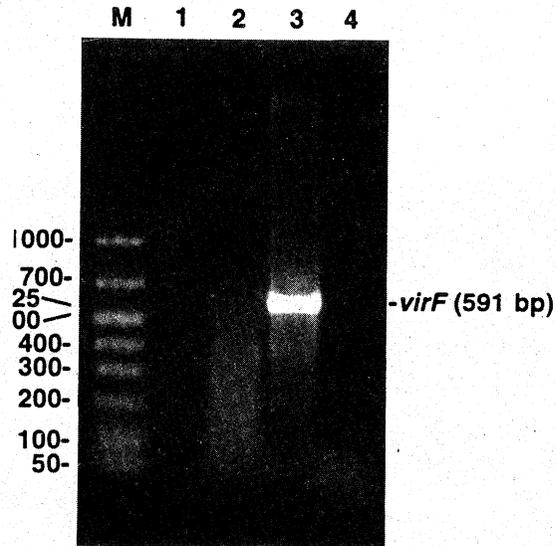


FIG. 2. PCR ANALYSIS OF DNA FROM PLASMIDLESS CELLS FOR DETECTION OF PLASMID DNA FOR SPECIFIC AMPLIFICATION OF 591 BP PRODUCT WITH *virF* PRIMERS

Lanes; 1, negative control with no template; 2, absence of 591 bp from virulence plasmid with *virF* primers in P⁺ cells grown on BHA at 37C for 48 h; 3, 591 bp product from virulence plasmid with *virF* primers from P⁺ cells grown at 37C for 24 h; 4, absence of 591 bp product from virulence plasmid with *virF* primers in P⁺ cells grown on CR-BHO at 37C for 48 h; M, 50 to 1000 bp ladder marker.

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