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**EFFECT OF ENRICHMENT PROCEDURES ON THE STABILITY
OF THE VIRULENCE PLASMID AND VIRULENCE-ASSOCIATED
CHARACTERISTICS IN *YERSINIA ENTEROCOLITICA*¹**

SAUMYA BHADURI

*U.S. Department of Agriculture, Agricultural Research Service
Eastern Regional Research Center, 600 East Mermaid Lane
Philadelphia, PA 19118*

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ABSTRACT

This report presents information on the effects of five current enrichment procedures including trypticase soy broth, phosphate buffered saline, peptone enriched phosphate buffered saline, phosphate buffered saline with sorbitol and bile salts and MacConkey broth on the stability of virulence plasmid in Yersinia enterocolitica. Virulence assays using crystal violet binding, low calcium response, Congo red uptake, hydrophobicity by latex particle agglutination, and autoagglutination as well as plasmid DNA analysis showed that Y. enterocolitica did not lose the virulence plasmid during isolation at 28C and the cells remained virulent. Moreover, postenrichment treatment with alkali to reduce competing microflora, when used in conjunction with these five enrichment techniques, did not cause the loss of the virulence plasmid, thus providing a way to selectively isolate this pathogen.

INTRODUCTION

The association of human illness with the consumption of food contaminated with *Y. enterocolitica* is well documented (Doyle and Cliver 1990; Kapperud 1991; Kwaga and Iversen 1991). Since yersiniae can grow at low temperatures,

¹Reference 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.

refrigerated foods are potential vehicles for the growth of the organisms (Doyle and Cliver 1990; Kapperud 1991). Strains of all serotypes implicated in human disease harbor a plasmid of molecular weight of 40 to 45 MDa, which is directly involved in the virulence of this bacterium (Kapperud 1991; Portnoy and Martinez 1985). A number of temperature dependent phenotypic characteristics associated with the virulence plasmid have been described and are used to detect plasmid-bearing virulent strains of *Y. enterocolitica* (Bhaduri 1990a,b, 1993; Bhaduri *et al.* 1987, 1990, 1991; Doyle and Cliver 1990; Kapperud 1991; Kwaga and Iversen 1991; Portnoy and Martinez 1985; Robins-Browne *et al.* 1989; Weagant *et al.* 1992). When virulent *Yersinia* strains are cultivated *in vitro*, the virulence plasmid can be spontaneously lost during cell division, resulting in a mixed population of virulent and avirulent cells. Loss is facilitated by culturing at 37C (Bhaduri 1993; Doyle and Cliver 1990; Kapperud 1991; Portnoy and Martinez 1985) and by prolonged storage at 4C or during laboratory manipulation (Bhaduri 1993; Miller *et al.* 1989). This leads to the eventual overgrowth by plasmidless cells in the population leading to a completely avirulent culture. Consequently, loss of this plasmid results in the loss of virulence and the concomitant disappearance of associated phenotypic characteristics. Previously, we studied the stability of the virulence plasmid in *Y. enterocolitica* at elevated temperatures (Bhaduri *et al.* 1988), at various sodium chloride concentrations (Bhaduri and Mertz 1989), acidic pH (Bhaduri and Mertz 1989) and under anaerobic atmospheric conditions (Bhaduri and Turner-Jones 1993) by using the plasmid-associated crystal violet (CV) binding, Congo red (CR) uptake, low calcium response (Lcr) and hydrophobicity (HP) properties to detect the presence of the plasmid in the cells and as an indicator of virulence. Although a wide variety of techniques for the enrichment of *Y. enterocolitica* from food has been described in the literature (Gilmour and Walker 1988; Weagant *et al.* 1992), no systematic and detailed study has been done on the stability of the resident plasmid in *Y. enterocolitica* during enrichment. Moreover, due to the unstable nature of the plasmid (Bhaduri 1993; Doyle and Cliver 1990; Kapperud 1991; Kwaga and Iversen 1991; Portnoy and Martinez 1985; Miller *et al.* 1989), the possibility of plasmid loss during the course of a protracted enrichment procedure from food cannot be excluded. The present study was initiated to determine whether current enrichment procedures used for the isolation of *Y. enterocolitica* from food trigger the loss of the virulence plasmid. The presence or loss of virulence plasmid was monitored by using CV binding (Bhaduri 1993; Bhaduri *et al.* 1987), Lcr (Bhaduri *et al.* 1990; Bhaduri *et al.* 1991), CR uptake (Bhaduri 1993; Bhaduri *et al.* 1991), HP by latex particle agglutination (LPA) (Bhaduri *et al.* 1987), agglutination test (Bhaduri *et al.* 1987) and plasmid DNA analysis (Bhaduri *et al.* 1987).

TABLE 1.
EFFECT OF ENRICHMENT MEDIA ON THE STABILITY OF VIRULENCE PLASMID
AND VIRULENCE-ASSOCIATED CHARACTERISTICS IN *YERSINIA ENTEROCOLITICA*
GER (SEROTYPE O:3)

Enrichment media	Total number of	No. (%) of	Total number of	No. (%) of
	colonies on BHA	colonies which bound CV	colonies on CR-BHO	colonies showing Lcr and CR uptake
TSB	45	43 (96)	44	44 (100)
PBS	49	48 (98)	48	47 (98)
PepPBS	50	48 (96)	51	50 (98)
MAC	42	41 (98)	56	56 (100)
PBSSB	48	46 (96)	53	51 (96)
Control P ⁺	40	39 (98)	60	59 (98)
Control P ⁻	64	0	66	0

P⁺ cells were grown for 24 h at 28C in each enrichment media. The cells were diluted to a concentration of 10³ cells per ml determined by A₆₀₀ in BHI broth. The cells were then surface plated on BHA and CR-BHO and incubated at 37C for 24 h. Total cells were counted and the presence of the plasmid in the cells was detected by CV binding (appearance of dark violet colonies), Lcr (appearance of pinpoint colonies), CR uptake (appearance of red pinpoint colonies). The P⁺ and P⁻ cells grown in BHI broth were tested as control.

retained the resident plasmid (Table 2). The presence of the virulence plasmid in the cells was also confirmed by HP, autoagglutination and plasmid DNA analysis (data not shown). Growth rate of P⁺ GER strain (serotype O:3) was equivalent in all five enrichment broths held at 28C for 24 h. The step of postenrichment treatment with alkali, which probably aids in reducing the competing background flora, did not adversely affect the retention of the virulence plasmid. In general, the percentage (2%) of P⁺ cells converting to P⁻ cells under all conditions did not differ from the controls. Results similar to those obtained with GER (Serotype O:3) were obtained when EWMS (Serotype O:13) or PT18-1 (Serotype O:5, O:27)

TABLE 2.
EFFECT OF ENRICHMENT MEDIA AND POSTENRICHMENT ALKALI TREATMENT
ON THE STABILITY OF VIRULENCE PLASMID AND VIRULENCE-ASSOCIATED
CHARACTERISTICS IN *YERSINIA ENTEROCOLITICA* GER (SEROTYPE O:3)

Enrichment media	Total number of	No. (%) of	Total number of	No. (%) of
	colonies on BHA	colonies which bound CV	colonies on CR-BHO	colonies showing Lcr and CR uptake
TSB	50	49 (98)	54	53 (98)
PBS	43	43 (100)	47	45 (96)
PepPBS	45	44 (98)	48	45 (94)
MAC	52	51 (98)	66	66 (100)
PBSSB	54	53 (98)	51	50 (98)
Control P ⁺	48	48 (100)	62	61 (98)
Control P ⁻	38	0	64	0

P⁺ cells were grown for 24 h at 28C in each enrichment media and then treated with alkali (0.5% KOH in 0.5% NaCl) for 15 s. The cells were diluted to a concentration of 10³ cells per ml determined by A₆₀₀ in BHI broth. The cells were then surface plated on BHA and CR-BHO and incubated at 37C for 24 h. Total cells were counted and the presence of the plasmid in the cells was detected by CV binding (appearance of dark violet colonies), Lcr (appearance of pinpoint colonies), CR uptake (appearance of red pinpoint colonies). The P⁺ and P⁻ cells grown in BHI broth were tested as control.

or O:TAC (Serotype O:TACOMA) or WA (Serotype O:8), representing four other serotypes of *Y. enterocolitica*, were used to study the effect of enrichment media and procedures on the stability of the virulence plasmid (data not shown). These four serotypes also showed that 94–98% of cells retained the virulence plasmid with or without postalkali treatment in five enrichment broths. The data presented here are the results of one of several experiments that all showed similar responses. These results indicate that when P⁺ cells of *Y. enterocolitica* are subjected to five enrichment procedures, including postenrichment alkali treatment, nearly all the cells retain the resident plasmid and the associated virulence traits. Similarly,

the virulence plasmid was stable when P⁺ cells of *Y. enterocolitica* were held at elevated temperatures (Bhaduri *et al.* 1988), at various sodium chloride concentrations (Bhaduri and Mertz 1989), acidic pH (Bhaduri and Mertz 1989) and under anaerobic conditions (Bhaduri and Turner-Jones 1993). Of greater significance is the observation that cells of *Y. enterocolitica* held under these conditions retain the virulence plasmid and are more stable than previously thought. Hence, any plasmid-bearing *Y. enterocolitica* recovered from foods using the standard procedures should still contain the virulence plasmid, thus allowing the potential hazard from these isolates to be properly assessed. However, the nature and condition of food and the presence of competitive microflora in food may affect the stability of virulence plasmid during the enrichment of *Y. enterocolitica*. Further studies are required to determine the effects of these parameters on the stability of virulence plasmid in *Y. enterocolitica* during its enrichment.

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