Determination of Plasmid-Associated Hydrophobicity of *Yersinia enterocolitica* by a Latex Particle Agglutination Test†

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A quick and simple method was developed to distinguish hydrophobic from hydrophilic cells. The latex particle agglutination test is based on the hydrophobic interactions between cells and latex particles which result in the agglutination of the suspension mixture. There was a direct correlation between the expression of plasmid-associated cell surface properties and latex particle agglutination by *Yersinia enterocolitica*. Multivalent cation-induced agglutination of suspensions of washed cells of virulent *Y. enterocolitica* and latex particles is indicative of their amphipathic character. Electrostatic interaction may also play a role in the latex particle agglutination reaction.

Yersinia enterocolitica is now recognized as an important cause of bacterial gastroenteritis in humans (1, 19). In recent years, there has been considerable interest in the pathogenicity of the organism with the discovery of a resident plasmid and its association with the virulence of the organism (3, 9–11, 18). Direct proof for the involvement of the plasmid in the virulence of the organism was described recently by Heeseman and Laufs (4).

In our attempt to elucidate the pathogenicity of *Y. enterocolitica*, we were confronted with the annoying problem of plasmid instability. It has been suggested that to minimize the high rate of spontaneous curing of the plasmid, growth temperatures over 30°C should be avoided (18, 19). Yet growth at 37°C is essential for the organism to express its virulence characteristics in vitro (10, 19). One approach to circumvent the problem is to tag the virulence plasmid with an ampicillin transposon and selectively grow the ampicillin-resistant derivative (J. R. Dubel, R. V. Lachica, and D. L. Zink, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B 191, p. 55). Another approach is the use of an indirect screening method to indicate the presence of the plasmid. Such a method, referred to as the latex particle agglutination (LPA) test, is described in the present study. Evidence is presented which indicates that the LPA test predominantly determines cell surface hydrophobicity, a plasmid-associated property of *Y. enterocolitica* (R. V. Lachica and D. L. Zink, Infect. Immun., in press).

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MATERIALS AND METHODS

Bacteria and growth conditions. Plasmid-bearing (p^+) strains of *Y. enterocolitica* and their plasmidless (p^-) derivatives used in this study are listed in Table 1. The p^- strains were derived from cells of large colonies which invariably emerged from p^+ cultures growing at 37°C in tryptic soy agar (TSA) supplemented with 20 mM sodium oxalate and 20 mM $MgCl_2$ (MOX agar) (Lachica and Zink, in press). The cultures were stored as a cell suspension at −20°C in 50% glycerol and cultivated in tryptic soy broth at 22°C. Unless described otherwise, experimental conditions involved incubation of cultures at 37°C for 28 h. Cell suspensions were usually obtained from cultures growing on agar media such as TSA or brain heart infusion agar. When the effect of Ca^{2+} in the expression of cell surface properties was investigated, agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used in place of agar as the gelling agent in some media.

Plasmid screening. The presence of plasmid DNA in bacteria was determined as described by Kado and Liu (5).

Virulence and properties associated with virulence. As previously described (Lachica and Zink, in press), the tissue culture detachment assay (11) was used as an in vitro method for determination of virulence. A modified MOX agar (Lachica and Zink, in press) was used for the determination of calcium growth dependence. Autoagglutination (AA) was determined by the method of Laird and Cavanaugh (6) with Eagle minimal essential medium supplemented with 10% fetal calf serum.

Cell surface properties. Cell surface hydrophobicity was determined by the polystyrene plate adherence (PSA) method and the nitrocellulose filter (NCF) adsorption procedure, which have been described previously (Lachica and Zink, in press). The tendency of xylene and hexadecane to lyse the cells caused us to discontinue the use of the hydrocarbon affinity procedure (14). Cell surface charge was determined by the hydroxyapatite (HA) adherence procedure (Lachica and Zink, in press). Suspensions of latex particles were also examined for their affinity to NCF and HA.

Latex particles. Suspension of latex particles of various sizes (0.109 to 5.70 μm in diameter) were purchased from E. F. Fullam, Inc., Schenectady, N.Y. A set of these suspensions was washed in 0.9% saline (pH 7.2) by nitrocellulose filtration and suspended in saline at various concentrations. Another set was directly diluted with saline without prior washing.

RESULTS AND DISCUSSION

This investigation presents an LPA test for rapidly differentiating p^+ cells of *Y. enterocolitica* from their p^- derivatives. This test is based on the differential adherence of cells to the surface of the latex particles. It is comparable to the

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TABLE 1. LPA, surface properties, and virulence of plasmid-bearing strains of *Y. enterocolitica* and their plasmidless derivatives

Strain	Origin	Source	Serotype	Surface properties			
				LPA	Hydrophobic[a]	Anionic[b]	Virulence[c]
TAMU 58	Chocolate milk	D. L. Zink	O:8	+	+	+	+
TAMU 58-C		This study		–	–	–	–
TAMU 59	Chocolate milk	D. L. Zink	O:8	+	+	+	+
TAMU 59-C		This study		–	–	–	–
TAMU 191	Pig tongue	C. Vanderzant	O:5, O:27	+	+	+	+
TAMU 191-C		This study		–	–	–	–
E773	Pork	D. A. Schiemann	O:5, O:27	+	+	+	+
E773-C		This study		–	–	–	–
12697/76	Human	G. Kapperud	O:3	+	+	+	+
12697/76-C		This study		–	–	–	–
96-13/76	Swine	K. B. Pedersen	O:3	+	+	+	+
96-13/76-C		This study		–	–	–	–
78-513	Pork	W. H. Lee	O:1, O:2, O:3	+	+	+	+
78-513-C		This study		–	–	–	–
E750	Human	D. A. Schiemann	O:21	+	+	+	+
E750-C		This study		–	–	–	–
E752	Human	D. A. Schiemann	O:31	+	+	+	+
E752-C		This study		–	–	–	–

[a] Cell surface hydrophobicity was determined by NCF and PSA techniques.
[b] Surface charge was determined by the HA adherence method.
[c] HEp-2 monolayer detachment assay; calcium growth dependency and AA served as virulence indicators.

PSA method of Rosenberg (13), but without excessive use of water and potential hazards from aerosol formation.

Development of the LPA test. Preliminary experiments involved the use of washed cell and latex suspensions. Subsequently, the washing step for the latex particles was found to be unnecessary. Best results were obtained when the largest of the latex particles (5.70 μm in diameter) were diluted with saline to obtain a suspension of about 1.3×10^8 latex particles per ml. We also found the washing step for the cell suspensions unnecessary, provided that heavy suspensions ($>10^9$ per ml) were obtained with an inoculation loop directly from colonies growing on brain heart infusion agar plates. Thus, the LPA test consisted of mixing equal volumes (10 μl) of latex particles and cell suspensions on a clean microscope slide. A positive test was indicated by an immediate strong agglutination reaction which was easy to discern (Fig. 1A).

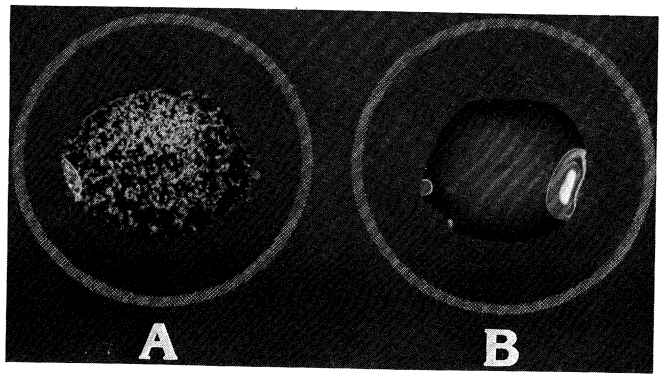

FIG. 1. (A) Plasmid-bearing cells of *Y. enterocolitica* previously grown on brain heart infusion agar at 37°C agglutinated a suspension of latex particles. (B) Plasmid-cured cells did not agglutinate the latex particle suspension.

Association of the LPA reaction and plasmid-mediated properties of *Y. enterocolitica*. To evaluate the utility of the LPA test in distinguishing virulent strains of *Y. enterocolitica*, we examined nine p^+ strains and their p^- derivatives representing six serotypes. All cell suspensions were har-

TABLE 2. Growth conditions regulating the expression of LPA, PSA, and surface properties of nine plasmid-bearing strains of *Y. enterocolitica*

Medium	LPA		PSA		Surface properties			
					Anionic[a]		Hydrophobic[b]	
	37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C
BHIO[c]	+	–	+	–	2+[d]	–	2+	–
BHIO[c] + 2.5 mM, $CaCl_2$	+	–	+	–	1+	–	1+	–
BHIA	+	–	+	–	1+	–	1+	–
TSO[c]	–	–	–	–	–	–	–	–
TSA	–	–	–	–	–	–	–	–

[a] Surface charge was determined by the HA adherence method (Lachica and Zink, in press).
[b] Adsorption to NCF procedure (Lachica and Zink, in press).
[c] Agar was replaced by agarose as the gelling agent.
[d] 1+ and 2+ indicate discernible differences in surface properties, as described in the text.

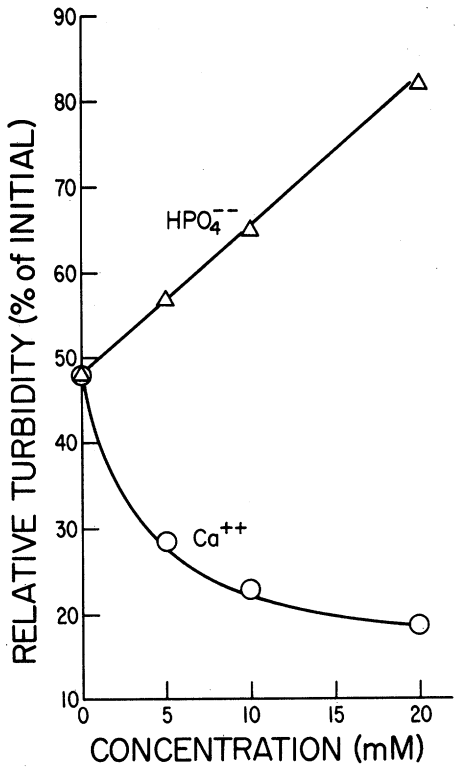

FIG. 2. Effect of added Ca^{2+} or HPO_4^{2-} in the adsorption of latex particles to HA.

vested from cultures grown on brain heart infusion agar. Table 1 shows that all the p^+ strains were LPA positive and virulent as determined by the in vitro assay of Portnoy et al. (11). They were also positive for calcium growth dependence and AA. The virulent strains all exhibited high surface charge and hydrophobicity as indicated by their adsorption to HA, NCF, and PSA. Loss of the resident plasmid brought about the loss of virulence, the reduction in cell surface properties, and the loss in the ability of the cells to agglutinate latex particles.

Growth conditions regulate the expression of all known plasmid-associated properties of *Y. enterocolitica* (9–11, 19). The properties are expressed when the p^+ cells are grown at 37°C. The absence of Ca^{2+} reduces the growth rate (3) but induces the synthesis of the V and W antigens (10) and outer membrane proteins (11). On the other hand, resistance to human serum and AA are Ca^{2+} independent (10). The altered surface properties of p^+ cells have been shown recently to be moderately affected by Ca^{2+} (Lachica and Zink, in press). Table 2 shows that growth conditions regulating the expression of LPA and PSA paralleled closely those conditions regulating the altered cell surface properties of p^+ cells. Consistent with our previous observations (Lachica and Zink, in press), Ca^{2+} had a moderate dampening effect on the expression of the surface properties of p^+ cells. However, Ca^{2+} had no apparent effect on the cell expression of LPA and PSA. This minor discrepancy may be attributed to the difference in the sensitivity of the methods. The p^+ cells grown on tryptic soy agarose or TSA were all negative for LPA and PSA and did not express increased cell surface charge or hydrophobicity. These results contrast with calcium growth dependence, which was not inhibited by TSA, the basal medium of the modified MOX agar (Lachica and Zink, in press). On the other hand, the expression of AA was more fastidious and required the use of Eagle minimal essential medium supplemented with 10% fetal calf serum. p^+ cells were considered to be in their virulent mode phase 1 ($vir1^+$) when they expressed their surface charge and hydrophobicity. None of the p^- strains inhibited surface charge and hydrophobicity; all were negative for LPA and PSA.

Surface properties of $vir1^+$ cells and latex particles. We consider hydrophobic interaction as the major force in the interaction between $vir1^+$ cells and latex particles which results in LPA. This is consistent with the notion that hydrophobic interaction is the dominant force in attachment to hydrophobic surfaces (2, 17). Von Hippel et al. (17) noted that only ions with appreciable nonpolar (hydrophobic) character bind to latex particles. Recently, Fletcher and Marshall (2) reported the low-energy (hydrophobic) surface of the polystyrene petri plate as determined by a bubble contact angle method. Predictably, we observed that NCF adsorbed over 90% of the latex particles during filtration. Moreover, there was complete inhibition of the LPA reaction with the addition of 0.05% Tween 80, indicating the disruption of the hydrophobic interactions between the $vir1^+$ cells and the latex particles by the nonionic detergent.

Electrostatic interaction may also play a role in the LPA reaction. This was indicated by the moderate reduction in the LPA reaction with the presence of 20 mM phosphate. As with the $vir1^+$ cells, the latex particles were also negatively charged as demonstrated by their affinity to HA. Figure 2 shows the enhanced adsorption of the latex particles with the addition of Ca^{2+}, whereas phosphate had the opposite effect. These are typical behavior patterns of anionic particles (12) and consistent with the observation that the negative charges of latex particles involve sulfate end groups (15, 16).

Multivalent cation-induced agglutination of $vir1^+$ cell and latex particle suspensions was another indication of their anionic property. The addition of 0.025 mM $CaCl_2$, $ZnSO_4$, and $FeCl_2$, but not $MgCl_2$ or NaCl, induced rapid agglutination of washed $vir1^+$ cell and latex particle suspensions. Neither p^- nor p^+ cells in their avirulent mode were induced to agglutinate with addition of the tri- or divalent cations. It was necessary to use washed cells to obtain consistent results.

$vir1^+$ cells and latex particles may be categorized as amphipathic colloids on the threshold of agglomeration. The addition of multivalent cations may bridge the anionic surfaces of the particles and cells and precipitate them out of suspension. Similarly, it is envisioned that in the LPA reaction, the negative charge on the $vir1^+$ cells and latex particles provide binding sites to which residual tri- or divalent cations can absorb to bridge the cells to the latex particles. This ionic interaction is then stabilized by hydrophobic groups adjacent to the site of bond formation. Similar mechanisms have been suggested in the adherence of oral streptococci to tooth surfaces (8, 12). This amphipathic colloidal nature of $vir1^+$ cells may explain, at least in part, the AA reaction described by Laird and Cavanaugh (6).

During the preparation of this report, Martinez (7) reported the hydrophobic nature of *Y. enterocolitica*, which substantiated our own observations in the present study and elsewhere (Lachica and Zink, in press). In addition, he described one strain (serotype O:Tacoma) in which expression of cell surface hydrophobicity was chromosomally mediated at 22°C but plasmid mediated at 37°C. The LPA test would be useful in a survey to determine whether this strain is unique or typical to that particular serogroup.

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