Adherence of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* to L-929 Cells: Association with Virulence Plasmid and Effects of Cultivation- and Adsorption-Temperatures

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ABSTRACT. Adhesive capacities of *Yersinia* strains to L-929 cells were compared in respect to the presence of virulence plasmid and the effects of temperature (25°C and 37°C) for bacterial cultivation and for adsorption with L-929 cells. *Yersinia pseudotuberculosis* IB carrying virulence plasmid adhered remarkably to L-929 cells when the organisms were cultivated at 37°C and adsorbed on L-929 cells at 37°C. Low degree of adherence was observed in the same organisms with three other combinations of cultivation- and adsorption-temperatures (37°C vs 25°C, 25°C vs 37°C and 25°C vs 25°C). Adhesive capacity to L-929 cells was low in *Y. pseudotuberculosis* IB mutant lacking virulence plasmid as compared with that in the parent strain. *Y. pseudotuberculosis* strains showed higher adhesive capacities than *Y. enterocolitica* strains. Furthermore, formalin-inactivated *Y. pseudotuberculosis* IB, both cultivated and adsorbed at 37°C, showed as high adhesive capacity as the live organism. The results suggested that the adhesive factor of *Yersinia* depended greatly on plasmid and was expressed more efficiently when the organisms were cultivated at 37°C and adsorbed with L-929 cells at 37°C.—**KEY WORDS:** adherence, cultivation-temperature, plasmid, *Yersinia*.

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The virulence of *Yersiniae* has been examined with various markers including pathogenicity to animal host, enterotoxin production, invasiveness in the intestinal tracts, and autoagglutination [12]. Recently, the close association between these markers and the presence of plasmid with 40- to 48-M dalton in *Yersiniae*, which provided clear evidence that the plasmid mediated the virulence of *Yersinia* were reported [8, 11]. However, further details on the relationship between the pathogenic effects of *Yersinia* to cells and the plasmid have not been fully elucidated yet.

We have also studied the role of plasmid of *Yersinia* using a mouse model, in which fecal excretion of the organisms and immunization of the mouse with the organisms by oral routes were chosen as the indicators of the expression of plasmid. It was suggested that the maintenance of fecal excretion of the organisms was due to the presence of viru-

lence plasmid in this organism [6, 13]. Furthermore, in many cases, bacterial adherence to epithelial cells *in vivo* was caused by the production of some kinds of surface antigens such as colonization factors encoded by plasmids, as in the case of *Escherichia coli* [2, 16]. Recently, also in the case of *Yersinia*, the expression of cell surface properties such as charge and hydrophobicity was reported to be associated with the plasmid, cultivation-temperature of 37°C and intestinal colonization [7]. The present study was undertaken to characterize the adhesive factor which is closely related to the virulence regulated by *Yersinia*'s plasmid, of which expression is dependent on temperature.

MATERIALS AND METHODS

Organisms and cultivation: The strains of *Yersiniae* used in this study were listed in

Table 1. *Yersinia* strains used in this study

Strain	Plasmid	Origin
<i>Y. pseudotuberculosis</i> IB	+	Brown rat
mutant IB ^{a)}	—	
<i>Y. pseudotuberculosis</i> IV A	+	Brown rat
<i>Y. pseudotuberculosis</i> IV B	+	Old World wood mouse
<i>Y. enterocolitica</i> O:3	+	Brown rat
<i>Y. enterocolitica</i> O:5B	+	Dog
<i>Y. enterocolitica</i> O:9	+	Dog
<i>Y. enterocolitica</i> O:6 ^{b)}	—	Brown rat

a) *Y. pseudotuberculosis* IB mutant was selected as the plasmid-lacking mutant as described in the text.

b) *Y. enterocolitica* O:6 was originally lacking plasmid when the organism was isolated.

Table 1. *Yersinia pseudotuberculosis* includes IB, IB mutant, IV A and IV B strains, and *Yersinia enterocolitica* includes O:3, O:5B, O:9 and O:6 strains. The strains were originated from dogs, mice and rats [5]. A plasmid lack mutant of *Y. pseudotuberculosis* IB strain was selected from a plasmid carrying parent strain in magnesium oxalate agar [3]. *Y. enterocolitica* O:6 strain was originally lacking the plasmid and Ca^{2+} independence when the organism was isolated. Each of the bacterial strains was inoculated into Trypticase Soy Broth (BBL) and incubated aerobically in either 37°C or 25°C for 48 hr. After incubation, the culture fluid was centrifuged at 4,000 rpm for 20 min and the bacterial pellet was suspended in aqueous solution containing 5% lactose and 50% calf serum to freeze. The bacterial suspension was frozen at -80°C prior to its use in the experiments. Presence or absence of the plasmid in the each strains was ascertained with the agarose gel electrophoresis as reported by Kado and Liu [4] and the results were shown in Table 1.

Formalin inactivation of *Y. pseudotuberculosis* IB strain: *Y. pseudotuberculosis* IB strain was cultured at 25°C or 37°C as described above, and then the formalin was added to the cultures (10^9 bacteria/ml) at a final concentration of 1%. After 24 hr of formaldehyde-treatment at 4°C, the inactivated organisms were centrifuged at 4,000 rpm

for 20 min, and the pellets were suspended in phosphate buffered saline (PBS), pH 7.2, and stored at 4°C until used.

Bacterial adherence test: L-929 cells were maintained in Eagle's minimum essential medium containing 8% fetal calf serum, 10% tryptose phosphate broth, 2 mM of L-glutamine and 60 µg/ml Kanamycine. Trypsinized cells (1×10^4 cells/0.05 ml) were seeded on each wells of Multitest slides (10 wells of 8 mm diameter, Flow Laboratories). The slides were placed in a petri dish and incubated at 37°C in humidified air with 5% CO₂ for 24 hr. Cell density reached the concentration of 5×10^4 cells/well. The cultured slides were washed three times with PBS (0.1 ml/well). Bacterial suspension was diluted with PBS at a concentration of 2×10^7 bacteria/ml and inoculated onto the L-929 cell monolayer formed on each well of the Multitest slides (0.05 ml/well). The slides were incubated for the adsorption on the cells in 5% CO₂ at 37°C or 25°C for 30 to 120 min. They were washed three times with PBS to remove free bacteria, fixed in methanol for 10 min and stained with Giemsa stain for 20 min. Usually one hundred of L-929 cells from each well were examined for the number of bacteria that attached to the cell surface under the light microscope with oil immersion. The number of adhesive organisms on the one hundred of L-929 cells were counted in five wells. The

Fig. 1. Effects of temperatures (25°C and 37°C) on adhesive capacity of *Y. pseudotuberculosis* IB (plasmid⁺) and IB mutant (plasmid⁻) to L-929 cells. *Y. pseudotuberculosis* IB (plasmid⁺) (A) and IB mutant (plasmid⁻) (B) were cultured at 37°C and 25°C for 48 hr and incubated with L-929 cells at 37°C and 25°C. Mean bacterial numbers that attached were shown in the following combinations, cultivation- and adsorption-temperatures of 37°C and 37°C (●—●), 37°C and 25°C (●---●), 25°C and 37°C (○—○) and 25°C and 25°C (○----○).

means of each, which was the number of adhesive organisms per cell, was calculated. And then the significance of difference of the means under several adhesive conditions was calculated by Student's *t* test. The indirect immunofluorescence antibody test was applied to confirm the bacterial attachment on the cell by using rabbit antiserum against *Y. pseudotuberculosis* IB and FITC-conjugated anti-rabbit IgG (Cappel).

RESULTS

1. Effect of cultivation- and adsorption-temperatures (25°C and 37°C) on adhesive capacities of *Y. pseudotuberculosis* IB (plasmid⁺) and its mutant (plasmid⁻) to L-929 cells.

Y. pseudotuberculosis IB carrying virulence associated plasmid (plasmid⁺) was cultivated at either 25°C or 37°C and adsorbed on the L-929 cells on the Multitest slides at either 25°C or 37°C. The number of bacteria

that attached to the L-929 cells were examined at intervals in four different combinations of cultivation-temperatures (25°C and 37°C) and adsorption-temperatures (25°C and 37°C) (Fig. 1,A). In the combination of the cultivation-temperature of 37°C and the adsorption-temperature of 37°C, the mean bacterial numbers that attached were 2.2 per cell at 60 min of adsorption, and they increased to 7.7 per cell at 120 min of adsorption. The mean numbers of attached bacteria were larger in the case of *Y. pseudotuberculosis* IB (plasmid⁺) cultivated at 37°C and more bacteria adsorbed at 37°C than in the other three combinations at 120 min of incubation ($P < 0.005$). There were no significant differences in the numbers of attached bacteria in the three combinations of the cultivation-temperature and the adsorption-temperature (25°C and 25°C, 25°C and 37°C, and 37°C and 25°C). *Y. pseudotuberculosis* IB mutant lacking virulence plasmid (plasmid⁻) was also cultivated at 25°C or 37°C and adsorbed

Fig. 2. Effects of cultivation-temperature (25°C and 37°C) on adhesive capacity of *Y. pseudotuberculosis* IV A and IV B and *Y. enterocolitica* O:3, O:5B, O:9 and O:6 strains to L-929 cells at 37°C. *Y. pseudotuberculosis* IV A(A) and IV B(B), and *Y. enterocolitica* O:3(C), O:5B(D), O:9(E) and O:6(F) were cultured at 37°C (●) and 25°C (○) and then adsorbed at 37°C with L-929 cells on Multitest slides.

with L-929 cells at 25°C or 37°C. The mean numbers of attached bacteria did not exceed more than 2.1 per cell at the cultivation-temperature of 25°C or 37°C and the adsorption-temperature of 25°C or 37°C after 120 min (Fig. 1,B). The patterns of adherence of *Y. pseudotuberculosis* IB (plasmid⁺) were compared with those of *Y. pseudotuberculosis* IB mutant (plasmid⁻) (Fig. 1,A and B). The mean numbers of attached bacteria in *Y. pseudotuberculosis* IB were significantly larger than those in *Y. pseudotuberculosis* IB mutant in all of the four combinations of cultivation- and adsorption-temperatures at 120 min of adsorption ($P < 0.05$). The results showed that *Y. pseudotuberculosis* carrying plasmid associated virulence had a high adhe-

sive capacity when it was cultivated at 37°C and adsorbed at 37°C with L-929 cells.

2. Effect of cultivation-temperatures (25°C and 37°C) on adhesive capacity of several strains of *Y. pseudotuberculosis* and *Y. enterocolitica* to L-929 cells at 37°C.

Several other strains of *Y. pseudotuberculosis* and *Y. enterocolitica* cultivated at 25°C or 37°C were examined for their adhesive capacity to L-929 cells at 37°C. The plasmid carrying virulent strains included *Y. pseudotuberculosis* IV A and IV B, *Y. enterocolitica* O:3, O:5B and O:9, and *Y. enterocolitica* O:6 which originally lacked plasmid associated virulence was also included. The results are shown in Fig. 2. The mean numbers of attached bacteria to L-929 cells were signifi-

Fig. 3. Effect of formalin inactivation on adhesive capacity of *Y. pseudotuberculosis* IB (plasmid⁺) and IB mutant (plasmid⁻) to L-929 cells at 37°C. *Y. pseudotuberculosis* IB (plasmid⁺) was cultured at 37°C (●) and 25°C (○), and IB mutant (plasmid⁻) was also cultured at 37°C (▲) and 25°C (△). These bacterial cultures were inactivated with formalin, as described in the test, and absorbed with L-929 cells at 37°C.

cantly higher in *Y. pseudotuberculosis* IV A cultivated at 37°C than in the same organisms cultivated at 25°C after 120 min of adsorption (6.6 ± 0.6 bacteria/cell vs. 2.3 ± 0.3 bacteria/cell, $P < 0.005$, Fig. 2,A). Degrees of adhesive capacity of *Y. pseudotuberculosis* IV B, *Y. enterocolitica* O:3, O:5B and O:9 were higher in the organisms cultivated at 37°C than in those cultivated at 25°C ($P < 0.05$ – 0.005). The plasmid-lack *Y. enterocolitica* O:6 adhered poorly to L-929 cells, and there was no significant difference in the adhesion patterns among the organisms cultivated at 37°C and those cultivated at 25°C (Fig. 2,F). However, the *Y. pseudotuberculosis* attached to L-929 cells at a significantly higher rate than in *Y. enterocolitica* O:3, O:5B and O:9 ($P < 0.05$).

3. Effect of formalin inactivation on adhesive capacity of *Y. pseudotuberculosis* IB (plasmid⁺) and IB mutant (plasmid⁻) to L-929 cells at 37°C.

To elucidate some of the characteristics of the adhesive factors of *Yersiniae*, *Y. pseudotuberculosis* IB and IB mutant were cultivated at 37°C or 25°C and inactivated with formalin, and then examined for their adhesive capacity to L-929 cells at 37°C. *Y. pseudotuberculosis* IB (plasmid⁺) cultivated at 37°C had a higher adhesive capacity even after formalin-inactivation than did the other three formalin-inactivated organisms, which included *Y. pseudotuberculosis* IB (plasmid⁺) cultivated at 25°C and IB mutant (plasmid⁻) cultivated at 37°C or 25°C ($P < 0.005$, Fig. 3). Formalin-inactivated *Y. pseudotuberculosis* IB (plasmid⁺) cultivated at 25°C adhered to L-929 cells at a low rate, like formalin-inactivated IB mutant (plasmid⁻).

The results showed that the adhesive capacity was expressed even after *Y. pseudotuberculosis* IB was inactivated by formalin, and that the adhesive factor of the organism was formalin-resistant and maintained after the lack of viability.

DISCUSSION

This study demonstrated that virulent strains of *Y. pseudotuberculosis* and *Y. enterocolitica* carrying plasmid had high adhesive capacity to L-929 cells when the organisms were grown at 37°C and adhered with L-929 cells at 37°C. This phenomenon was not observed among the plasmid lacking avirulent strains of *Y. pseudotuberculosis* and *Y. enterocolitica*. The adhesive capacities of these strains were low even after the organisms were cultivated at 37°C and adhered with L-929 cells at 37°C. Vesikari *et al.* [15] also reported that the adhesive capacity of *Yersinia* was enhanced when the organisms were adhered with HeLa cells and HEp-2 cells at 37°C as compared with those adhered at

room-temperature. The cultivation-temperature of 22–25°C was reported to cause higher adhesion of *Yersinia* to mammalian cells than that of 37°C [9, 10], which disagreed with our results. If a high level of bacterial adherence is needed for infection of this organism in the host, it may be reasonable to assume that the optimal temperature for this bacterial adherence corresponds to the body temperature of the mammalian host.

The adhesive capacities of *Y. pseudotuberculosis* IB, IV A and IV B were higher than those of *Y. enterocolitica* O:3, O:5B and O:9. Most strains of *Y. pseudotuberculosis* were more pathogenic to the mammalian host than the strains of *Y. enterocolitica* [8]. Thus, the degree of adhesive capacity may reflect the pathogenicity of *Yersinia* spp. strains. This is also supported from the results in that virulence associated plasmid lacking *Y. pseudotuberculosis* IB mutant and *Y. enterocolitica* O:6 showed low adhesive capacity with less pathogenicity.

The adhesive capacity of *Y. pseudotuberculosis* IB (plasmid⁺) cultivated at 37°C was expressed even after inactivation of the organism with formalin. Our results also indicated that the adhesive factors were more efficiently produced during cultivation of the organisms at 37°C than at 25°C, and that production of the factors was closely associated with virulence-associated plasmid. It was reported that enteropathogenic *Escherichia coli* possessed the colonization factor antigen (CFA) associated with plasmid and that the organism could proliferate and colonize in the intestine of the host by the aid of CFA [2, 16]. Recently, it was also reported that in *Yersinia*, the expression of the cell surface properties, such as the charge and hydrophobicity, was associated with the plasmid, growth temperature of 37°C and colonization in the intestine [7]. Therefore, the adhesive factor in this study may have characteristics similar to the cell surface properties in *Yersinia* and CFA in *E. coli*, because

they are associated with the plasmid, enteropathogenicity and/or the growth temperature of 37°C.

The same adhesive capacities of *Yersinia* to fibroblast cells were observed as those in several kinds of epithelial cell lines [1, 12, 14, 15]. Our study demonstrated that the adhesive capacity was greatly affected according to the *Yersinia* species, the presence of plasmid and the cultivation- and adsorption-temperatures. These results suggest that the adhesive capacity of *Yersinia* may be based on the virulence of *Yersinia* strains, and that this adhesive capacity plays a role as a virulence marker of *Yersinia*.

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要 約

L-929 細胞に対する *Yersinia pseudotuberculosis* と *Yersinia enterocolitica* の吸着性：吸着性と病原性プラスミドとの関連，吸着性と菌の増殖温度との関連および吸着における温度の影響：杉山芳宏・高島郁夫・橋本信夫（北海道大学獣医学部獣医公衆衛生学講座）——エルシニア属菌の病原性解析の一環として，培養細胞を用いて感染の第一段階である細胞吸着と病原性との関連性を検討した。エルシニア属菌の細胞吸着性はカルシウム依存性プラスミドの保有により増強された。また 37°C で細胞吸着を行った場合，プラスミドを保有する 37°C 増殖菌の細胞吸着性は 25°C 増殖菌のそれに比べて著しく強かった。しかもこの吸着性は，*Y. pseudotuberculosis* の方が *Y. enterocolitica* よりも高いことからエルシニア属菌の毒力の指標になると判断された。同様の現象はホルマリン不活化菌でも認められ，エルシニア属の 37°C 培養プラスミド保有菌においてホルマリン耐性の吸着増強因子の産生が示唆された。