

# Lack of correlation between haemagglutination and adherence to epithelial cells in *Yersinia pseudotuberculosis*

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**Summary.** *Yersinia pseudotuberculosis* was examined for its haemagglutinating activity and adherence to cultured epithelial cells (HEp-2) in relation to possession of a virulence (VW) plasmid and to growth conditions. VW-lacking (VW<sup>-</sup>) bacteria were isolated from ten VW<sup>+</sup> strains of each serovar which, after they were grown on CFA plates at 37°C, agglutinated the erythrocytes from five different species. In contrast to the bacteria possessing the plasmid (VW<sup>+</sup>) half of the VW<sup>-</sup> bacteria, grown on CFA plates at 37°C, did not agglutinate any of the erythrocytes used and the other half agglutinated only human erythrocytes. Furthermore, when grown on CFA plates at 25°C, neither VW<sup>+</sup> nor VW<sup>-</sup> bacteria showed a haemagglutinating activity. When the bacteria were grown in CFA broth, only two strains grown at 25°C did not agglutinate any of the erythrocytes tested. The VW<sup>+</sup> and VW<sup>-</sup> bacteria of the remaining strains, grown either at 25°C or 37°C, showed relatively high haemagglutinating activity. Adherence to HEp-2 cells did not correlate with haemagglutinating activity in *Y. pseudotuberculosis*; the VW<sup>+</sup> bacteria grown at 37°C adhered to HEp-2 cells more efficiently than either the VW<sup>-</sup> derivatives or the VW<sup>+</sup> bacteria grown at 25°C, regardless of the growth medium. These results indicate that some of the haemagglutinins detected on *Y. pseudotuberculosis* are not involved in the adherence to HEp-2 cells.

## Introduction

Several haemagglutinins and cell surface appendages in *Yersinia* spp. have been detected (MacLagan and Old, 1980; Kapperud and Lassen, 1983; Skurnik, 1984; Kapperud *et al.*, 1985a). The first haemagglutinin associated with fimbriae morphologically similar to the type I fimbriae of other enterobacteria was detected in non-pathogenic *Yersinia* spp. (MacLagan and Old, 1980; Skurnik, 1984) and *Y. pseudotuberculosis* (Skurnik, 1984). This haemagglutinin was expressed when the bacteria were grown at 25°C, but not at 37°C, and agglutinated the erythrocytes from various animal species. A second type of haemagglutinin was detected in *Y. enterocolitica* serovar O10 and was associated with the presence of fimbriae on the cell surface (Aleksic *et al.*, 1976; MacLagan and Old, 1980). This haemagglutinin agglutinated only chicken erythrocytes. The expression of the haemagglutinin was independent of the growth temper-

ature. A third type of haemagglutinin has been detected only in pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* strains and was associated with fine fibrillae on the cell surface (Lachica *et al.*, 1984; Kapperud *et al.*, 1985a). The expression of the haemagglutinin was highly restricted; it was detected when the bacteria were grown in tissue culture media at 37°C but not in brain heart infusion broth nor at 25°C in any culture medium.

The virulence of *Yersinia* spp. has been associated with a (40–50) × 10<sup>6</sup>-mol. wt plasmid (VW plasmid) (Gemski *et al.*, 1980a and b; Ferber and Brubaker, 1981). This plasmid is associated with several virulence-associated characteristics of *Yersinia* spp: production of VW antigens (Ferber and Brubaker, 1981; Straley and Brubaker, 1981 and 1982) and specific outer-membrane proteins (Portnoy *et al.*, 1981; Bolin *et al.*, 1982); cytotoxicity (Portnoy *et al.*, 1981; Vesikari *et al.*, 1981); low LD50 for mice (Gemski *et al.*, 1980a; Portnoy *et al.*, 1981); Ca<sup>2+</sup>-dependent growth at 37°C (Gemski *et al.*, 1980a; Portnoy and Falkow, 1981); and autoagglutination (Laird and Cavanaugh, 1980; Vesikari *et al.*, 1981).

In this paper, we show that some of the haemagglutinating activity does not necessarily

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correlate with the ability to adhere to epithelial cells in *Y. pseudotuberculosis*.

## Materials and methods

### Bacteria

A total of 61 strains of *Y. pseudotuberculosis* of various serovars and origins shown in table I was examined. The VW<sup>-</sup> derivatives were obtained as Ca<sup>2+</sup>-independent derivatives selected on magnesium oxalate agar plates which consisted of Trypticase Soy Broth (BBL, USA), 20 mM MgCl<sub>2</sub>, 20 mM sodium oxalate, and agar (Nakarai, Kyoto, Japan) 1.5% (Higuchi and Smith, 1961).

### Media

CFA broth was prepared according to the method of Evans *et al.* (1977): it comprised Casamino acids (Difco, USA) 1%, Yeast extract (Daigo, Osaka, Japan) 0.15%, MgSO<sub>4</sub> 0.005%, and MnCl<sub>2</sub> 0.0005%. Yeast extract (YE) broth consisted of meat extract (Mikuni, Tokyo, Japan) 2%, Polypepton (Daigo) 1.5%, and Yeast extract 1.5%. CFA and YE agar plates were prepared by adding agar at a concentration of 1.5% to CFA and YE broths, respectively. Nutrient broth and nutrient agar plates were prepared as described previously (Kawaoka *et al.*, 1983). Heart infusion broth and Eagle's Minimal Essential Medium (MEM) were purchased from Nissui (Tokyo, Japan).

### Plasmid DNA isolation

Plasmid DNA was isolated by the method of Portnoy *et al.* (1981).

### Haemagglutination (HA) test

Screening of HA positive strains was performed as described by MacLagan and Old (1980). The bacterial suspension (0.1 ml, 10<sup>9</sup> bacteria) was mixed with 0.1 ml of erythrocyte 1% suspension in a test tube (12 × 105 mm). After incubation for 1 h at 37°C, HA was read.

Titration of the HA activity was performed as follows; the concentration of bacteria was adjusted with PBS to give E<sub>600</sub> 1.4 at a 1 in 20 dilution. After serial two-fold dilution, 25 µl of the bacterial suspension was mixed with 50 µl of erythrocyte 1.5% suspension. HA was read after incubation for 1 h at 37°C. Each strain was tested with the erythrocytes from man (blood groups A, AB, B, O), guinea pig, sheep, cow, and chicken.

The HA inhibition tests in the presence of monosaccharides were performed as follows: the bacterial suspension (25 µl) containing 4 HA units (1 HA unit = the smallest amount of haemagglutinin that gives complete haemagglutination in the HA test system described above) was mixed with 25 µl of monosaccharide 5% solution. After incubation for 1 h at 37°C, 50 µl of erythrocyte 1% suspension was added to it and incubated

for 1 h at 37°C. Phosphate-buffered saline (PBS) (0.01M, pH 7.2) was used as a diluent in all tests.

### Tissue culture infection

The procedure used was a modification of the method of Lee *et al.* (1981). HEp-2 cells were grown in 5 ml of MEM containing newborn calf serum 5% on 22-mm<sup>2</sup> cover slips in 70-mm diameter petri dishes. HEp-2 cells were grown at 37°C in a CO<sub>2</sub> 5% atmosphere. Before infection, the monolayers (approximately 10<sup>6</sup> cells/dish) were washed three times with PBS. They were then infected with 10<sup>8</sup> bacterial cells. After 90 min, the cover slips were rinsed with PBS, fixed in methanol for 5 min, and stained with Giemsa stain for 15 min. The number of bacteria adhering to the epithelial cells was counted by light microscopy (× 1000). In each experiment, 50 cells were examined.

### Cell-surface hydrophobicity

Cell-surface hydrophobicity was determined by the latex particle agglutination (LPA) test of Lachica and Zink (1984b). A 1 in 100 dilution of latex particle suspension (0.81 µm diameter, Difco) was mixed with bacteria on a glass slide. A positive reaction was indicated by immediate agglutination.

### Electronmicroscopy

The bacteria were negatively stained with uranyl acetate 0.5% w/v as described previously (Kawaoka *et al.*, 1982) and were examined for the presence of cell-surface appendages with a Hitachi H-500 transmission electronmicroscope at a gun voltage of 75 kV.

## Results

### Haemagglutination

A total of 61 strains of *Y. pseudotuberculosis* of different serovars and origins (table I) was examined for haemagglutinating activity. All strains grown on CFA plates at 37°C agglutinated erythrocytes from at least one of five different species of animal. Approximately half of the strains grown at 25°C on CFA plates showed HA activity only with guinea-pig, cow, or sheep erythrocytes.

The haemagglutinin of *Y. pseudotuberculosis* was further characterised with 10 representative strains of different serovars which agglutinated all erythrocytes tested and were all VW-plasmid positive. To examine the effect of the presence of the VW plasmid on HA activity, the VW<sup>-</sup> derivatives of these strains were isolated. The presence and absence of the plasmid were confirmed by extraction of the plasmid DNA. When grown at 25°C on

**Table I.** Origins and serovars of *Y. pseudotuberculosis*

Origin of strain	Number of strains of <i>Y. pseudotuberculosis</i> serovar*													
	1a	1b	2a	2b	2c	3	4a	4b	5a	5b	6	7	8	total
Man		2	4	1	3	1		2	2	2	1			18
Rabbit	1		1	1		1				2	1			7
Guinea-pig	1										3			4
Rat							2						1	3
Dog		1		1	1		2	1	1			1		8
Monkey		1						1						2
Cow				1										1
Cat				1	1	1		1						4
Pig						6								6
Duck									1					1
Pigeon			1											1
Unknown	2						1				3			6
Total	4	4	6	5	5	9	5	5	4	4	8	1	1	61

\* The serovars were as defined by Tsubokura *et al.* (1984).

CFA agar plates, none of the strains agglutinated any erythrocytes tested (table II). All of the VW<sup>+</sup> strains grown at 37°C on CFA plates showed relatively strong HA activity (table II). Half of the VW<sup>-</sup> derivatives grown on CFA plates at 37°C did not show HA activity with any of the erythrocytes tested, whereas the other half of the VW<sup>-</sup> derivatives did not show HA activity with erythrocytes

from cow, chicken, guinea-pig, and sheep but retained activity with those from man (table II).

When the bacteria were grown in CFA broth, the majority of the strains (8 out of 10) showed HA activity regardless of the presence or absence of the VW plasmid, though the cells grown at 37°C showed higher HA activity than the cells grown at 25°C (table II). Only two strains showed no HA

**Table II.** HA activity of *Y. pseudotuberculosis* strains grown on CFA agar and in CFA broth at 25°C and 37°C\*

Strain (serovar)	Presence of VW plasmid	Growth temperature (°C)	HA activity with given erythrocytes of cells grown			
			on CFA agar		in CFA broth	
			Human (AB)†	Sheep‡	Human (AB)	Sheep
K260-1 (1b)	+	25	—	—	—	—
1924-1 (4a)	—	37	++	++	++	++
	—		—	—	+++	+++
Endo (2a)	+	25	—	—	+ ~ ++	++
Cow2 (2b)	—		—	—	++	++
Moriya (5a)	+	37	++	+++	+++	+++
	—		—	—	+++	+++
Miyake (2b)	+	25	—	—	++	++
Hayashi (2c)	—		—	—	++	++
TP1040 (3)	+	37	++	++	+++	+++
Pa3593 (4b)	—		+ ~ ++	—	+++	+++
Inoue (5b)	—					

— = <2 units of HA activity; + = 1–8 units; ++ = 16–64 units; +++ = ≥128 units.

\* The bacteria were tested after cultivation for 4 days.

† Similar results were obtained with human A, B, and O erythrocytes.

‡ Similar results were obtained with cow, guinea-pig, and chicken erythrocytes.

activity when grown at 25°C. The dramatic loss of HA activity, detected in the VW<sup>-</sup> derivatives grown on CFA plates, was not observed in the cells grown in CFA broth.

Different growth media were examined for expression of the haemagglutinin of *Y. pseudotuberculosis* with both VW<sup>+</sup> and VW<sup>-</sup> bacteria of the 10 representative strains. The extent to which the media supported the expression of HA was as follows: CFA broth > YE broth > heart infusion broth > nutrient broth. There was no HA observed with the cells grown in MEM containing 10% newborn calf serum at 37°C nor at 25°C, which is contrary to previous findings (Kapperud *et al.*, 1985b).

All monosaccharides detected in animals (galactose, glucose, mannose, fucose N-acetyl galactosamine, N-acetyl glucosamine, and N-acetyl or N-glycolyl neuraminic acid) were tested for their inhibitory activity to HA by *Y. pseudotuberculosis*. No HA inhibition was detected by any of the monosaccharides tested.

Stability of the haemagglutinin of *Y. pseudotuberculosis* to heat treatment was examined. The cells grown on CFA agar plates retained HA activity after incubation for 30 min at 65°C but lost HA activity after incubation at 100°C for 10 min, whereas the haemagglutinin of the cells grown in CFA broth was heat labile; these bacteria lost HA activity after incubation for 10 min at 65°C.

The results indicate that HA activity is commonly detected in *Y. pseudotuberculosis* and that the presence of the VW plasmid is associated with HA activity when the bacteria are grown on CFA plates at 37°C, whereas the effect of the VW plasmid on HA activity is not observed when the bacteria are grown in CFA broth.

#### *Adherence to HEP-2 cell*

To examine the correlation between HA activity and ability of the bacteria to adhere to epithelial cells, the effects of growth conditions and the presence of the VW plasmid on the adhesion of *Y. pseudotuberculosis* were examined with HEP-2 cells. In all strains, the VW<sup>+</sup> parents grown at 37°C had higher adhesive activity than did the VW<sup>-</sup> derivatives or the VW<sup>+</sup> bacteria grown at 25°C (table III). The HA activity of *Y. pseudotuberculosis* did not correlate with adhesion of the bacteria to HEP-2 cells; in spite of the fact that most VW<sup>-</sup> derivatives grown in CFA broth showed as much HA activity as the VW<sup>+</sup> parents did, the adhesive activity of these strains to HEP-2 cells was markedly reduced when the VW plasmid was lost. The

difference in adhesive activity between the cells grown in CFA broth and those grown on CFA plates was not so prominent as the difference in the HA activity.

The results indicate that the VW plasmid of *Y. pseudotuberculosis* encodes the factor(s) involved in the adherence to HEP-2 cells, and that some of the haemagglutinin expressed on the bacterial cells grown in CFA broth at 37°C did not mediate adhesion to HEP-2 cells.

#### *Cell-surface hydrophobicity of Y. pseudotuberculosis*

Cell-surface hydrophobicity was examined by the method of Lachica and Zink (1984b) with latex particles. All VW<sup>+</sup> strains were LPA positive when grown at 37°C either on CFA plates or in CFA broth, whereas most of the strains did not show LPA when they were grown at 25°C. On the other hand, most VW<sup>-</sup> derivatives did not show LPA in any growth condition.

The results indicate that the cell-surface hydrophobicity of *Y. pseudotuberculosis* correlates with the presence of VW plasmid as has been shown in *Y. enterocolitica* (Lachica and Zink, 1984a) and is associated with the adhesion to HEP-2 cells but not with HA activity.

#### *Electronmicroscopic examination*

The ten strains of *Y. pseudotuberculosis* selected for detailed examinations and their VW<sup>-</sup> derivatives were examined for cell surface appendages after growth in different conditions. Fimbriae or fibrillae were not detected on the cell surface of any strains examined.

#### **Discussion**

Haemagglutinating activity was often detected in *Y. pseudotuberculosis* in this study. The effect of the VW plasmid on expression of the haemagglutinin varied with the growth media used; the expression of the haemagglutinin correlated with the presence of the VW plasmid when the bacteria were grown on CFA agar plates but not when the bacteria were grown in CFA broth. In contrast, the adherence of *Y. pseudotuberculosis* to HEP-2 cells correlated with the presence of the VW plasmid regardless of the medium in which the bacteria were grown. These results suggest that some of the haemagglutinin detected on the VW<sup>-</sup> cells grown in CFA broth is not involved in the adherence of *Y. pseudotuberculosis* to HEP-2 cells. This is similar to the findings obtained with *Y. enterocolitica* (Old

**Table III.** Adherence of *Y. pseudotuberculosis* to HEP-2 cells\*

Strain (serovar)	Presence of VW plasmid	Mean number (SD) of adherent bacteria on a HEP-2 cell† when cells were grown at			
		25°C		37°C	
		on CFA agar	in CFA broth	on CFA agar	in CFA broth
K260-1 (1b)	+	38 (13.5)	31 (13.3)	74 (32.3)	48 (22.3)
	—	7 (5.7)	2 (2.3)	2 (4.8)	3 (2.3)
Endo (2a)	+	20 (5.6)	25 (7.1)	32 (11.4)	38 (9.7)
	—	7 (3.9)	8 (3.5)	17 (6.0)	11 (4.2)
Cow2 (2b)	+	37 (16.7)	37 (13.5)	44 (13.1)	57 (20.2)
	—	9 (7.3)	8 (6.2)	12 (6.9)	15 (7.9)
Miyake (2b)	+	18 (7.6)	15 (6.1)	32 (12.6)	40 (12.4)
	—	4 (3.2)	3 (4.2)	10 (4.7)	7 (4.2)
Hayashi (2c)	+	28 (8.4)	23 (10.3)	58 (10.4)	64 (20.3)
	—	3 (5.3)	7 (4.5)	20 (8.5)	25 (7.8)
TP1040 (3)	+	31 (8.7)	43 (10.4)	80 (28.8)	73 (16.8)
	—	9 (3.6)	16 (6.2)	26 (7.3)	21 (7.1)
1924-1 (4a)	+	28 (9.1)	33 (14.8)	45 (15.9)	40 (15.9)
	—	10 (4.5)	2 (2.3)	15 (7.3)	7 (3.9)
Pa3593 (4b)	+	22 (5.9)	24 (8.7)	31 (9.3)	59 (20.0)
	—	3 (3.1)	14 (7.9)	9 (8.5)	18 (6.3)
Moriya (5a)	+	22 (9.9)	32 (15.1)	45 (12.1)	67 (26.1)
	—	6 (4.6)	6 (5.0)	11 (5.9)	12 (5.3)
Inoue (5b)	+	21 (5.0)	11 (4.9)	33 (6.9)	33 (7.1)
	—	4 (2.9)	1 (0.8)	11 (3.9)	5 (3.2)

\* The bacteria were tested after cultivation for 4 days.

† The bacteria which invaded in the cells were also included.

and Robertson, 1981); the haemagglutinins of *Y. enterocolitica* do not necessarily explain adherence to HEP-2 cells. However, we should not conclude that the haemagglutinin of *Y. pseudotuberculosis* is unimportant for *Y. pseudotuberculosis* infection, because HEP-2 cells may not necessarily represent the cells involved in infection *in vivo*. Other cells should also be examined.

At least two different haemagglutinins have been reported in *Y. pseudotuberculosis*. Kapperud *et al.* (1985a) found a haemagglutinin and very fine fibrillae on the cell surface of *Y. pseudotuberculosis* grown in tissue culture media at 37°C but not at 25°C. This haemagglutinin is associated with the presence of the VW plasmid and agglutinates only guinea-pig erythrocytes. The haemagglutinin of *Y. pseudotuberculosis* reported by Skurnik (1984) is expressed at 25°C either on nutrient agar or in nutrient broth and is associated with thin or thick fimbriae or both. Although these haemagglutinins differ from the one detected in the present study in

their specificity for different erythrocytes, the presence of appendages on the cell surface, and the effect of the VW plasmid and growth condition, these differences could be due to slight differences in the growth conditions.

The effect of the presence of the VW plasmid on HA activity differed between the cells grown on CFA plates and in CFA broth and the stability of the haemagglutinins to heat differed between the cells grown on CFA agar plates and those grown in CFA broth. Furthermore, different HA patterns were observed when the bacteria were grown on CFA agar, showing heterogeneity of the haemagglutinin of *Y. pseudotuberculosis*. Another possibility is that the difference was a consequence of quantitative differences of the haemagglutinin in response to the different growth conditions.

The adherence of *Yersinia* spp. to various epithelial cells has been investigated. Some *Y. enterocolitica* adhere to epithelial cells when grown at 25°C but not at 37°C (Okamoto *et al.*, 1980; Old

and Robertson, 1981) and some adhere equally well after cultivation at 25°C or at 37°C (Lee *et al.*, 1981; Old and Robertson, 1981). Because the VW<sup>-</sup> derivatives showed adhesive ability with epithelial cells (Portnoy *et al.*, 1981; Schiemann, 1981; Heesemann *et al.*, 1983), the plasmid had not been considered to be involved in the adherence of pathogenic *Y. enterocolitica*. Heesemann *et al.* (1984), however, reported that non-pathogenic *Y. enterocolitica* acquired the ability to adhere to epithelial cells after acquisition of the VW plasmid from pathogenic *Y. enterocolitica*. The VW plasmid, therefore, appears to encode the factor(s) involved in the adherence of *Y. enterocolitica* to epithelial cells. Bölin *et al.* (1982) reported that *Y. pseudotuberculosis* grown at 25°C adhere to HeLa cells to a greater extent than the cells grown at 37°C and that the VW plasmid was not involved in this adherence. These results differ from those obtained in our study. As Bölin *et al.* (1982) examined only one strain, a strain similar to the one that Bölin *et al.* used might not have been included in our study and *Y. pseudotuberculosis* may be heterogenous in dependency of the adherence to epithelial cells on growth temperature and the presence of the VW plasmid.

Cell-surface hydrophobicity correlated with the adherence to HEp-2 cells but not with the HA activity of *Y. pseudotuberculosis* cells grown in CFA broth. Cell-surface hydrophobicity seems to originate from the specific outer-membrane proteins encoded by the VW plasmid and contributes to

autoagglutination in *Y. enterocolitica* (Martinez, 1983; Lachica and Zink, 1984a; Lachica *et al.*, 1984). The haemagglutinin detected in pathogenic *Y. enterocolitica* was expressed on the cell surface of the VW<sup>+</sup> bacteria grown at 37°C and was, therefore, considered to confer hydrophobic property on the cells (Lachica *et al.*, 1984a). The haemagglutinin associated with fimbriae detected in non-pathogenic *Y. enterocolitica* strains with cell-surface hydrophobicity (Kihlstrom and Magnusson, 1983). In contrast, the haemagglutinin expressed on *Y. pseudotuberculosis* grown in CFA broth did not confer hydrophobicity to the cells.

The VW plasmid encodes specific outer-membrane proteins expressed at 37°C but not at 25°C. One of these proteins, P1, is involved in serum resistance and autoagglutination of *Y. enterocolitica* (Skurnik *et al.*, 1984; Balligand *et al.*, 1985). Kapperud *et al.* (1985a) suggested that one of these outer-membrane proteins, HMWP (probably identical to P1) was fine fibrillae and was involved in HA. There are at least eight different outer-membrane proteins encoded by the VW plasmid and expressed at 37°C but not at 25°C. The haemagglutinin detected on the VW<sup>+</sup> cells grown on CFA agar plates at 37°C could be one of these proteins. Studies are in progress to identify and purify the haemagglutinin(s) of *Y. pseudotuberculosis* detected in this study.

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