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Vwa$^+$ Phenotype of *Yersinia enterocolitica*†

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Expression of the Vwa$^+$ phenotype of *Yersinia pestis* in vitro is known to reflect maximum induction of virulence (or V and W antigens) at 37°C with concomitant restriction of cell division. Both phenomena are potentiated by 20 mM Mg^{2+} and prevented by cultivation at 26 or 37°C with 2.5 mM Ca^{2+}. We have now compared this classic plasmid-mediated phenotype with those of Vwa$^+$ *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* which, unlike *Y. pestis*, produce ancillary outer membrane peptides unrelated to the V and W antigens. All of 10 wild-type strains of *Y. enterocolitica* (serotypes O:3, O:4,32, O:8, O:9, O:15, and O:21) exhibited a nutritional requirement for Ca^{2+} at 37°C and produced significant V antigen. Like *Y. pseudotuberculosis*, autoagglutination of Vwa$^+$ *Y. enterocolitica* was dependent upon prior growth at 37°C but was not influenced by Ca^{2+}. Autoagglutination of *Y. pestis* was never observed. Resistance of *Y. enterocolitica* to 10% human serum was typically dependent upon prior growth at 37°C, either with or without added Ca^{2+}, and carriage of a Vwa plasmid. In contrast, serum resistance of *Y. pseudotuberculosis* was temperature but not plasmid dependent and that of *Y. pestis* was constitutive.

Many of the more severe bacterial diseases of man are caused by facultative intracellular parasites (38). *Yersinia pestis*, the causative agent of bubonic plague, is considered the type species of this group by virtue of its classical role in identifying virulence factors required for extracellular growth (8, 10). However, the most important determinant of this species is the ability to promote maximum expression (9) of the V and W (or virulence) antigens (Vwa$^+$) of Burrows and Bacon (11), a property possibly required for intracellular survival. Mutation to Vwa$^-$ in *Y. pestis* results in a 10^6- to 10^7-fold increase in the 50% lethal dose in experimental animals (10).

V antigen is a 90-megadalton (mDal) protein and W antigen is a 140 mDal lipoprotein (35). Their synthesis is coordinately induced at 37 but not 26°C in synthetic medium simulating intraleukocytic fluid with respect to Ca^{2+} (not added) and Mg^{2+} (~20 mM). This environment selectively prevents in vitro division of Vwa$^+$ cells; addition of that concentration of Ca^{2+} present in plasma (~2.5 mM) permits growth while coordinately repressing the V and W antigens (8). Recent work showed that restriction promoted by Ca^{2+} deprivation is a consequence of an ordered stepdown of macromolecular synthesis initiated by reduction of adenylate energy charge and shutoff of stable RNA synthesis (16, 51); these reactions were not mediated by regulatory MS nucleotides (16). The first detected event (50) in this sequence was accumulation of cytoplasmic V antigen (44, 45). The origin of lipoidal W antigen has not been determined, although a source within the envelope seems probable. *Y. pestis* does not produce detectable Vwa$^+$-dependent ancillary outer membrane peptides (44, 45).

At 37°C, wild-type cells of *Yersinia pseudotuberculosis* are also Vwa$^+$ (6, 12), produce ancillary outer membrane peptides (6, 46), and may undergo spontaneous autoagglutination (6). Although only highly virulent *Yersinia enterocolitica* WA has yet been shown to be Vwa$^+$ (14), wild-type cells of this species generally exhibit the attendant nutritional requirement for Ca^{2+} (43), produce ancillary outer membrane peptides (5, 20, 40), and undergo autoagglutination (34). Dependence upon a 41- to 45-mDal plasmid associated with expression of Ca^{2+} dependence was first shown for these species (23, 24); this result was later extended to *Y. pestis* (4, 21). Recent findings indicate that induction of a soluble antigen of *Y. enterocolitica* that is probably related to the ancillary outer membrane peptides (M. P. Doyle, personal communication) is temperature dependent but, unlike the V and W antigens, not significantly influenced by Ca^{2+} (20). In addition, the Vwa$^+$ phenotype of *Y. enterocolitica* was reported to promote damage to cultured mammalian cells (40), adhesion to cultured cells (47), and resistance to the

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antibacterial action of serum (39). The purpose of this report is to define the Vwa$^+$ phenotype of *Y. enterocolitica* in context with that already characterized for *Y. pestis*.

MATERIALS AND METHODS

Bacteria. A nonpigmented (32) but otherwise virulent isolate of *Y. pestis* KIM (7), wild-type *Y. pseudotuberculosis* PB1/+ (12), and virulent *Y. enterocolitica* WA (13) were used as Vwa$^+$ type strains. Salient features of these and other yersiniae used in this study are shown in Table 1. Among the other strains, *Y. pestis* EV76 has been used in many contexts (8, 10), and growth of *Y. pseudotuberculosis* MD31 in cultivated cells has been described (41).

Cultivation. Bacteria stored at −20°C in buffered glycerol (3) were inoculated onto slopes of tryptose blood agar base (Difco Laboratories, Detroit, Mich.) and incubated at 26°C for 24 h (*Y. pseudotuberculosis* and *Y. enterocolitica*) or 48 h (*Y. pestis*). Organisms were removed in 0.033 M potassium phosphate buffer, pH 7.0 (phosphate buffer), and inoculated into a chemically defined liquid medium (50). Two transfers were made in this medium, aerated in Erlenmeyer flasks (10% [vol/vol]), before inoculation of cultures used in experiments.

Calcium dependence. Nutritional dependence upon Ca^{2+} at 37°C was determined by plating on tryptose blood agar base supplemented with 0.02 M sodium oxalate and 0.02 M $MgCl_2$ (29). When differences in colony size of *Y. enterocolitica* on this medium were insufficient to isolate Vwa$^-$ mutants, a defined solid selective medium was utilized. This medium contained the inorganic salts, vitamins, and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer component of Zahorchak and Brubaker (50), 20 mM $MgCl_2$, 12.5 mM L-glutamic acid, 10 mM potassium gluconate, 1.0 mM L-methionine, 1.0 mM L-threonine, 1.0 mM L-isoleucine, 1.0 mM L-valine, and 0.85% Ionagar no. 2 (Oxoid Ltd., Sheffield, England); the medium was adjusted to pH 7.0 with NaOH.

Plasmids. Native plasmid DNA was prepared by the procedure of Casse et al. (15) and electrophoresed as described previously (21). This method was insufficient to routinely reveal the second larger Vwa plasmids (~80 mDal) reported by Kay et al. (33).

Pesticin. Homogenous pesticin was prepared by the method of Hu and Brubaker (31) and assayed by the procedure used by Ferber et al. (22).

V antigen biosynthesis. V antigen was assayed after aeration of the bacteria for 6 h in chemically defined medium (50) after their inoculation at an optical density (at 620 nm) of 0.1. The cells were harvested by centrifugation (40,000 × *g* for 10 min), suspended in 0.05 M Tris-hydrochloride buffer, pH 7.8, and disrupted by sonification for 1 min. After removal of debris by centrifugation, the resulting extracts were assayed for V antigen by gel diffusion, using monospecific antisera (35); one unit is defined as the least amount capable of yielding a visible band of precipitate. Results are presented in terms of specific activity, defined as units per milligram of protein. The latter was determined by the method of Lowry et al. (36). Sufficient protein was present in all assays to detect V antigen at a specific activity of 0.05.

Autoagglutination. The ability of all yersiniae to undergo spontaneous agglutination was determined as described previously for *Y. pseudotuberculosis* (6). The method involved growing the organisms in a medium composed of 0.8% casitone (Difco), the salt solution of Higuchi and Carlin (28), 2.5 mM $CaCl_2$, and 10 mM D-glucose; after preparation, the medium was adjusted to pH 7.0 with NaOH. After growth into late-log phase, the cells were harvested by centrifugation (40,000 × *g* for 10 min), suspended at an optical density (at 620 nm) of about 10 in phosphate buffer, and observed for autoagglutination at room temperature.

Serum sensitivity. The ability of pooled normal human sera to kill yersiniae was determined by the method of Pai and DeStephano (39), except that phosphate buffer was substituted for gelatin in Hanks balanced salt solution. Serum was stored at −70°C and thawed before dilution at a final concentration of 10% (vol/vol). Suspensions of bacteria in phosphate buffer either with or without serum were incubated at 37°C with gentle aeration, and samples were removed at appropriate intervals for determination of viability.

RESULTS

In preliminary studies, V antigen was detected in all of 15 tested wild-type isolates of *Y. enterocolitica*. Of these, five strains of serotype O:3 and five isolates of other serotypes were retained for further study. Also included in this study were known Vwa$^+$ strains of *Y. pestis* and *Y. pseudotuberculosis*. Isogenic Vwa$^-$ mutants of all yersiniae were obtained by selection for Ca^{2+} independence at 37°C, and the presence of plasmids in these mutants and their parents was determined. In every case, loss of a 41- to 45-mDal plasmid was associated with mutation to Ca^{2+} independence (Table 1). These isogenic pairs were then characterized with regard to V antigen production, autoagglutination, sensitivity to serum, and inhibition by pesticin.

V antigen production. Regardless of species, all Ca^{2+}-dependent strains produced V antigen when aerated without Ca^{2+} for 6 h at 37°C (Table 1). The specific activity of the *Y. enterocolitica* serotype O:9 strain was unusually low, the serotype O:15 isolate produced detectable V antigen at both 26 and 37°C, and one serotype O:3 strain also produced some V antigen at 37°C with 4.0 mM Ca^{2+}. The antigen was never detected in cells grown at 26°C with 4.0 mM Ca^{2+}.

Autoagglutination. Autoagglutination of Vwa$^+$ *Y. pseudotuberculosis* and *Y. enterocolitica* usually occurred after growth at 37°C with Ca^{2+} and was sometimes observed in cells grown at 26°C (Table 1). Both Vwa$^+$ strains of *Y. pseudotuberculosis* and the isogenic set of Vwa$^+$ and Vwa$^-$ *Y. enterocolitica* C35 underwent autoagglutination after growth at 26 but not 37°C. Cells of *Y. pestis* never exhibited autoagglutination; this observation was extended to 25 additional Vwa$^+$ isolates of this species.

Sensitivity to serum. Vwa$^+$ cells of *Y. entero-*

TABLE 1. Some properties associated with expression of the temperature-dependent requirement of yesiniae for Ca^{2+}

Species	Strain[a]	Variety or serotype[b]	Ca^{2+} requirements[c]	Plasmids detected (mDa1)[d]	V antigen (sp. act.)[e]	Autoagglutination after growth at:[f]	
						26°C	37°C
Y. pestis	EV76	*orientalis*	+	6, 42, 65	2.6	0	0
			0	6, 65	0	0	0
	KIM	*mediaevalis*	+	6, 42, 65	2.0	0	0
			0	6, 65	0	0	0
Y. pseudotuberculosis	PB1	IB	+	45	1.5	+	+
			0	0	0	0	0
	MD31	III	+	42	2.7	+	0
			0	0	0	0	0
Y. enterocolitica	E675	O:3	+	48	1.2	0	+
			0	0	0	0	0
	C32	O:3	+	41	0.3	0	0
			0	0	0	0	0
	C33	O:3	+	41	2.0	0	+
			0	0	0	0	0
	C34	O:3	+	41	0.07	0	+
			0	0	0	0	0
	C35	O:3	+	45	3.8 (0.07)	+	+
			0	0	0	+	+
	E701	O:4, 32	+	43	3.2	0	+
			0	0	0	0	0
	WA	O:8	+	41	2.0	0	+
			0	0	0	0	0
	E705	O:9	+	45	0.8	0	+
			0	0	0	0	0
	C36	O:15	+	45	1.5 (0.7)	0	+
			0	0	0	0	0
	E736	O:21	+	43	1.7	0	+
			0	0	0	0	0

[a] Strains E675, E701, E705, and E736 were obtained from D. A. Schiemann and are of North American origin; strains C32 to C36 are European isolates received from A. Mellado.

[b] *Y. pestis* biotypes are from Devignat (19); serotypes of *Y. pseudotuberculosis* and *Y. enterocolitica* were as defined by Wauters et al. (49) and Thal and Knapp (46), respectively.

[c] Determined at 37°C on magnesium oxalate agar (29).

[d] Plasmid sizes determined as previously described (21, 45).

[e] Expressed as units (see text) per milligram of protein after 6 h of growth at 37°C without Ca^{2+}. Parentheses indicate V antigen titer after 6 h of growth at 37°C with 4.0 mM Ca^{2+} (strain C35) or at 26° without Ca^{2+} (strain C36); production at 26°C with 4.0 mM Ca^{2+} was never detected.

[f] +, Significant autoagglutination by 30 min; 0, no agglutination.

colitica grown at 37 but not 26°C were resistant to the antibacterial activity of 10% serum (Table 2). Serum resistance in this species, however, was not dependent upon actual induction of the Vwa^+ phenotype by starvation for Ca^{2+} at 37°C. In contrast, serum resistance in *Y. pseudotuberculosis* was associated with prior growth at 37°C but not upon ability to produce virulence antigens (Fig. 1; Table 2). Serum resistance of *Y. pestis* was constitutive in the sense that it was neither temperature nor plasmid dependent (Table 2).

Sensitivity to pesticin. With a preparation of pesticin containing 60,000 U/ml when assayed against *Y. pseudotuberculosis* PB1, cells of *Y. enterocolitica* WA serotype O:8 and the serotype O:21 isolate proved to be 30 times less sensitive. The serotype O:4,32 isolate was 60 times less sensitive, and the remaining isolates of *Y. enterocolitica*, including the highly virulent serotype O:3 strains, were not significantly inhibited. No differences in resistance to pesticin were observed between isogenic Vwa^+ and Vwa^- pairs.

DISCUSSION

The results of this study showed that all tested wild-type strains of *Y. enterocolitica* produced V antigen while undergoing restriction in Ca^{2+}-deficient medium. This finding indicates that

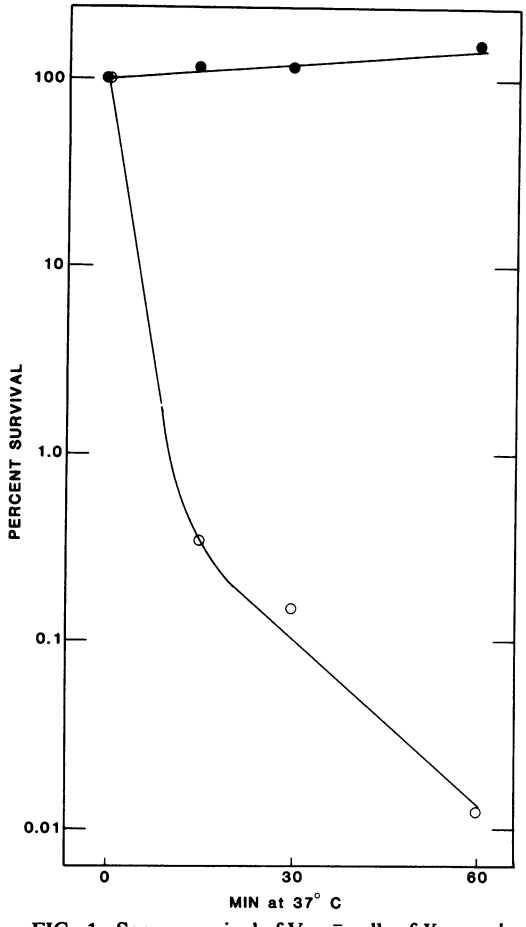

FIG. 1. Serum survival of Vwa$^-$ cells of *Y. pseudotuberculosis* PB1. Cells were incubated at 37°C in 10% (vol/vol) serum after 6 h of growth with 4.0 mM Ca^{2+} at 26 (○) and at 37°C (●).

wild-type *Y. enterocolitica*, like *Y. pestis* and *Y. pseudotuberculosis*, is Vwa$^+$ and thus shares a common mechanism of virulence. However, further study showed a number of important differences in the Vwa$^+$ phenotype of *Y. pseudotuberculosis* and *Y. enterocolitica* when compared with that previously established for *Y. pestis*. An understanding of these differences may be requisite to defining mechanisms used by *Y. pseudotuberculosis* and *Y. enterocolitica* to colonize the gut as opposed to mechanisms of *Y. pestis* involved in promoting systemic disease.

One distinction shown in this report was the inability of Vwa$^+$ cells of *Y. pestis* to undergo autoagglutination regardless of growth temperature or the presence of exogenous Ca^{2+}. This result is contrary to the findings of Laird and Cavanaugh (34) but consistent with the notion that autoagglutination is correlated with expression of a hydrophobic outer membrane component (6). Further study may show that this component is associated with or identical to the ancillary outer membrane peptides of *Y. pseudotuberculosis* and *Y. enterocolitica*. Even if *Y. pestis* could produce these structures, the presence of antigen 4 and especially capsular fraction 1 would be sufficient to maintain the bacteria in suspension (17). These findings indicate a loose correlation between autoagglutination and carriage of Vwa plasmids by *Y. enterocolitica* and *Y. pseudotuberculosis* and no relationship between autoagglutination and expression of virulence antigens.

Sensitivity to normal human serum provided another major difference among Vwa$^+$ phenotypes. Serum resistance in *Y. enterocolitica* was always temperature and Vwa plasmid dependent, in accord with the initial findings of Pai and DeStephano (39), but was independent of prior growth under restrictive conditions as claimed by these authors. In contrast, serum resistance of *Y. pseudotuberculosis* was temperature dependent but Vwa plasmid independent, and that of the *Y. pestis* strains examined was constitutive. Further work will be required to define the nature of serum sensitivity in yersiniae, which is probably mediated by complement (39; unpublished data). Nonspecific fixation of complement to gram-negative bacteria can often be prevented by lipopolysaccharide (2, 25, 37), but little is known about the structures that account for serum resistance. In this context, it is probably significant that the predominant sugars in O-groups of many *Y. enterocolitica* serotypes are deoxyhexoses (30, 48), whereas those of *Y. pseudotuberculosis* contain even more hydrophobic dideoxyhexoses (18, 42); profound temperature-dependent changes in *Y. enterocolitica* O-group content have been described (1, 27).

Strains of the more virulent *Y. enterocolitica* serotypes have been reported to be especially sensitive to pesticin (26). A correlation between virulence and inhibition by homogenous pesticin was not observed in this study. Of interest, however, was the finding that the standard *Y. pseudotuberculosis* indicator strain PB1 was 30 times more sensitive to the bacteriocin than were the most sensitive *Y. enterocolitica* isolates (serotypes O:8 and O:21).

A major finding of this study was that only synthesis of V antigen (and thus presumably W antigen) was temperature dependent and repressed by Ca^{2+} in all species of yersiniae. Autoagglutination and serum resistance of *Y. pseudotuberculosis* and *Y. enterocolitica* was temperature dependent but not influenced by exogenous Ca^{2+}. Serum resistance was constitutive in *Y. pestis*, which did not undergo autoagglutination. It is now recognized that expression of the ancillary outer membrane peptides of *Y.*

TABLE 2. Serum sensitivity of Vwa^+ and Vwa^- yersiniae after 6 h of growth with or without Ca^{2+} at 26 and 37°C

Species	Strain	Vwa	Percent survival (1 h in 10% serum at 37°C)			
			Prior growth at 26°C		Prior growth at 37°C	
			Without Ca^{2+}	4.0 mM Ca^{2+}	Without Ca^{2+}	4.0 mM Ca^{2+}
Y. pestis	EV76	+	67	46	79	65
		0	61	52	93	79
	KIM	+	39	24	43	19
		0	13	15	36	57
Y. pseudotuberculosis	PB1	+	1.2	0.7	239	235
		0	1.9	3.7	120	113
	MD31	+	6.6	0.09	128	154
		0	1.5	0.5	180	114
Y. enterocolitica	E675	+	ND[a]	ND	ND	ND
		0	<0.01	0.3	0.04	0.1
	C32	+	<0.01	0.01	86	93.5
		0	0.02	0.09	0.45	0.5
	C33	+	0.13	<0.01	44	71.3
		0	<0.01	0.02	0.6	0.27
	C34	+	0.2	1.6	0.8	9.4
		0	0.2	0.2	<0.01	<0.01
	C35	+	9.3	0.6	125	292
		0	0.1	0.2	31	42
	E701	+	11.0	6.1	207	231
		0	0.2	0.3	0.03	0.04
	WA	+	0.03	0.05	95	450
		0	0.01	0.01	0.04	0.01
	E705	+	0.6	0.2	74	1.4
		0	0.02	0.01	0.04	0.01
	C36	+	42	0.07	180	100
		0	0.03	0.01	0.1	0.08
	E736	+	0.03	0.04	67	167
		0	0.02	0.04	0.05	0.01

[a] ND, Not determined due to erratic growth at 37°C in synthetic medium.

enterocolitica is also temperature dependent but a related soluble antigen is not repressed by Ca^{2+} (20) and that the ancillary outer membrane peptides of *Y. pseudotuberculosis* are only "moderately" inhibited by Ca^{2+} (5). The possibility thus exists that the ancillary outer membrane peptides per se promote Ca^{2+}-independent autoagglutination, host cell adhesion, and host cell damage in tissue culture. They probably do not influence serum sensitivity since both Vwa^+ and Vwa^- *Y. pseudotuberculosis* are resistant to serum after growth at 37°C. In conclusion, it seems probable that definition of the role of Vwa plasmids in promoting disease in *Y. pseudotuberculosis* and *Y. enterocolitica* will depend upon distinguishing between effects mediated by their unique ancillary outer membrane peptides and those caused by V and W antigens held in common with *Y. pestis*. Expression of the former is temperature dependent but evidently not regulated by Ca^{2+}, as opposed to production of virulence antigens, which is dependent upon both variables.

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