

Correlation of the Suicide Phenomenon in *Aeromonas* Species with Virulence and Enteropathogenicity

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Certain strains of mesophilic aeromonads (*Aeromonas hydrophila*, *A. sobria*, and *A. caviae*), when grown in broth containing 0.5% glucose, undergo growth inhibition concomitant with acetate accumulation. Because these strains are nonviable after 24 h, this phenomenon is termed suicide. We investigated suicidal strains of *Aeromonas* species as a means of understanding animal virulence and enteropathogenicity. To assess virulence, batches of five white mice were inoculated intraperitoneally with 10^7 cells (washed) of suicidal and nonsuicidal strains of *A. hydrophila* and *A. sobria* and suicidal strains of *A. caviae*. The three nonsuicidal strains of *A. sobria* tested showed lethality as early as 12 h and were uniformly fatal within 36 h postinoculation. After 36 h, the three suicidal strains killed only 1 of 15 mice inoculated. Four *A. hydrophila* strains tested which showed the suicide phenomenon at 37°C were variably lethal (40 to 100%). None of three suicidal strains of *A. caviae* were lethal. Enteropathogenicity was studied by orally inoculating three white mice each with the same *Aeromonas* strains (10^8 cells, in skim milk) and assessing diarrhea and intestinal fluid accumulation. Diarrhea and fluid accumulation were present in all mice inoculated with two nonsuicidal strains of *A. sobria* and in 4 of 12 mice given four suicidal strains of *A. hydrophila*. Two suicidal strains each of *A. sobria* and *A. caviae* failed to elicit any gastrointestinal disturbances. These data suggest that the suicide phenomenon may explain strain-specific (*A. sobria* and *A. hydrophila*) and species-specific (*A. caviae*) virulence and enteropathogenicity.

Mesophilic *Aeromonas* species (*A. hydrophila*, *A. sobria*, and *A. caviae*) have been the focal point of intensive investigation regarding their roles as gastrointestinal tract pathogens. Despite numerous studies in which various extracellular products such as cytotoxic (6, 16) and cytotoxic enterotoxins (22) and hemolysins (3, 21) have been identified, controversy still exists regarding *Aeromonas* enteropathogenicity.

Contradictory reports have appeared as to whether or not all three mesophilic aeromonads are enteric pathogens. For instance, while several studies support the role of only *A. hydrophila* and *A. sobria* as intestinal pathogens (7, 24), others have also advanced *A. caviae* as a cause of diarrheal disease (2, 9, 19).

To sort out this dilemma, various investigators have probed the virulence potential of mesophilic aeromonads by assessing mouse pathogenicity by oral and intraperitoneal administration (4, 5, 11, 12) and by correlating the human diarrheagenic potential of *A. hydrophila* and *A. sobria* strains with virulence-associated phenotypic markers (4, 24). Results of these studies, however, have been equally dichotomous. Animal virulence studies have revealed a strain-specific lethality for *A. sobria* and *A. hydrophila* and a lack of virulence among *A. caviae* strains (4, 11), and no correlation could be established between phenotypic markers of virulence and human enteropathogenicity (14, 18).

To date, no unifying attribute of mesophilic aeromonads has been uncovered to account for the discrepant biologic activities which have been noted. However, what has been overlooked in all of these endeavors is the role of the suicide phenomenon in *Aeromonas* species (H. Namdari, Ph.D. thesis, University of Rhode Island, Kingston, 1987), which could account for the apparent lack of concordance among various investigations. We propose that this acetic acid-induced self-killing phenomenon among mesophilic aeromo-

nads, which is expressed in vitro and probably in vivo, accounts for the numerous dichotomous observations regarding species-specific and strain-specific human enteropathogenicity and animal virulence properties of *Aeromonas* species.

MATERIALS AND METHODS

The 26 *Aeromonas* strains tested were recovered from clinical sources ($n = 12$) or freshwater environments ($n = 14$), and their species were determined by the criteria of Janda et al. (13). Among the properties for which we tested were aerogenicity, acetylmethylcarbinol production (Voges-Proskauer [VP]), fermentation of salicin, esculin, and arabinose, and hemolytic activity on Trypticase soy agar (BBL Microbiology Systems) supplemented with 5% sheep blood. Thirteen of these strains, twelve of which were from human sources, were selected for further study.

The suicidal capacity of each isolate was determined by inoculation of duplicate tubes of Nutrient Broth (Difco Laboratories) containing 0.5% glucose followed by incubation of individual tubes at 30 or 37°C for 24 h. Strains showing the suicide phenomenon spontaneously pelleted, while those lacking this characteristic showed uniform broth turbidity. Suicidal activity was further confirmed by subculturing the pelleted organisms to sheep blood agar.

To assess the role of the suicide phenomenon in *Aeromonas* species as a factor in species- and strain-specific virulence, white mice were challenged by the intraperitoneal and oral routes with suicidal and nonsuicidal *Aeromonas* strains. For the former, groups of five 3-month-old Swiss mice were injected with individual 0.1-ml saline suspensions containing 10^8 CFU of suicidal or nonsuicidal strains of *A. sobria* ($n = 6$), *A. hydrophila* ($n = 4$), or *A. caviae* ($n = 3$) per ml. Suspensions were prepared by harvesting the 16-h growth (35°C incubation) from glucose-free Trypticase soy agar containing 5% sheep blood in sterile saline and washing the cells three times. The cell density was adjusted to 10^8 CFU/

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TABLE 1. Salient characteristics of 13 *Aeromonas* strains studied for animal virulence^a

Species and strain no.	Source	Suicidal characteristics at ^b :		Sheep blood hemolysis	VP	Esculin
		37°C	30°C			
<i>A. sobria</i>						
14, 171, 206	Blood, stool	NS	NS	+	+	-
181, 228, 176	Ulcer, colon, peritoneal fluid	S	S	+	+	-
<i>A. hydrophila</i>						
85, 9902	Environment, blood	S	NS	+	+	+
223, 222	Bullae, stool	S	NS	+	+	+
<i>A. caviae</i>						
199, 251, 194	Stool, lung	S	S	-	-	+
	Unknown	S	S	-	-	+

^a Additional characteristics include anaerogenic fermentation of glucose (*A. caviae*) and hydrogen sulfide production from cysteine (*A. sobria* and *A. hydrophila*).

^b NS, Nonsuicidal; S, suicidal.

ml (0.38 optical density units at 435 nm) and confirmed by colony count. Mice were observed for 14 days. Animals that died were subjected to postmortem analysis, and cultures of heart blood and various internal organs were aseptically prepared. Blood cultures from surviving mice were obtained by cardiac puncture at 48 h and after 14 days. Mice inoculated with 0.1 ml of sterile saline served as controls.

To determine the gastrointestinal effect of oral administration of suicidal or nonsuicidal *Aeromonas* strains, bacterial suspensions containing 10⁹ CFU were prepared in skim milk. Groups of three *Aeromonas*-free (as determined by stool culture) Swiss mice were deprived of water for 24 h and inoculated orally with 0.1 ml of the skim milk suspension by inserting a cut-off 18-gauge needle attached to a tuberculin syringe into each animal's mouth, from which they drank freely. Postinoculation, the animals were separated and observed for 14 days for evidence of diarrhea, duration of *Aeromonas* excretion, and mortality. Duration of excretion was determined by collecting fecal samples at 6, 18, 24, and 48 h. Stools were emulsified in sterile saline and plated onto *Aeromonas* differential medium (20). Animals which died in the course of infection or sacrificed animals (those showing evidence of diarrhea) were surgically examined for intestinal fluid accumulation; in addition, organ and blood cultures were prepared as described above. Animals inoculated with skim milk alone served as controls.

RESULTS

Table 1 lists the suicidal characteristics of the 13 strains of *A. sobria*, *A. hydrophila*, and *A. caviae* correlated with two phenotypic markers (hemolytic activity and VP reaction) of virulence (1, 7). *A. sobria* and *A. hydrophila* were hemolytic and VP positive, whereas *A. caviae* was nonhemolytic and VP negative.

A direct correlation was observed between the suicidal capacity of *A. sobria* and *A. caviae* and mouse virulence irrespective of the route of inoculation. By the intraperitoneal route, the three nonsuicidal strains of *A. sobria* killed 14 of 15 (93%) of inoculated mice within 36 h; 4 mice were dead as early as 12 h postinoculation (Table 2). In all instances, organisms were easily visible in touch preparations of the cut surface of internal organs and in Gram-stained smears of heart blood. In contrast, the three suicidal strains of *A. sobria* were virtually avirulent, killing only 1 of 15 (6.6%) intraperitoneally inoculated mice. Necropsy cultures performed on surviving mice sacrificed 48 h and 14 days postinoculation were negative for the suicidal strains. The three *A. caviae* strains which were suicidal at 37 and 30°C were avirulent for white mice and could not be recovered from internal organs and heart blood of mice sacrificed after 48 h.

TABLE 2. Mouse virulence of suicidal and nonsuicidal strains of *Aeromonas* species as assessed by intraperitoneal inoculation

Species	Strain no.	Suicidal characteristics at 30°C ^a	No. of mice injected	No. (%) dead after:		Presence of <i>Aeromonas</i> species at necropsy ^b
				12 h	36 h	
<i>A. sobria</i>	14	NS	5	1 (20)	5 (100)	+
	171	NS	5	2 (40)	5 (100)	+
	206	NS	5	1 (20)	4 (80)	+
	181	S	5	0 (0)	1 (20)	-
	228	S	5	0 (0)	0 (0)	-
	176	S	5	0 (0)	0 (0)	-
<i>A. hydrophila</i> ^c	85	NS	5	2 (40)	5 (100)	+
	9902	NS	5	2 (40)	4 (80)	+
	222	NS	5	0 (0)	2 (40)	+
	223	NS	5	0 (0)	2 (40)	+
<i>A. caviae</i>	199	S	4	0 (0)	0 (0)	-
	251	S	4	0 (0)	0 (0)	-
	194	S	4	0 (0)	0 (0)	-

^a NS, Nonsuicidal; S, suicidal.

^b *Aeromonads* were recovered from heart blood, spleen, liver, and kidneys of expired animals.

^c *A. hydrophila* was suicidal at 37°C.

TABLE 3. Enteropathogenicity for mice of suicidal and nonsuicidal *Aeromonas* strains

Species	Strain no.	Suicidal characteristics at 30°C ^a	No. of mice orally injected	No. dead after 24 h	Occurrence of diarrhea ^b	Fluid accumulation in expired animals ^b	Recovery of <i>Aeromonas</i> species from feces of surviving mice after 24 h ^b
<i>A. sobria</i>	14	NS	3	3	+	+	—
	171	NS	3	2	+	+	—
	181	S	3	0	—	—	—
	176	S	3	0	—	—	—
<i>A. hydrophila</i>	85	NS	3	2	+	+	—
	9902	NS	3	1	+	+	—
	223	NS	3	1	+	+	—
	222	NS	3	0	—	0	—
<i>A. caviae</i>	199	S	3	0	—	0	—
	194	S	3	0	—	0	—

^a NS, Nonsuicidal; S, suicidal.

^b +, Positive; —, negative; 0, none.

A. hydrophila strains, which are suicidal only at 37°C, showed a strain variability with regard to mouse lethality. Isolate nos. 85 and 9902 were 90% lethal 36 h postinoculation, whereas the remaining two strains (222, 223) killed 4 out of 10 (40%) inoculated mice within 36 h. Interestingly, in contrast to *A. hydrophila* strains 85 and 9902, which killed mice as early as 12 h postinoculation, strains 223 and 222 killed two mice each only after 36 h. Postmortem examinations of mice which died in the course of infection were positive for the inoculated strains, whereas similar examinations of sacrificed surviving mice were negative. Identical data (not shown) were obtained for suicidal and nonsuicidal mesophilic *Aeromonas* species from environmental sources.

Oral inoculation of mice also revealed virulence or enteropathogenicity as a function of the suicidal capacity of the test *Aeromonas* strain (Table 3). With the two nonsuicidal *A. sobria* isolates (nos. 14 and 171) tested, four of six inoculated mice died within 24 h. Before death, these mice had diarrheal stools, and on postmortem examination, fluid accumulation was noted in their intestinal tracts (Fig. 1). Aspiration and direct microscopic examination of the fluid content revealed innumerable motile bacilli. Polymorphonuclear leukocytes were not observed on wet preparations or in Gram-stained smears. *A. sobria* was isolated in pure cultures from the antemortem diarrheal stools and the aspirated fluid. One mouse each inoculated with strains 14 and 171, respectively, survived oral administration. These mice showed evidence of diarrhea as manifested by mushy stool pellets and had positive cultures for the nonsuicidal *A. sobria*.

Suicidal strains of *A. sobria* and *A. caviae* were nonlethal and nondiarrheagenic. Necropsy of one mouse sacrificed after 24 h failed to show fluid accumulation in the intestinal tract (Fig. 1). In the four surviving mice, excretion of the orally administered strains terminated 18 h postinoculation.

The four suicidal strains of *A. hydrophila*, as noted previously, showed strain variability regarding virulence and enteropathogenicity. Postmortem examination of two of four mice expiring within 24 h showed evidence of intestinal fluid accumulation as noted for nonsuicidal *A. sobria*. Only one of the eight surviving mice had a diarrheal stool, and in no instance was the inoculated strain recovered from mouse feces after 24 h.

DISCUSSION

The suicide phenomenon in *Aeromonas* species ensues when certain strains are inoculated into broth media containing 0.5% glucose. Accumulation of acetic acid and other

short-chain fatty acids as a result of suppression of the tricarboxylic acid cycle reduces the pH of the medium, thereby inactivating the electron transport chain, resulting in cessation of growth and death of the organisms (10; Namdari, Ph.D. thesis).

The suicide phenomenon, which occurs within 18 to 24 h of growth, is strain variable among *A. sobria* isolates, temperature dependent (37°C) among *A. hydrophila* isolates, and uniformly observed among *A. caviae* isolates irrespective of temperature (H. Namdari, personal observation).

In the present study, mouse lethality and enteropathogenicity could be correlated with the suicidal tendency of the inoculated strain. Nonsuicidal strains of *A. sobria* showed an overall 93% lethality rate, as contrasted to a 6.6% rate for its suicidal counterpart. None of the three suicidal *A. caviae* strains tested were lethal, while *A. hydrophila* was strain variable and delayed with regard to mouse virulence. The mesophilic strains tested could be therefore divided into high, intermediate, and avirulent depending on their suicidal



FIG. 1. Distinct distension of and fluid accumulation in intestine of a mouse which died 24 h after oral challenge with 10^8 CFU of nonsuicidal *A. sobria* (bottom) as contrasted to normal intestinal tract of sacrificed mouse similarly challenged with suicidal *A. sobria* (top).

TABLE 4. Correlation between suicide phenomenon and VP and MR reactions in *Aeromonas* species

Species	Suicidal characteristic ^a	VP	MR
<i>A. sobria</i>	NS	+	-
<i>A. sobria</i>	S	+	+
<i>A. caviae</i>	S	-	+
<i>A. hydrophila</i>	NS	+	-
<i>A. hydrophila</i>	S	+	+

^a NS, Nonsuicidal; S, suicidal.

tendency. Janda et al. (11) and Daly et al. (7), using a mouse virulence assay, and Wakabayashi et al. (23) and Kou (15), using a fish (Japanese loach and common carp) model, also showed a species- and strain-specific distribution in degree of virulence among motile aeromonads. *A. sobria* and *A. hydrophila* showed a range of lethality from high to avirulent, whereas *A. caviae* was uniformly nonlethal. No explanation, however, for the observed gradation in animal virulence was offered by these authors.

We propose, on the basis of our data, that the differences between *Aeromonas* species with regard to animal virulence are a function of their suicidal capacity. The data are clear for suicidal and nonsuicidal *A. sobria* and suicidal *A. caviae*. For *A. hydrophila*, which is suicidal at 37°C but not at 30°C and shows strain-variable animal virulence, we suggest that individual strains may intrinsically activate the suicide phenomenon depending upon local factors, such as gradations in the temperature of the inoculated animal (e.g., fish) and pH, as a function of either the metabolic activity of the inoculated bacterium or the resident gastrointestinal tract flora. For instance, *A. hydrophila*, which is nonsuicidal at 30°C, is highly virulent for the Japanese loach when experimentally inoculated intramuscularly and maintained at 16 to 20°C in static water (23), a temperature range favoring suppression of the suicide phenomenon. Interestingly, even under these conditions, *A. caviae*, which is suicidal irrespective of temperature, is avirulent.

To further strengthen the concept of a metabolic regulatory mechanism underscoring the suicide phenomenon, we propose that suicidal activity is mediated through shifts in or exclusivity in the mixed-acid (versus oxidative phosphorylation) or butylene glycol (acetylmethylcarbinol) or both pathways. Nonsuicidal strains of *A. sobria* are VP positive, but methyl red (MR) (mixed-acid fermentation) negative, whereas suicidal strains are both VP and MR positive (Table 4). *A. caviae* strains, which are uniformly suicidal and avirulent, utilize the mixed-acid pathway exclusively, thereby favoring acetate accumulation irrespective of environmental temperature. Subsequent lowering of pH either by metabolic activity or by resident flora in the gastrointestinal tract could potentially result in *A. caviae* killing.

Since *A. hydrophila* strains are suicidal only at 37°C, fluctuations in temperature may favor activation of either pathway. We have shown that 40 strains of *A. hydrophila* tested from human and environmental sources are uniformly VP positive at 30 and 37°C but MR negative at 30°C and MR positive at 37°C. Thus, absence or suppression of the mixed-acid fermentation pathway at 30°C with a concomitant loss of suicidal capacity could easily explain the uniform marked virulence of *A. hydrophila* for fish at the lower temperatures but variable virulence for mice. Thus, animal virulence as a function of the suicidal propensity of the strain or species would embody correlates of intrinsic metabolism for *A. sobria* (and *A. caviae*), and would be mediated through a

temperature-dependent regulatory mechanism for *A. hydrophila*.

Several investigators have correlated animal and fish virulence of *A. hydrophila* and *A. sobria* strains with the presence of an array of surface proteins constituting a single thermostable serogroup (8, 12). Such strains do not spontaneously autoagglutinate but pellet after boiling (12) and are resistant to the bactericidal action of fresh normal mammalian serum (12, 17). *Aeromonas* strains which spontaneously autoagglutinate (suicidal strains) lack these surface components and are avirulent. We hypothesize that the presence of peripheral surface proteins and animal virulence are inextricably linked to the suicide phenomenon, which coincides with these data as well.

H. Namdari and V. J. Cabelli (submitted for publication) have shown that nonsuicidal *A. sobria* and *A. hydrophila* strains, although themselves potentially susceptible to the lethal action of acetic acid, have an increased tolerance to acetic acid compared with suicidal strains. As the presence of surface protein confers serum resistance, presumably by blocking the action of complement, tolerance to acetic acid accumulation may also be related to decreased permeability of this metabolite into strains possessing the peripheral protein matrix. Acetic acid impermeability could also account for the strain variability in animal virulence noted with *A. hydrophila*, even though this species is suicidal at 37°C. On the basis of the data of Janda et al. (12), a peripheral cell-associated layer is variably present in *A. hydrophila* and *A. sobria* and absent in *A. caviae*. These data are superimposable on our data correlating the suicide phenomenon with animal virulence.

The role of mesophilic *Aeromonas* species in gastrointestinal disease is still in question. For instance, Morgan et al. (18) failed to induce diarrhea in 55 of 57 human volunteers orally administered up to 10¹⁰ CFU of two strains biochemically conforming to *A. hydrophila* and three esculin-negative strains of *A. sobria*. All five strains were VP positive and hemolytic and produced cytotoxin and cholera toxin cross-reactive factor. Of additional interest was the lack of shedding of three of the test strains despite oral administration of 10⁷ to 10¹⁰ CFU to these volunteers. These investigators therefore suggested that additional virulence properties of *Aeromonas* species should be sought before establishing human enteropathogenicity.

We believe that *A. hydrophila* and *A. sobria* but not *A. caviae* possess human and animal enteropathogenicity and that assessment of this feature in experimental studies must take into consideration the suicidal propensity of the test strain. In the present study, we could induce diarrheal illness with gastrointestinal fluid accumulation with nonsuicidal *A. sobria* and *A. hydrophila* but not with suicidal isolates of these species or *A. caviae*. The recovery of *A. caviae* predominantly from the feces of children less than 2 years of age (1) could reflect survival of this aeromonad in modified gastrointestinal tract flora not resulting in marked reduction in pH. Indeed, in one series, 3 of the 36 infants for whom details were given suffered from failure to thrive secondary to formula intolerance.

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