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The Suicide Phenomenon in Motile Aeromonads

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Certain strains of motile *Aeromonas* species, including all those of *Aeromonas caviae* examined, were shown to be suicidal. When they were grown in the presence of glucose at both 30 and 37°C, there was rapid die-off of the organisms after 12 h of incubation, and viable cells generally could not be recovered after 24 h. It was shown that this phenomenon was due to the production of relatively high levels of acetic acid by these strains, even during growth under highly aerobic conditions, and to the greater susceptibility of these strains to acetic acid-mediated death. Suicide did not occur when the pH was maintained above 6.5 or in the presence of high concentration of P_i. These observations were consistent with our inability to isolate suicidal *Aeromonas* spp. from acidic lakes in New England and with their recovery from alkaline waters in Israel and from sewage. Suicidal aeromonads appear to be better adapted than the nonsuicidal biotypes to anaerobic growth in low-nutrient environments.

The genus *Aeromonas* includes three facultatively anaerobic, mesophilic, motile species, *Aeromonas sobria*, *Aeromonas hydrophila*, and *Aeromonas caviae* (4, 8). Unlike *A. hydrophila* and *A. sobria*, *A. caviae* typically is anaerogenic, is H_2S negative, and does not produce acetoin as a fermentation product. It ferments lactose, although at times slowly, and cellobiose.

Because the mesophilic *Aeromonas* spp. are indigenous to freshwater environments, multiplying therein under appropriate conditions of temperature, pH, and nutrient (especially phosphorus [10, 12]) loading, their levels can be used as an index of the trophic state of fresh waters (10). These *Aeromonas* spp., although infrequently found in human feces, constitute a major portion of the facultatively anaerobic microbial flora in sewage (9). They also are able to multiply in drinking water distribution systems when sufficient nutrients are present (5). There are few reports on the distribution of different *Aeromonas* species or biotypes in aquatic environments. Schubert (12) reported that the majority of anaerogenic strains exist in high-nutrient waters, especially "sewage water," whereas the aerogenic biotypes are predominant in oligotrophic waters.

Certain *Aeromonas* strains, when inoculated into tubes of nutrient broth containing glucose and bromcresol purple, rapidly produce enough acid to reduce the pH of the medium well below the pK of the indicator, 6.3, with minimal growth as seen by faint turbidity. We have termed this observation the suicide phenomenon because, in most instances, viable cells could not be recovered from the fermentation tubes 24 h after inoculation. The large majority of the suicidal strains tested were identified biochemically as *A. caviae*.

The metabolic end product(s) responsible for the suicide phenomenon and the environmental conditions which foster or inhibit it were examined as the first step in determining the ecologic niche of *Aeromonas* spp. and the biochemical basis for adaptation of these organisms to it.

MATERIALS AND METHODS

Cultures. *Aeromonas* strains used in this study were isolated from sewage and freshwater samples collected in

New England and Israel. After isolation by the mA method (9), *Aeromonas* isolates were identified to the species level by the following biochemical tests: esculin hydrolysis, L-arabinose utilization, fermentation of salicin, and production of acetoin from glucose, gas from glucose, and H_2S from cysteine (4, 8).

Distribution of mesophilic aeromonads in New England lakes. Water samples from five lakes in New England were examined for distribution of mesophilic aeromonads, particularly *A. caviae* and suicidal biotypes. One of the lakes was oligotrophic, and two each were mesotrophic and eutrophic with respect to limnetic quality. The lakes were similar, however, with regard to acidity; in none did the pH of the water exceed 5.8. The water samples were collected at or near the deepest portions of the lakes to increase the probability that the isolates represented the autochthonous *Aeromonas* populations and not those derived from wastewater discharges or stormwater runoff. Samples of the prechlorinated effluents from two secondary treatment plants in Rhode Island also were examined for the presence of *A. caviae*.

Media and solutions. Nutrient broth-glucose (NBG) medium was prepared by supplementing nutrient broth with 0.5% glucose. The ingredients were mixed and autoclaved at 121°C for 15 min.

Modified Davis minimal salt glucose (DMSG) medium was prepared by dissolving 7 g of K_2HPO_4, 2 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 1 g of $(NH_4)_2SO_4$ in 1 liter of deionized water. After it was autoclaved at 121°C for 15 min, the solution was cooled to room temperature and supplemented with a filter-sterilized 20% glucose solution to a final concentration of 0.5%.

Growth of aeromonads in NBG and DMSG media. Duplicate 50-ml Erlenmeyer flasks, each containing 10 ml of NBG or DMSG medium, were inoculated with about 10^3 CFU from an overnight nutrient broth culture of the test organism. The cultures were incubated at 30°C. One of each pair of flasks was left as a stationary culture, and the other was shaken at 250 rpm on a GIO Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Samples from each flask were spread plated in duplicate on nutrient agar immediately after inoculation of the flasks and at 6-h intervals for 48 h. Colonies were counted after the plates were incubated

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at 30°C for 24 h. The pH of each culture was determined every 3 h for 24 h.

Gas-liquid chromatography. Concentrations of the short-chain volatile and nonvolatile fatty acids that accumulated in the spent medium from NBG and DMSG cultures were determined by gas-liquid chromatography (Capco Clinical Analysis Products Co.). A stainless-steel column (0.6 cm in diameter, 15 cm long) containing 10% OV-351 with 1% H_3PO_4 on a 100/120-mesh Chromosorb WHP support was used. The thermal conductivity detector was set at 95 mA, the column was run isothermally at 135°C, and the flow rate of the helium carrier was 120 cm^3/min. The volatile and nonvolatile fatty acids were prepared for analysis by ethyl ether extraction and methylation procedures, respectively (3).

Effect of acidic metabolites on survival. NaCl solutions (0.6%) were supplemented with 6 μmol of either acetic, formic, propionic, succinic, or malic acid per ml. The solutions were adjusted to pH 5.3 by addition of filter-sterilized 1 N NaOH and dispensed in 8-ml quantities to sterile 15-by-150-mm test tubes. The tubes were seeded with about 10^6 CFU from overnight cultures of the suicidal and nonsuicidal *Aeromonas* strains. Samples from each tube were spread plated on nutrient agar plates after 0, 2, 6, 12, and 24 h of incubation at 30°C.

pH requirement for the suicide phenomenon. Triplicate 500-ml sidearm flasks containing 50 ml of NBG were inoculated with approximately 10^7 CFU of the suicidal strains. The medium in one of the three flasks also contained 0.004 g of bromthymol blue (Sigma Chemical Co., St. Louis, Mo.) per ml. The cultures were shaken as described above during incubation at 30°C. The pH of the test culture was maintained between 6.5 and 7.0 by aseptic addition of sterile 1 N NaOH, whereas that of the control was allowed to decrease. The need for addition of NaOH and the quantity required was determined from examination of the flask with the bromthymol blue-containing medium. When the color reached that comparable to a pH of about 6.5, enough NaOH was added to bring the pH back to approximately 7.0; an equal quantity of the base was added to the test culture lacking bromthymol blue. Optical density, pH, residual glucose, and acetic acid were determined after incubation for 0, 6, 12, and 24 h. Residual glucose was determined enzymatically (11).

Effect of phosphate on the lethality of acetic acid. Three solutions containing 0.3% NaCl, 6 μmol of acetic acid per ml, and KH_2PO_4 to a final concentration of 0.1, 0.01, or 0.001 M were prepared. Each was adjusted to pH 5.3 by addition of 1 N NaOH, filter sterilized, and dispensed in 8-ml quantities to sterile 15-by-150-mm test tubes. Control solutions containing no acetic acid but supplemented with 0.1 and 0.001 M phosphate were similarly prepared. The tubes were inoculated with about 10^5 CFU from overnight cultures of the suicidal and nonsuicidal *Aeromonas* strains. Samples from each tube were spread plated on nutrient agar plates after 0, 6, 12, and 24 h of incubation at 30°C.

RESULTS

Distribution of *Aeromonas* strains and suicidal biotypes. *A. caviae* could not be isolated from any of the New England lakes, and none of the *Aeromonas* isolates from these bodies of water were suicidal at 30°C. Although most of the *Aeromonas* isolates recovered from each of the lakes were identified as *A. sobria*, the percentage of *A. hydrophila* isolates increased as the water became more oligotrophic.
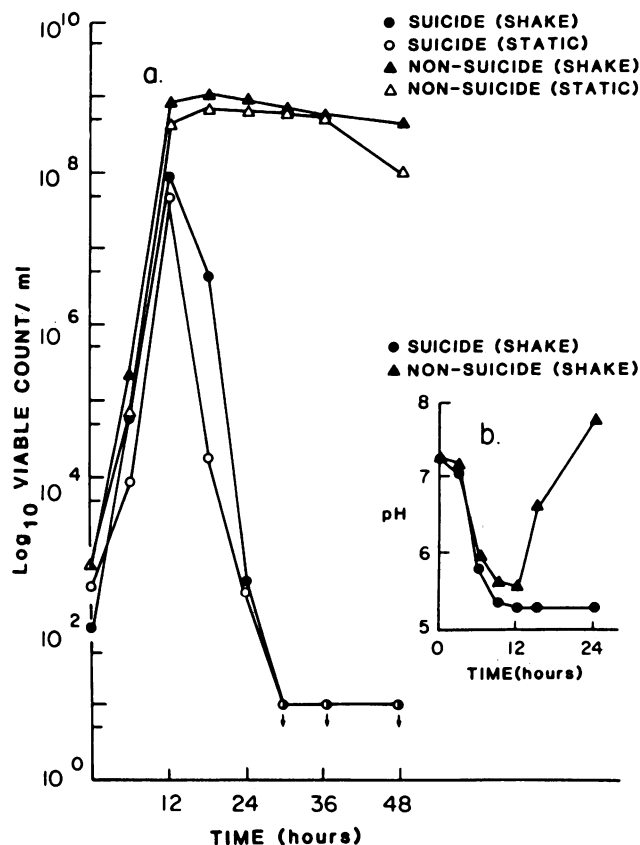

FIG. 1. Growth of suicidal (OP2) and nonsuicidal (2BT) *Aeromonas* strains in NBG medium incubated at 30°C. (a) Viable count; (b) pH of the culture.

(The trophic state of these lakes was presented in an earlier report [10].) *A. caviae* was recovered from lake, river, drinking water, and sewage samples from Israel but only from sewage samples in New England.

All 44 *A. caviae* strains examined were suicidal at both 30 and 37°C and in standing and shake cultures; all fermented lactose and cellobiose, although lactose fermentation was delayed in many strains. The *A. hydrophila* isolates were suicidal only at 37°C in standing cultures. Some of the lactose-positive and lactose-negative strains of *A. sobria* were suicidal at both 30 and 37°C and in both standing and shake cultures.

Growth of suicidal and nonsuicidal strains. Four *Aeromonas* strains were used in this experiment, performed to better define the suicide phenomenon. Y1 and OP2 were *A. caviae* strains; Y1L⁻ and 2BT were *A. sobria* and *A. hydrophila* isolates, respectively, which were nonsuicidal at 30°C. Since the results obtained with each pair were similar, only the data for OP2 and 2BT are shown in Fig. 1. The maximum cell densities achieved by the suicidal strains in the NBG static cultures were less than those reached by the nonsuicidal strains. Whereas the viable counts in the 2BT and Y1L⁻ cultures remained relatively constant for the subsequent 36 h, those in the OP2 and Y1 cultures decreased rapidly to undetectable levels during the following 18 h. Essentially the same results were obtained with the shake and the static cultures (Fig. 1a). The pH in the NBG shake cultures of the suicidal strains decreased rapidly between the 3rd and 6th h of growth, reaching a minimum value of about 5.3 after 9 h. With the nonsuicidal strains, the pH decreased more slowly

TABLE 1. Production of acidic end products by suicidal and nonsuicidal *Aeromonas* strains
grown in nutrient broth cultures at 30°C for 24 h

		Measurement							
		Optical density		pH		End product (μmol/0.1 optical density unit)			
Strain	Type					Standing			Acetic acid (shake)[a]
		Standing	Shake	Standing	Shake	Acetic acid	Succinic acid	Propionic acid	
OP2	Suicidal	0.15	0.7	5.2	5.3	4.6	3.8	3.3	0.8
OP85	Suicidal	0.15	ND[b]	5.2	ND	3.9	3.6	3.3	ND
Y1	Suicidal	0.17	0.6	5.3	5.3	3.6	3.1	3.2	1.0
1BT	Nonsuicidal	0.77	1.9	5.6	7.4	0.3	0.3	0.7	UD[c]
2BT	Nonsuicidal	0.82	1.8	5.6	8.0	0.5	0.3	0.6	UD
1ST	Nonsuicidal	0.77	ND	5.7	ND	0.6	0.3	0.6	ND
X	Suicidal	0.16	0.7	5.2	5.3	4.0	3.5	3.3	
X	Nonsuicidal	0.79[d]	1.83[e]	5.60[e]	7.7[e]	0.5[d]	0.3[d]	0.6[d]	

[a] Acetic acid was the only end product accumulated under shaking conditions.
[b] ND, Not determined.
[c] UD, Undetected.
[d] Significantly different from value for suicidal strains at $P < 0.001$.
[e] Significantly different from value for suicidal strains at $P < 0.01$.

to about 5.6 and then increased during the subsequent 12 h (Fig. 1b).

Growth characteristics of the strains in DMSG were similar to those noted above. In DMSG, however, the lag periods and the decrease in pH were more protracted with both the suicidal and nonsuicidal strains. In addition, the maximum densities achieved by the suicidal strains were higher and the death rates were somewhat lower in DMSG than in NBG, presumably because of the phosphate buffer in

DMSG. An increase in pH after incubation of the nonsuicidal strains for 12 h in DMSG indicated secondary catabolism of an acidic end product.

Metabolic end products. Acetic, succinic, and propionic acids were the only acidic end products produced when the aeromonads were grown in NBG static cultures (Table 1). Concentrations relative to biomass of these acids as measured by optical density were significantly higher with suicidal than with nonsuicidal strains. Neither lactic acid, as
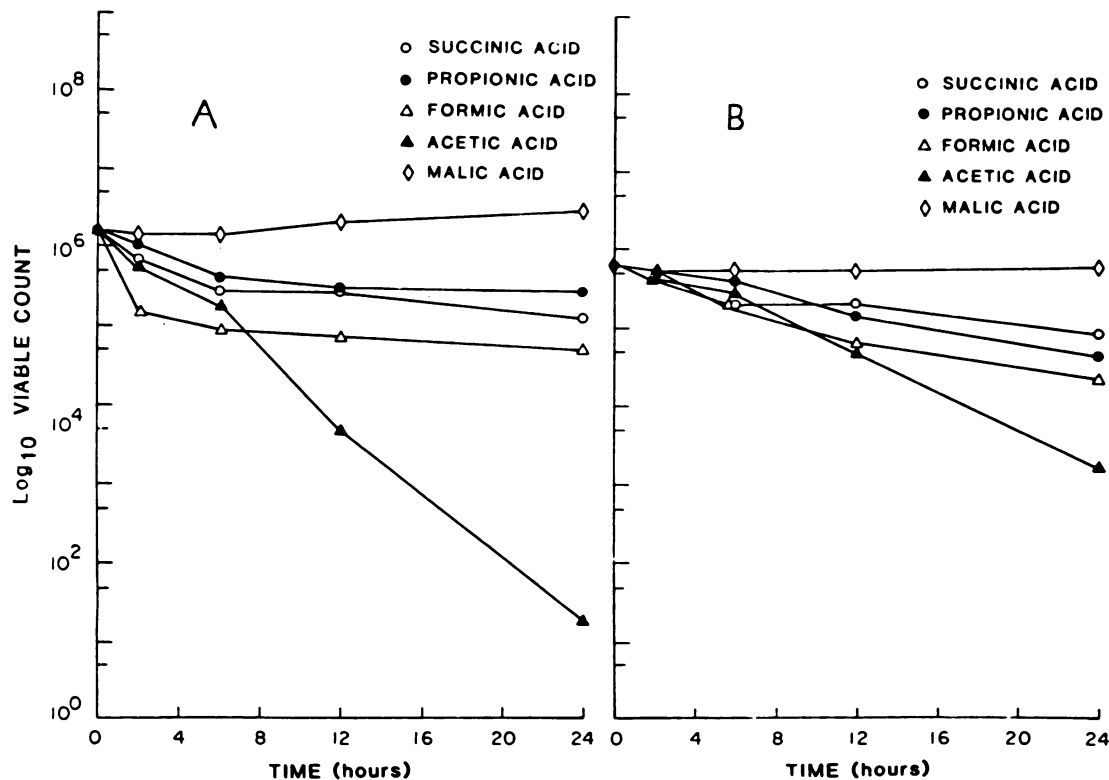

FIG. 2. Survival of suicidal (Y1) (A) and nonsuicidal (Y1L⁻) (B) aeromonads in the presence of weak organic acids. Concentration of acids, 6 μmol/ml; pH adjusted to 5.3; temperature, 30°C.

TABLE 2. Comparison of cell growth, production and secondary metabolism of acetic acid, and utilization of
glucose in pH-neutralized shake cultures of a suicidal *Aeromonas* strain

Time (h)	Measurement							
	pH		Optical density		Acetic acid (μmol/ml)		Glucose (μmol/ml)	
	Neutral[a]	Control	Neutral	Control	Neutral	Control	Neutral	Control
0	6.8	6.8	0.0	0.0	0.50	0.50	26.7	26.7
6	6.5–7.0	5.4	0.95	0.72	18.0	0.96	11.1	22.2
12	6.5–7.0	5.3	1.6	0.76	23	4.0	0.11	21.7
24	6.5–7.0	5.3	1.8	0.76	6.3	4.0	UD[b]	21.7

[a] pH adjusted (neutralized) to 6.5 to 7.0 by the addition of base (see text).
[b] UD, Undetectable.

determined by gas chromatography, nor formic acid, as determined colorimetrically (13), was detected in suicidal or nonsuicidal cultures. Acetic acid was the only acidic end product detected in shake cultures of the suicidal strains. It was not found in those of the nonsuicidal isolates, which suggested that acetic acid was the acidic end product catabolized as a secondary metabolite.

Subsequent experiments showed that the suicidal strains of *Aeromonas* spp. were capable of growing in DMSG containing 0.5% glucose under anaerobic conditions, whereas the nonsuicidal strains and *Escherichia coli* failed to do so. The acidic end products from the suicidal cultures were acetic, succinic, and propionic acids (data not shown).

Effect of end products on survival. Nonsuicidal but not suicidal cells survived equally well at NaCl concentrations of between 0.30 and 0.85%. An NaCl concentration of 0.6% was selected for the survival experiments since, even at pH 5.3, there was no decrease in the viable recovery of both the suicidal and nonsuicidal strains over the 24-h observation period.

Results obtained with one pair of strains are shown in Fig. 2. Acetic acid was markedly more bactericidal for the suicidal strains (e.g., OP2) than were any of the other compounds tested. The nonsuicidal aeromonads were also susceptible to the cidal effect of acetic acid but to a much lesser extent than were the suicidal strains.

Effect of pH and phosphate on the suicide phenomenon. When the pH of a shake culture of a suicidal strain was maintained between 6.5 and 7.0, there was no inhibition of growth even though there was more than a 10-fold increase in the concentration of acetic acid (Table 2). The cells remained metabolically active, utilizing the glucose and, when glucose was expended, the produced acetate was metabolized.

Although phosphate at a concentration of 0.01 M delayed death of the cells in the presence of acetic acid at pH 5.3, a concentration of about 0.1 M was required to negate this effect.

DISCUSSION

The data obtained identify two conditions necessary for accelerated death to take place in *Aeromonas* strains: production of acetic acid and susceptibility to its lethal effects at a low pH. The second aspect of the phenomenon, the toxicity of volatile fatty acids in their undisassociated form, has been described in a number of organisms and studied by several investigators (2, 6, 7) and will not be considered herein. Acetic acid-mediated death among the suicidal strains and its absence at neutral pH and in the presence of high phosphate concentrations is consistent with our inability to recover *A. caviae* or suicidal *A. sobria* biotypes from the acidic lakes in New England. (The concentration of acetic acid in the water at the single New England lake examined in this manner was 2.5 μmol/ml.) Since *A. caviae* strains are both anaerogenic and suicidal, we speculate that the observation of Schubert (12) that aerogenic aeromonads predominate in oligosaprobic waters may have been due to the inability of *A. caviae* to survive in such waters. Oligosaprobic lakes would tend to be more acidic because of their lower phosphate levels and buffering capacities.

A. caviae strains typically are anaerogenic, do not produce acetoin, and ferment cellobiose and lactose, albeit slowly in some cases. There is a need to determine whether there is a genetic, regulatory, or biochemical association of these characteristics with either of the two aspects of the suicide phenomenon, acetic acid production and sensitivity to its effects.

It may be reasonably assumed that suicidal aeromonads have both a citric acid cycle and the electron transport chain to produce energy by oxidative phosphorylation, since acetate is utilized as a secondary metabolite once the glucose is expended and acetate, but not succinate or propionate, is produced as an end product under aerobic conditions. The most interesting questions, both biochemically and ecologically, are why and how an aquatic bacterium growing aerobically in the presence of an available sugar would utilize a much less efficient method of producing energy. Amarasingham and Davis (1) made essentially the same observation with *E. coli*. They suggested that this was a mechanism by which *E. coli* deprived certain competing bacteria in the colon of glucose and other available sugars by converting them to acetate for subsequent utilization. We too believe that the production of acetate under aerobic conditions, in this case by suicidal aeromonads, must satisfy a requirement beyond that for energy and that it is an ecological adaptation.

Two possible functions of the acetic acid produced by the suicidal strains are suggested by certain physiological and ecologic characteristics of the organisms. The first is mobilization of phosphate from its insoluble form in calcium-rich alkaline lakes. This explanation is consistent with the observation that *Aeromonas* levels in fresh waters correlate with their trophic conditions (10). The second is release of nutrients from associated algae. This explanation is consistent with the observation that *A. caviae* levels in water from Lake Kinneret in Israel were highest in the spring, when there is a *Pyridinium* bloom.

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