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Tn5-Induced Protease-Deficient Strains of *Aeromonas hydrophila* with Reduced Virulence for Fish

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Protease-deficient strains of *Aeromonas hydrophila* TF7 were induced by transposon Tn5 mutagenesis, with *Escherichia coli* 1830(pJB4JI) as the Tn5 donor. The parent strain has the cell surface characteristics associated with virulence for fish, and as it produces a single metalloprotease, mutants could be distinguished by direct plating on brain-heart infusion skim milk agar. Mutants Pd-7 and Pd-10 still produced metalloprotease, but at reduced levels and only after prolonged incubation. The activities of other exoenzymes and hemolysin were unaffected, and the mutants autoagglutinated in broth, indicating that the cell surface characteristics of *A. hydrophila* TF7 had been retained. Unlike the parent strain, the mutants did not produce lesions or mortalities in rainbow trout (*Salmo gairneri*) when 5×10^6 CFU were injected intramuscularly. The bacterial cells were completely cleared from the site of the injection and the organs within 7 days. For 60-g rainbow trout held at 10°C, the 50% lethal dose of Pd-10 was $>10^7$ CFU, compared with 8.1×10^5 CFU for the parent strain. The mutants were significantly more susceptible than the parent strain to the bactericidal effect of fresh normal trout serum in vitro. Mutants Pd-7 and Pd-10 grew as well as the parent on M9 salts-glucose medium but more slowly on heat-inactivated fish serum. Thus, protease appears to be able to contribute to the establishment of *A. hydrophila* infection in fish both by overcoming initial host defenses and by providing nutrients for cell proliferation.

Aeromonas hydrophila, an opportunistic pathogen associated with hemorrhagic septicemia of fish, produces a variety of extracellular products (ECPs), including enterotoxins, hemolysins, and proteases (12). Correlations between extracellular enzyme activities and virulence of *A. hydrophila* strains have been used to suggest that proteolytic enzymes may be important for pathogenic activities (8). Crude preparations of ECPs can produce mortality and tissue damage when injected into rainbow trout (1), channel catfish (22), and carp (25). In attempts to identify the significant toxic component of ECPs, purified or semipurified proteases were injected into fish, with necrosis resulting (9, 22). However, tissue damage and mortality can also occur with injections of purified hemolysin (23). Allan and Stevenson (1) argued that protease alone could not be the sole toxic factor of ECPs, as preparations from a protease-deficient mutant of *A. hydrophila* were not less toxic than those from the parent strain.

The limitation of all experiments involving injection of ECPs is that they disregard the potential synergy and interactions of various virulence factors that would occur during infection by a living bacterium, such as proteolytic activation of aerolysin toxin (7). Trust (24) notes that the significance of putative virulence factors can only be clearly demonstrated by virulence tests with deficient mutants. Thus, the question of the role of proteases in infections of fish by *A. hydrophila* should be addressed by comparing the virulence of protease-deficient mutants with that of the parent. Chemical mutagens have been used to produce protease-deficient mutants of *A. hydrophila* (1, 6), but more than one extracellular component may be affected, as in the case of the pleiotropic export mutants of Howard and Buckley (6). The protease-deficient derivative of *A. hydrophila* NRC505 that was used by Allan and Stevenson (1) produced more hemolysin than the parent and was sensitive to a broader range of bacteriophages (R. Stevenson and B. J. Allan, unpublished results), suggesting an alteration in surface characteristics. Such changes are likely to result in reduced virulence of *A. hydrophila* (4, 14).

Mutagenesis with transposons, such as Tn5, provides a better approach to obtaining isogenic mutants for virulence studies. Tn5 mutagenesis has generated cell surface mutants of the fish pathogen *Aeromonas salmonicida* (2); it has also been used to obtain protease mutants of *Pseudomonas aeruginosa* (21). Selecting protease-deficient mutants of *P. aeruginosa* presented a problem because the bacterium produces more than one exoprotease (21). This was also a potential problem with *A. hydrophila*, as multiple proteases have been reported (15, 22). In a study of 47 strains, we found that 57% produced both a heat-stable metalloprotease and a heat-labile serine protease (11). However, 40% of the isolates produced only the heat-stable metalloprotease. For this study, a parent strain was selected from this group, allowing direct selection of protease-deficient mutants from skim milk agar. These Tn5-induced mutants of *A. hydrophila* TF7 have reduced virulence for fish, and their characteristics suggest that protease aids infectivity both by overcoming initial host defenses and by providing nutrients for growth.

MATERIALS AND METHODS

Bacterial strains and growth. The Tn5 donor was *Escherichia coli* 1830 carrying plasmid pJB4JI (3). The conjugal recipient was *A. hydrophila* TF7, originally obtained from R. Lallier, University of Montreal, Ste. Hyacinthe, Quebec (10). Cultures were routinely grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 30°C for *E. coli* and 25°C for *A. hydrophila*. When required, antibiotics (Sigma Chemical Co.) were added at the following final concentrations: kanamycin, 100 μg/ml; gentamicin, 5 μg/ml; and ampicillin, 50 μg/ml. Stock cultures, including Tn5 mutants,

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were maintained at −70°C in a suspension of 5% (wt/vol) dextran–7.5% sucrose–1% sodium glutamate.

Protease detection. Protease production was detected as zones of clearing around colonies grown on BHISMA (brain-heart infusion agar with skim milk powder added at a final concentration of 1.5% [wt/vol]). ECPs of *A. hydrophila* and mutants were collected from BHISMA by washing cells and products from dialysis tubing overlays with 5 ml of 0.1 M sodium phosphate buffer, pH 7.2, and removing cells by centrifugation as described before (1). Protease activity in the ECPs was assayed by hydrolysis of Azocasein (Sigma), incubating 0.1 ml of a 10% (wt/vol) stock solution with 0.1 ml of ECP sample (or dilution) and 2.3 ml of buffer for 30 min at 37°C. The reaction was stopped with 2.5 ml of 10% (wt/vol), trichloroacetic acid, and after 30 min at 22°C, the precipitate was removed by centrifugation. Equal volumes of the supernatant and 1 M NaOH were mixed, and the absorbance was read at 450 nm, with a pre-boiled sample serving as the blank.

Antisera raised against the purified metalloprotease of *A. hydrophila* NRC505 (11) was used to detect protease by immunoprecipitation. Radial immunodiffusion plates were prepared containing 1% (vol/vol) serum in 1% (wt/vol) agarose. ECP samples of 10 μl were added to 4-mm wells, and the reaction was allowed to develop for 18 h at 37°C.

Tn*5* mutagenesis. Conjugal transfer was performed by using a filter mating procedure essentially as described by Beringer et al. (3), except that mating was performed at 25°C for 24 h on BHISMA. The mating ratio of the donor *E. coli* 1830(pJB4JI) to *A. hydrophila* TF7 (Amp^r) was adjusted to 1:2, with a total of about 10^8 CFU for both donor and recipient during mating. After incubation, cells were resuspended in 10 ml of saline, and appropriate dilutions were plated onto BHISMA plus kanamycin (100 μg/ml) and ampicillin (50 μg/ml) to select Tn*5* transconjugants of *A. hydrophila*. Colonies appearing after 2 days of incubation at 25°C were replica-plated on BHISMA plates by using sterile velvet pads, and colonies that did not produce a zone of hydrolysis were picked as potential protease-deficient mutants.

Mutant screening. Selected mutants were screened for production of exoenzymes by inoculating agar plates by means of a Repliplate multi-inoculator. The test plates contained BHISMA and dialyzed BHISMA media (20), TSA with 1% (wt/vol) elastin (Sigma Chemical Co.), Frazier gelatin agar with gelatin at 1% (wt/vol) concentration in TSA, starch agar and DNase agar (both from Difco), and blood agar with 5% (vol/vol) rabbit erythrocytes added to cooled TSA. For Tween 80 agar, Polysorbate 80 was added at a final concentration of 1% (vol/vol) to agar base containing 10 g of peptone, 5 g of NaCl, and 0.1 g of $CaCl_2$ per liter. Plates were incubated at 25°C for 48 h (72 h for elastase) and then examined for clear zones or opaque halos, depending on the medium used. Motility was tested in semisolid motility-sulfide agar (Difco). Surface characteristics of the strains were tested by precipitation after boiling and serum sensitivity as described by Mittal et al. (14). Lipopolysaccharide patterns were examined by polyacrylamide gel electrophoresis and silver staining by the procedure of Hitchcock and Brown (5).

Growth rates of selected mutants and the parent strain were compared by following changes in optical density measurements at 550 nm with a Spectronic 70 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.). Each strain was first grown overnight in M9 minimal salts medium (13) with 0.4% (wt/vol) glucose and 0.01 M $(NH_4)_2SO_4$. ($CaCl_2$ was omitted.) The culture was washed three times in saline and inoculated into either M9 base with 0.4% glucose and 0.01 M $(NH_4)_2SO_4$ or M9 base with 50% (vol/vol) trout serum that had been heat-inactivated at 45°C for 20 min. The cultures were incubated at 25°C for 9 h, with OD_{550} readings made at hourly intervals.

Fish tests. Rainbow trout (*Salmo gairdneri*) weighing approximately 60 g were purchased from a commercial hatchery and maintained in flowing well water (10°C). Groups of 10 trout were placed in 75-liter tubs containing 55 liters of water and supplied with aeration. Maintenance and experimental procedures were in accordance with the principles of the Canadian Council on Animal Care and the Guidelines for the Care and Use of Animals in Research and Teaching, University of Guelph (1985). Fish were anesthetized for handling by being placed in water containing MS222 (3-aminobenzoic acid ethyl ester; Sigma Chemical Co.).

To study the progress of infection, each culture was injected into the dorsal muscle of eight rainbow trout with a 26-gauge needle. Each fish was injected with 0.1 ml containing 5×10^6 CFU; control fish were injected with saline. The fish were held for 7 days, with two fish sacrificed from each group at intervals. The tissue around the injection site (approximately 5 g) was aseptically cut out, homogenized, and diluted, and then plated on BHISMA plates for viable counts. Kidney tissue was also homogenized and counts were determined.

For 50% lethal dose (LD_{50}) determinations, five groups of 10 fish each were injected intramuscularly with 0.1-ml doses of washed cells adjusted to the required concentrations. Fish were monitored for 7 days. For mortality studies and for all fish at the end of the experiment, the muscle tissue at the injection site was streaked on BHISMA plates. The LD_{50} values were calculated by the method of Reed and Muench (17).

Survival in trout serum. Rainbow trout blood was collected from the caudal sinus, and serum was separated from the clot by centrifugation at 4°C. Bacteria grown in TSB at 15°C for 48 h were collected by centrifugation (Eppendorf microcentrifuge, 1 min) and washed three times with 0.1 M sodium phosphate buffer containing 0.5 mM $CaCl_2$ and $MgCl_2$. The cell suspension was adjusted to about 10^7 CFU/0.1 ml, and this volume was added to 1.5 ml of fresh normal trout serum and 1.4 ml of TSB, giving a final serum concentration of 50%. Tubes were incubated at 15°C, and 0.1-ml samples were removed hourly for serial dilutions and plate counts on BHISMA.

RESULTS

Selection of parent strain. *A. hydrophila* TF7 was chosen as the parent strain because it has a single extracellular protease, a heat-stable metalloprotease, as shown by immunoprecipitation and activity tests (11). In addition, TSB cultures auto-agglutinated after 24 and 48 h of growth, rapidly sedimented after steaming for 1 h and vortexing, and could grow in 50% fresh normal rabbit serum at 30°C, indicating the presence of cell surface characteristics associated with virulence (14). In plate tests, the strain produced other extracellular activities, including DNase, amylase, lipase, and hemolysin.

Tn*5* mutagenesis. The transfer frequency for plasmid pJB4JI (Km^r) was estimated as 1.4×10^{-2} transconjugants per input donor cell after overnight membrane mating. Spontaneous Km^r Amp^r in *A. hydrophila* TF7 occurred at a frequency of 1.3×10^{-5}. The spontaneous mutation rate of

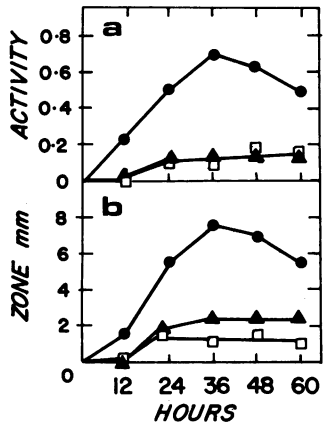

FIG. 1. Protease production by *A. hydrophila* TF7 and Tn*5*-induced protease-deficient mutants measured by (a) azocasein hydrolysis and (b) radial immunodiffusion. For each time point, ECPs were prepared from overlay plates in 5 ml of buffer, and 10-μl samples were tested for azocasein hydrolysis and for the diameter of the immunoprecipitation zone (minus 3.5 mm for the well). Symbols: ●, TF-7; ▲, Pd-7; □, Pd-10.

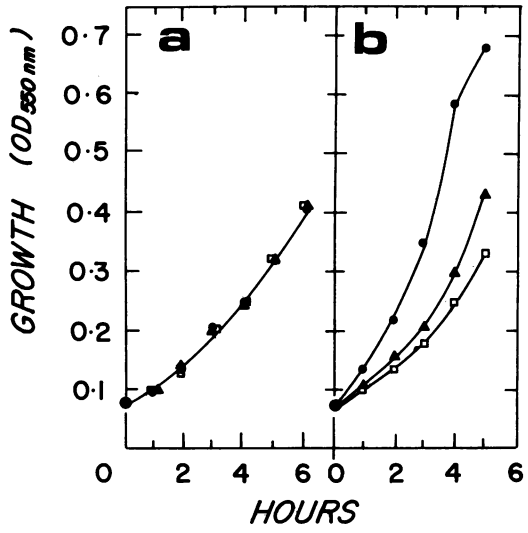

FIG. 2. Growth rate comparisons of *A. hydrophila* TF7 and its protease-deficient mutants in (a) M9 medium with glucose and ammonium sulfate, and (b) M9 salts base plus 50% heat-inactivated trout serum. Cells were grown in M9 medium at 25°C, washed in M9 medium, and then incubated in the test medium at 25°C. Growth of the cultures was monitored by OD_{550} readings at hourly intervals. Symbols: ●, parent strain TF7; ▲, Pd-7; □, Pd-10.

E. coli 1830(pJB4JI) to ampicillin resistance was less than 9.1×10^{-9}. Among 5,000 Km^r Amp^r transconjugants, potential protease-deficient colonies were found with a frequency of 3.2%. These colonies did not produce a zone of hydrolysis on BHISMA containing ampicillin and kanamycin, although some colonies reverted to a protease-producing phenotype when replica-plated onto BHISMA without antibiotics. From 160 protease-deficient isolates, 10 were selected because they maintained the protease-deficient phenotype after repeated transfers on BHISMA without antibiotics. Isolates Pd-1 to Pd-10 were all oxidase positive, sensitive to gentamicin, motile, and autoagglutinated, consistent with the parent characteristics.

Protease production by mutants. On BHISMA, eight of the mutant strains had no zone of clearing around the colonies, while faint, reduced zones showed around colonies of Pd-4 and Pd-7 after 48 h. On dialyzed BHISMA, a more sensitive indicator medium, some clearing was seen with all mutants except Pd-1. Gelatin and elastin were hydrolyzed by all mutants, but at a much reduced level compared with the parent stain. Spontaneous kanamycin-resistant mutants of TF7 showed the same zones of activity as the parent strain on skim milk, gelatin, and elastin agars. Of the 10 protease-deficient mutants, Pd-4, Pd-7, and Pd-10 produced the same amount of hemolytic activity, DNase, amylase, and lipase as strain TF7. The other mutants showed reduced zones of activity, suggesting that they might have a more general export deficiency.

Mutants Pd-7 and Pd-10, which were chosen for use in subsequent experiments, produced negligible amounts of protease after 12 h, and the maximum activity attained was less than that of the parent strain (Fig. 1a). The enzyme produced by the mutants was serologically identical to that of the parent strain in immunodiffusion tests (Fig. 1b), indicating that activity was not due to induction or derepression of a second enzyme. The loss of protease from parental cultures after 36 h is consistent with the pattern of production seen with other strains of *A. hydrophila* (1) and may be due to autolysis.

Other characteristics of mutants Pd-7 and Pd-10. When the protease-deficient mutants and the parent were grown on BHISMA at 25°C overnight, the colonies of the parent TF7 were larger and were surrounded with halos or clear zones, while the colonies of the mutant were smaller and gave no clear zones. When protease production was not required, as in M9 medium with glucose and ammonium salts, the mutants grew as well as the parent strain did (Fig. 2a). When heat-inactivated rainbow trout serum replaced the glucose and ammonium, the protease-producing parental strain had the growth advantage (Fig. 2b). The mutants resembled the parents in aggregation in broth after boiling and in the lipopolysaccharide patterns seen after polyacrylamide gel electrophoresis (data not shown), indicating that the protease deficiency was not a result of a general cell surface alteration.

Fish challenge studies. Within 24 h, the eight fish injected with 5×10^6 CFU of *A. hydrophila* TF7 had a blanched and edematous appearance at the injection site. Swelling and necrosis increased, and the fish developed problems in balancing and swimming. Two fish were sampled on the first day; all others had died by the second day. In the groups of fish injected with the same dose of either of the two mutants, no swelling or blanching was seen during a 7-day observation period, and no fish died except those sacrificed for scheduled samplings.

The tissue taken at 24 h from the injection sites of two fish from each group contained about 10^4 CFU of *A. hydrophila* for both the parent and mutant strains. By day 2, the injection site of fish receiving TF7 was liquefied and hemorrhagic, and the number of bacteria in the tissue around the injection site had increased to about 2.1×10^7 CFU, compared with 10^3 CFU in a comparable tissue sample from fish injected with the mutants. In addition, no external swelling was seen on fish injected with the mutants. When necropsies were performed on the fish injected with TF7, all had internal lesions, including petechial hemorrhage in the pyloric ceca. The kidney was infected in four fish, with 10^3 CFU/g of tissue. In fish injected with either Pd-7 or Pd-10, the number of organisms isolated from the muscle around the

TABLE 1. Determination of LD_{50} in rainbow trout for *A. hydrophila* TF7 and its Tn*5*-induced protease-deficient mutant Pd-10[a]

Dose (CFU)	No. dead/10 fish injected[b]	
	TF7	Pd-10
1×10^7	10	3
5×10^6	10	0
1×10^6	5	1[c]
5×10^5	4	0
1×10^5	0	0

[a] Cells were grown on TSA at 15°C for 48 h and then collected and washed three times in saline before adjustment to the required dilutions. The doses, in 0.1-ml volumes, were injected into the dorsal muscle. Fish were held for 7 days at 10°C. Fish that died were necropsied and the muscle tissue and organs were cultured on BHISMA plates. Fish weights ranged from 54 to 82 g (average, 60 g).

[b] LD_{50} values (8.1×10^5 for TF7 and $>10^7$ for Pd-10) were determined from cumulative deaths by the method of Reed and Muench (17) with interpolation to 50% based on logarithmic values of doses. None of the control (saline-injected) fish died.

[c] One fish died on day 4, with no swelling at the injection site and no *A. hydrophila* recovered, and was considered to have died of an unrelated cause.

injection site decreased, from 10^3 CFU on days 2 and 4 to none detectable after 7 days for either mutant. The mutants remained protease deficient during the experiment; no protease-positive strains were found when muscle samples were plated on BHISMA. No bacteria were isolated from control fish injected with saline.

In the subsequent experiment, the LD_{50} for the wild-type TF7 was 8.1×10^5 CFU for 60-g rainbow trout injected intramuscularly and held at 10°C for 7 days. The LD_{50} for the mutant Pd-10 was greater than 10^7 CFU (Table 1). *A. hydrophila* TF7 was isolated from the lesions of fish injected with the parent strain. After 7 days, the mutant Pd-10 could not be isolated from the injection site.

Serum sensitivity. In the fish tests, the protease-deficient mutants appeared to have a reduced ability to sustain an infection in vivo. The slower growth rate of these strains in heat-inactivated serum (Fig. 2b) suggested that lack of protease activity might result in reduced availability of nutrients. However, the mutants were also more sensitive to the killing effect of normal, unheated trout serum (Fig. 3). In serum, viable counts of the protease-producing parent strain also decreased, but to a much lesser extent. After 5 h, growth of *A. hydrophila* TF7 resumed, but serum killing continued for both Pd-7 and Pd-10.

DISCUSSION

By use of Tn*5* mutagenesis, we were able to obtain suitable protease-deficient strains of *A. hydrophila* for virulence tests on fish. Detection of mutants by direct plating on skim milk agar (BHISMA) was possible because the parent strain, *A. hydrophila* TF7, had previously been shown to have only one major extracellular protease (11). In parallel with results described for Tn*5*-induced protease-deficient mutants of *P. aeruginosa* (21), there was a fairly high rate of spontaneous kanamycin resistance in *A. hydrophila*. A second similarity to *P. aeruginosa* was that the antibiotics in the differential medium inhibited production of zones of clearing (26), requiring additional transfers in order to select authentic protease-deficient isolates.

Protease-deficient isolates produced smaller colonies on BHISMA and grew more slowly in medium with serum (Fig. 2b), suggesting that they were nutritionally restricted on the complex medium. As strains of *A. hydrophila* produce proteolytic activity at a relatively late stage of growth in culture (1), the reduced amounts of protease activity present in ECPs of the mutants and evident on plate tests may reflect the slower growth. However, the Tn*5*-induced alteration does appear to relate specifically to the production of protease activity, as the growth rate of the mutants was the same as that of the parent strain on media in which protease production presented no advantage (Fig. 2a). In addition, the mutants chosen had normal or near-normal levels of other extracellular activities, suggesting that they are unlikely to be pleiotropic export mutants, such as are generated by chemical mutagenesis. None of our mutants were completely deficient in protease, perhaps because direct plating of the mutants on BHISMA may have served as a reverse selection against strains with complete loss of protease. As the Tn*5*-generated mutations were not genetically characterized, we do not know whether the insertion is in the region of the protease gene itself or at another site. Our aim in this study was to determine whether protease could be shown to play a role in pathogenesis, making subsequent genetic analysis pertinent. Further analysis of the mutants Pd-7 and Pd-10 may provide a means of obtaining completely protease-deficient strains.

For the purpose of virulence comparisons, mutants that were reduced in, rather than completely devoid of, proteolytic activity were still suitable. It was much more critical that the mutagenesis not alter other potential virulence factors, particularly the cell surface features considered indicative of virulence for fish (14). The nature of the mutations induced by Tn*5* mutagenesis and the observed characteristics of mutants Pd-7 and Pd-10 suggest that these strains have a relatively restricted alteration, affecting the amount of protease produced. The protease-deficient strain *A. hydrophila* NRC505 obtained previously by nitrosoguanidine treatment (1) had significant alterations in the cell surface and increased sensitivity to normal trout serum (unpublished observations). In contrast, Sakai (19) was able to obtain a nitrosoguanidine-induced protease-deficient mutant of *A. salmonicida* which had no apparent changes in its surface characteristics, including resistance to serum killing. It is not know whether these two aeromonads have very

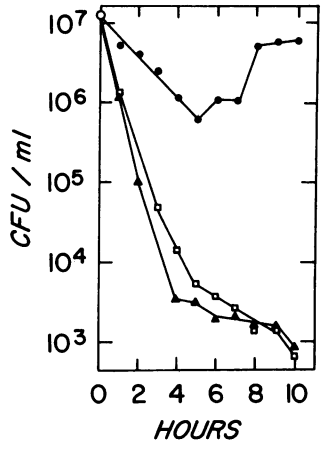

FIG. 3. Survival of *A. hydrophila* TF7 and protease-deficient mutants in normal trout serum. Cultures were grown at 15°C for 2 days before inoculating into a 1:1 mixture of TSB and fresh rainbow trout serum. Mixtures were incubated at 15°C for 10 h and sampled hourly by plating dilutions on BHISMA. Symbols: ●, TF7; ▲, Pd-7; □, Pd-10.

similar pathogenic mechanisms in fish, but in this study the protease-deficient mutants of *A. hydrophila* TF7 obtained by Tn*5* mutagenesis also lost resistance to normal trout serum (Fig. 3). This change appears to be directly linked to the contribution of protease to protection by complement inactivation (K.-Y. Leung, Ph.D. dissertation, University of Guelph, Ontario, 1987). Thus, in the host, proteases may contribute to the survival of *A. hydrophila* in the presence of nonspecific serum defenses, including the rapid killing effect of serum (Fig. 3).

The slower growth rate of the protease-deficient mutants on complex medium may also contribute to reduced virulence. In the tissues and body fluids of a host animal, bacteria capable of proteolytic digestion should have a survival advantage, similar to the situation in vitro (Fig. 2b). Protease may also assist the spread of bacteria in the tissues, incidentally producing the necrotic effects observed in fish injected with TF7, and it may also activate toxins (7). Each of these specific actions of protease may contribute to virulence, but the overall effect will also depend on the amount of protease produced in the host tissue and the multiplying effect of the various activities in combination. Thus, in vivo tests are essential in resolving the contribution of protease to infection.

For virulence tests with fish, a waterborne immersion challenge is generally considered most appropriate, as it does not circumvent the natural barriers faced by a pathogen, However, environmental stresses are an important factor contributing to outbreaks of disease due to *A. hydrophila*, and in order to be effective, experimental challenges with this organism almost always involve injection of bacteria (16, 18). For our study, cells were injected into the dorsal muscle to simulate a natural situation in which a wound becomes infected with *A. hydrophila*. Mittal et al. (14) reported the LD_{50} for strain TF7 as $10^{4.5}$ for intramuscular injection into rainbow trout, presumably for 10- to 15-cm fish held at 18°C, as reported later by Lallier et al. (10). For larger fish, at the lower water temperature of 10°C, our LD_{50} value of 8.1×10^5 CFU for TF7 is not untoward, although it would indicate a weakly virulent strain by the criteria of Mittal et al. (14). However, the parent strain was quickly able to produce lesions and establish a systemic infection, evidenced by isolation of the organism from the kidney. With this challenge system, it appears that even a reduction in the amount of protease produced has the effect of reducing the virulence of *A. hydrophila* for fish. Both Pd-7 and Pd-10 were essentially avirulent, producing no necrosis and being rapidly cleared from the muscle at the site of injection. The LD_{50} value of greater than 10^7 CFU obtained for Pd-10 (Table 1) indicates an avirulent strain (14). Clearly, the mutants are reduced in virulence despite possessing cell surface characteristics indicative of virulence. The protease-deficient strains did not produce host tissue damage when injected into fish muscle tissue, but this is not evidence that the protease is the major toxic substance of ECP. As Pd-7 and Pd-10 grew poorly in tissue and were cleared within a few days, the total in vivo production of all tissue-damaging components of ECP would be correspondingly reduced in comparison with the parent strain.

In vitro (Fig. 2), the reduced amount of protease produced by the mutants resulted in a slower rate of growth on heat-inactivated serum, but not complete starvation. In contrast, in the presence of active serum, the reduction in protease levels produced had immediate consequences for cell survival (Fig. 3). Thus, we suggest that protease plays a critical role in the early stages of the infective process, protecting the bacterial cells against complement-mediated killing or other serum bactericidal effects. Subsequently, proteolytic activity would act to provide nutrients for continued growth and proliferation. These functions could presumably be carried out by either the heat-stable metalloprotease studied here or by the additional serine protease produced by many strains of *A. hydrophila* (1). It is not apparent why the two enzyme types are produced. However, the apparent role of protease activity in establishing infection has implications for attempts to immunize fish against *A. hydrophila* (18). As proteases may be important protective antigens, the methods used to grow and prepare cells should ensure that they are present in vaccines.

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