

## Defined Deletion Mutants Demonstrate that the Major Secreted Toxins Are Not Essential for the Virulence of *Aeromonas salmonicida*

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**The importance of the two major extracellular enzymes of *Aeromonas salmonicida*, glycerophospholipid:cholesterol acyltransferase (GCAT) and a serine protease (AspA), to the pathology and mortality of salmonid fish with furunculosis had been indicated in toxicity studies. In this study, the genes encoding GCAT (*sata*) and AspA (*aspA*) have been cloned and mutagenized by marker replacement of internal deletions, and the constructs have been used for the creation of isogenic *sata* and *aspA* mutants of *A. salmonicida*. A pSUP202 derivative (pSUP202sac) carrying the *sacRB* genes was constructed to facilitate the selection of mutants. The requirement of serine protease for processing of pro-GCAT was demonstrated. Processing involved the removal of a short internal fragment. Surprisingly, pathogenicity trials revealed no major decrease in virulence of the *A. salmonicida*  $\Delta$ *sata*::*kan* or *A. salmonicida*  $\Delta$ *aspA*::*kan* mutants compared to the wild-type parent strains when Atlantic salmon (*Salmo salar* L.) were challenged by intraperitoneal injection. Moreover, using a cohabitation model, which more closely mimics the natural disease, there was also no significant decrease in the relative cumulative mortality following infection with either of the deletion mutants compared to the parent strain. Thus, although these two toxins may confer some competitive advantage to *A. salmonicida*, neither toxin is essential for the very high virulence of *A. salmonicida* in Atlantic salmon. This first report of defined deletion mutations within any proposed extracellular virulence factor of *A. salmonicida* raises crucial questions about the pathogenesis of this important fish pathogen.**

The fish disease furunculosis derived its name from the characteristic lesions or furuncles formed on the surface of fish as a result of chronic infection with *Aeromonas salmonicida*. Early demonstration that injection of crude preparations of products secreted from the bacterium were lethal and reproduced typical symptoms of the disease (including darkening of the skin, hemorrhaging at the base of the fins, a bleeding vent externally, and necrotic lesions and general liquefaction of tissues internally) led to a search for the toxin(s) responsible for furunculosis. Formation of classic hemorrhagic furuncles has been reproduced following intramuscular injection of a combination of the two major secreted enzymes, the AspA serine protease (9) and glycerophospholipid:cholesterol acyltransferase (7) (GCAT) (14, 18). More importantly, a high-molecular-weight GCAT–lipopolysaccharide (LPS) complex (50% lethal dose [LD<sub>50</sub>] of 45 ng of protein/g of fish) is considered to be the major lethal toxin secreted by *A. salmonicida* (4, 14, 24). Alone, the GCAT-LPS complex resulted in only very limited tissue necrosis, and the cause of death appeared to be respiratory failure. Thus, the above two extracellular enzymes have been implicated as the most important secreted products in *A. salmonicida* pathogenesis. In addition to tissue destruction, proposed roles of serine protease include nutrient acquisition (14, 29) and activation of protoxins, in particular GCAT (13, 21).

GCAT is an unusual bacterial lipase in that it can use cholesterol as an acyl acceptor (7). The protein is synthesized as a preproenzyme of 37.4 kDa (21). Following proteolytic activation of pro-GCAT, the surface activity of the enzyme increases, permitting hydrolysis of membrane phospholipids (19), in turn resulting in lysis of fish erythrocytes, although not directly of mammalian erythrocytes (13, 14). The same enzyme activity has been detected in spent culture supernatants from several members of the closely related genera *Aeromonas* and *Vibrio* (25). Recent computer analysis suggests that this enzyme may belong to a new family of lipases (5, 16). Although the biochemical properties and activity of *A. salmonicida* GCAT (AsGCAT) (7) and later *A. hydrophila* GCAT (AhGCAT) (5, 19, 21) have been studied in detail, no GCAT-deficient mutant has yet been created to ascertain the importance of this unusual enzyme to the pathogenesis of any bacterium. Serine protease-deficient mutant strains created by chemical mutagenesis have been used to examine the role of this protease in virulence, with conflicting results (12, 29). Indeed, in view of the economic importance of *A. salmonicida* to the salmonid farming industry, surprisingly little is known about the factors essential to the pathogenesis of this microorganism.

In this study, isogenic *sata* and *aspA* deletion mutants of *A. salmonicida* were created to define the relative roles of these two secreted toxins in furunculosis. Further evidence to support the role of serine protease in pro-GCAT processing was provided. However, the unexpected result that deletion of GCAT had little effect on virulence underscores the need for more extensive studies with defined genetic mutants to identify factors important to *A. salmonicida* pathogenesis.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic	Source or reference
<b>Strains</b>		
<i>A. salmonicida</i>		
AS1102	Type strain, A-layer negative	NCIMB 1102
644Rb	Virulent isolate, Nal <sup>r</sup> , A-layer positive	35
MT1326	Virulent isolate, A-layer positive	3
CB3	Undefined Tn5 mutant, decreased synthesis of extracellular proteins including GCAT and serine protease	6
644RbΔG	Δ <i>satA</i> :: <i>kan</i> , serine protease, A-layer positive	This study
644RbΔSP	Δ <i>aspA</i> :: <i>kan</i> , pro-GCAT, A-layer positive	This study
MT1326ΔG	Δ <i>satA</i> :: <i>kan</i> , serine protease, A-layer positive	This study
MT1326ΔSP	Δ <i>aspA</i> :: <i>kan</i> , pro-GCAT, A layer positive	This study
<i>E. coli</i>		
HB101	Δ( <i>gpt-proA</i> )62 <i>leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20 xyl-5 mtl-1 recA13</i>	1
DH5α	<i>recA1grrA relA1 Δ(lacIZYA-argF)U169 deoR [φ80dlac lacZΔM15]</i>	1
S17-1	<i>thi pro hsdR hsdM<sup>+</sup> recA [RP4 2-Tc::Mu-Km::Tn7 (Tp<sup>r</sup> Sm<sup>r</sup>) Tra<sup>+</sup>]</i>	32
<b>Plasmids</b>		
pUC18/19	Cloning and sequencing	1
pBSK	Bluescript SKII, cloning and sequencing	Stratagene
M13mp18/19	Sequencing and mutagenesis	1
pSUP202	Mobilizable suicide vector (Amp <sup>r</sup> Tet <sup>r</sup> Cam <sup>r</sup> ColE1 <i>ori</i> Mob <sup>+</sup> )	32
pSM20	Levansucrose confers sucrose sensitivity ( <i>sacRB<sup>+</sup></i> Amp <sup>r</sup> )	31
pRAT1	Kanamycin resistance determinant, lacking transcriptional terminator, on 1.436-kb <i>HaeII</i> fragment from Tn903	D. Evans, University of Reading
pMMB66HE	Broad-host-range expression vector (Amp <sup>r</sup> )	17
pSUP202sac	Mobilizable suicide vector, confers Suc <sup>c</sup> (Mob <sup>+</sup> <i>sacRB<sup>+</sup></i> Tet <sup>r</sup> )	This study
pUC18G	<i>satA</i> on 1.2-kb <i>PstI</i> fragment, cloned in <i>PstI</i> site of pUC18 in reverse orientation to <i>plac</i>	This study
pUC19S	<i>satA</i> on 5.5-kb <i>SacI</i> fragment in <i>SacI</i> site of pUC19	This study
pUC19SΔG	pUC19S with Kan cassette replacing an internal fragment of <i>satA</i>	This study
pSUP202sacΔG	<i>SacI</i> fragment from pUC19SΔ <i>satA</i> :: <i>kan</i> inserted in pSUP202sac	This study
pBSKSP	<i>aspA</i> cloned in pBSK	This study
pBSKΔSP	pBSKSP with Kan cassette replacing small internal fragment of <i>ΔaspA</i>	This study
pSUP202sacΔSP	Δ <i>aspA</i> :: <i>kan</i> on <i>PstI</i> fragment from pBSKSPΔ <i>aspA</i> :: <i>kan</i> in <i>PstI</i> site of pSUP202sac	This study
pMMBG	<i>satA</i> subcloned on 1.2-kb <i>PstI</i> fragment in <i>PstI</i> site of pMMB66HE, under <i>tac</i> control	This study
M13mp18G+/-	<i>satA</i> on 1.2-kb <i>PstI</i> fragment in forward or reverse orientation in <i>PstI</i> site of M13mp18	This study
pMMBG228-Ala	pMMBG encoding mutation of Gly-228 to Ala-228	This study

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The properties and source of all bacterial strains and plasmids used in this study are listed in Table 1.

**Media and growth conditions.** *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani broth or agar (1). *A. salmonicida* strains were grown at 15 to 18°C in tryptic soy agar (TSA) or broth (tryptic soy broth [TSB; Oxoid]) supplemented with Davis minimal medium (6). Media used for screening or selection included egg yolk emulsion (EYE) (20% [vol/vol] egg yolk in 10% [wt/vol] NaCl), egg yolk agar (EYA) (10% [vol/vol] EYE in salt-free nutrient agar), skim milk (50% [vol/vol]) in salt-free nutrient agar (SMA), sucrose (15% [wt/vol]) in TSA (TSAS), and Congo Red (50 μg/ml) in TSA (CRA). Antibiotics were added at the following final concentrations: for *E. coli*, ampicillin (50 μg/ml), tetracycline (10 μg/ml), and kanamycin (25 μg/ml); for *A. salmonicida*, ampicillin (100 μg/ml), tetracycline (2 μg/ml), kanamycin (40 μg/ml), and nalidixic acid (40 μg/ml).

**DNA isolation, manipulation, and sequencing.** Chromosomal DNA was isolated as previously described (11). Plasmid and cosmid DNA was routinely prepared from *E. coli* DH5α by alkali lysis, and pMMB66 derivatives were isolated by the boiling method (1). Fragments were isolated with Prep-a-gene (Bio-Rad). Restriction endonucleases, DNA-modifying enzymes, and polymerases were used as specified by the manufacturers (Gibco BRL and Boehringer Mannheim) and by routine procedures (1). Southern blotting was carried out under standard conditions with probes incorporating digoxigenin-dUTP (DIG-dUTP) created by random hexanucleotide priming or by PCR with specific primers as described (DIG labelling kit; Boehringer Mannheim). Oligonucleotides were supplied by Genosys Ltd. DNA sequencing was performed with a Sequenase version 2.0 kit (Amersham) or by cycle sequencing with an *fmol* DNA sequencing kit (Promega Corp.).

**Cloning and sequencing of the GCAT gene (*satA*).** (i) **1.2-kb *PstI* fragment.** *A. salmonicida* AS1102 chromosomal DNA was digested to completion with *PstI*. Fragments in the 0.8- to 1.4-kb size range were purified from a TAE agarose (1) (0.75%) gel and ligated into *PstI*-digested, dephosphorylated pUC18. Transfor-

mants in *E. coli* HB101 were screened for clearing on EYA plates. Two positive transformants were confirmed by PCR with primers based on the sequence of the gene encoding *A. hydrophila* GCAT (EMBL accession no. X07279). The cloned gene was sequenced in part in pUC18G, and the sequencing was completed in M13mp18G. Although the gene was cloned in the reverse orientation with respect to the *lac* promoter in pUC18G, weak activity was observed on EYA plates and by acyltransferase activity in cell lysates.

(ii) **pLAFR3 cosmid bank.** Clones from an *A. salmonicida* AS1102/pLAFR3 cosmid bank (22) were lifted, denatured on a positively charged nylon membrane, and screened with a 724-bp *satA* probe, created by PCR incorporation of DIG-dUTP (Boehringer Mannheim) with pUC18G as the template and primers SMR10 (5' GCGACAACCTCTCCGATACCGG) and SMR13 (5' GGCGTTC CTGCGGACTGAAG) as described previously (22) (DIG kit). Restriction and Southern analysis of one positive cosmid (p7G7) revealed that the *satA* gene was located on the 10-kb *EcoRV*, 6-kb *NotI*, 5.5-kb *NcoI*, and 5.5-kb *SacI* fragments and that it was centrally located on the *SacI* fragment.

**Cloning of *aspA*.** *aspA* was cloned, in a pMMB66HE derivative, after PCR amplification of the gene from an AS1102 genomic DNA template (100 ng) with tailed primers (200 ng each) SP1 (5' GCGAGTCGACATGGAGTTATAAAT GAAAAACATCG) and SP2 (5' CCGGATCCATCAGGAACGGCTGCGTC GTGACC), based on the published sequence (EMBL accession no. X67043). Cycle conditions were 95°C for 1 min followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min and a single step of 72°C for 10 min with *Pfu* DNA polymerase (Stratagene). For subsequent manipulation, the *aspA* gene was transferred on a 2.0-kb *EcoRI-HindIII* fragment from pMMBSP to *EcoRI-HindIII*-digested pBluescript SKII to form pBSKSP.

**Marker replacement mutagenesis.** (i) **pSUP202sac suicide vector.** The suicide vector pWS233 (31), which carries the sucrose sensitivity *sacRB* genes, appeared to replicate in *A. salmonicida*. Therefore, to facilitate the selection of double-crossover mutants following marker exchange mutagenesis, the *sacRB* cassette was excised from pSM20 on a *PstI-EcoRI* fragment and ligated into the large *PstI-EcoRI* fragment of pSUP202 to create pSUP202sac. pSUP202sac had lost

the functional *bla* and *cam* genes but retained tetracycline resistance and the *Pst*I and *Eco*RI cloning sites (see Fig. 1). Although strains of *A. salmonicida* expressing *sac*RB were resistant to 5% (wt/vol) sucrose, sucrose sensitivity was achieved with 15% (wt/vol) sucrose.

(ii) **Conjugation and mutant selection.** Mutated genes (Fig. 1) were conjugated into *A. salmonicida* 644Rb or strain MT1326 from *E. coli* S-17 on the suicide vector pSUP202sac by filter mating (32) at 15°C for 2 days. *A. salmonicida* primary integrants were then selected in the presence of tetracycline and kanamycin. Nalidixic acid was also added for strain 644Rb; for MT1326, selection against *E. coli* was based solely on growth on TSA at 15°C for 3 days. Selected primary integrants were grown in TSB plus kanamycin prior to growth for 3 to 4 days on TSA containing 15% (wt/vol) sucrose (TSAS) and kanamycin to select for double-crossover mutants. Alternatively, double-crossover mutants were selected directly by growth on TSAS plus kanamycin at 15°C for 4 to 5 days.

**Site-directed mutagenesis of Gly-228 of GCAT.** Site-directed mutagenesis was carried out on M13mp18G with the mismatch (mutation in boldface) oligonucleotide 5'-CTACGACGCCGGCTATGTGTG by the method of Kunkel (23). The mutation was confirmed by the presence of an additional *Ngo*AIV restriction site (underlined) and sequencing, prior to transfer of the mutation on a 0.86-kb *Bam*HI-*Bgl*II fragment to similarly restricted pMMB66G to form pMMB66G228-Ala.

**In vitro processing of proGCAT and SDS-polyacrylamide gel electrophoresis (PAGE).** (i) **Immunoblots.** The source of proGCAT was an overnight (18-h) culture supernatant of *A. salmonicida* CB3/pMMBG following a 30-min induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The source of protease was 60-h culture supernatant from MT1326 $\Delta$ G. Following incubation of 0.9 ml of a pro-GCAT sample at 25°C with 100  $\mu$ l of protease, proteins were precipitated with 10% trichloroacetic acid (TCA), electrophoresed on Laemmli sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels, and analyzed by immunoblotting with an ECL detection kit (Amersham) and 1:10,000 dilution of rabbit anti-pro-GCAT serum.

(ii) **Identification of the C-terminal fragment of processed GCAT.** Induction of *A. salmonicida* CB3/pMMBG was extended to 3 h. Proteins in the recovered culture supernatant were concentrated by precipitation with ammonium sulfate (65% saturation) at 4°C, and pro-GCAT was isolated by high-pressure liquid chromatography on a Superose 12 column (bed volume, 24 ml) equilibrated with 20 mM Tris-HCl (pH 7.5)-0.15 M NaCl at a flow rate of 0.5 ml/min. pro-GCAT was recovered, >98% pure, between the 33- and 35-min fractions. For processing, 3  $\mu$ g of purified pro-GCAT was incubated with 5  $\mu$ l of overnight culture supernatant from AS1102 $\Delta$ G, as a source of serine protease, in a final volume of 100  $\mu$ l at 25°C for 1 h, at which time serine protease was inhibited by the addition of phenylmethylsulfonyl fluoride (PMSF; final concentration, 6.25 mM). The protein was then concentrated by TCA precipitation and analyzed on an SDS-16% polyacrylamide gel.

**Enzyme assays.** The AspA serine protease was assayed with azocasein as the substrate, as previously described (2). Typically, 0.1 to 0.5 ml of culture supernatant was used in a final reaction volume of 2 ml and activity was measured after 10, 20, 60, and 120 min at 37°C. Protease units were calculated as the change in absorbance at 436 nm per minute per milliliter of supernatant. General protease activity against Azocoll was determined from the mean of duplicate samples assayed as described previously (8). Acyltransferase activity of GCAT was monitored qualitatively by thin-layer chromatography of the neutral lipids following incubation of 1 ml of spent bacterial culture supernatant with 3.3 ml of EYE at 37°C for 16 h (25).

**Pathogenicity trials.** Fish trials were performed at the FRS Experimental Fish Production Unit, Aultbea, Scotland, where Atlantic salmon (*Salmo salar* L.) were raised in 1-m<sup>3</sup> aquaria containing approximately 450 liters of salt water for smolts and fresh water for parr, maintained at 14°C and exchanging at 4 liters/min. The fish were fed with BP Mainstream pellets at 2% of body weight per day. Both the fish stock and water source were certified disease free. Salmon parr, all siblings, were potential S2 fish, with an average weight of 100 g, and they were 12 to 17 months old at challenge. Smolts (17 months) were potential S1 fish, full siblings of the members of the first group, and had been in seawater (9 to 15°C) for 3 months prior to challenge. During challenge, the temperature was maintained at 14°C.

(i) **i.p. injection.** The fish were anesthetized by immersion in tricaine methane sulfonate solution (MS-222; Sigma), injected intraperitoneally (i.p.) with 0.1 ml of a given dilution of bacterial suspension or with phosphate-buffered saline (control), dye marked with Alcian blue (Sigma), and returned to the parent tank. A separate tank was used for each bacterial strain. Samples of head kidney from fish that died were plated onto TSA or SMA, and the kanamycin resistance of the recovered *A. salmonicida* was subsequently confirmed. The genotype of randomly selected samples was confirmed by PCR and Southern blotting analysis of isolated DNA.

(ii) **Cohabitation challenge.** Atlantic salmon smolts were maintained as above, and 10% of the population were injected with a potentially lethal dose (10<sup>5</sup> in 100  $\mu$ l of sterile phosphate-buffered saline [pH 7.4]) of the appropriate strain and returned to the parent tank, with two separate tanks being used for the wild-type strain and two for each mutant strain. The injected fish died within 2 to 3 days and were immediately removed. The uninjected cohabitants began to die after a further 4 to 5 days and were removed and screened as above. The fish were monitored for 21 days.

**Statistics.** The final LD<sub>50</sub> and effective doses giving 50% cumulative mortality (ED<sub>50</sub>) on a given day were calculated by logistic regression analysis, with 95% confidence intervals calculated from Fieller's theorem with Genstat 5 release 3.2 (Lawes Agricultural Trust, Rothamstead Experimental Station). Independence of tanks and strains (cohabitation trial) was determined by the chi-square test, and differences between groups were determined by Student's *t* test (36).

## RESULTS

**Cloning and properties of the GCAT gene (*satA*).** The gene encoding GCAT was initially cloned and sequenced from *A. salmonicida* AS1102 on a 1,176-bp *Pst*I fragment. Subsequently, to facilitate allelic replacement, it was cloned from a pLAFR3 cosmid (p7G7) on a 5.5-kb *Sac*I fragment (see Materials and Methods for details). The same 35.4-kDa pro-GCAT was produced and secreted in reasonable levels from both the *Pst*I and *Sac*I fragments following transfer of the fragments individually to the broad-host-range plasmid pMMB66HE and conjugation into *A. salmonicida* CB3. The DNA sequence of the cloned gene, which encoded a 335-residue protein including an 18-amino-acid signal sequence, was identical to that recently published (27) for *satA* from *A. salmonicida* FT449 (GenEmbl Accession no X70686). It had 93.7% amino acid identity to the AhGCAT precursor (SwissProt accession no. P10480), including the proposed active-site residues, Ser-16, Asp-116, and His-291 (5). The only notable difference between the two sequences was the conservation of tandem Gly residues but with a single residue shift: 225-CYGGSYV for AhGCAT and 225-CYDGGYV for AsGCAT.

**Marker replacement mutagenesis.** (i) ***A. salmonicida*  $\Delta$ *satA*::*kan* mutants.**  $\Delta$ *satA*::*kan* mutants (referred to as  $\Delta$ G) of two virulent isolates of *A. salmonicida* (strains MT1326 and 644Rb) were created by marker replacement mutagenesis as outlined in Fig. 1. Deletion of the 561-bp *Bgl*II-*Ngo*AIV fragment removed approximately half of the *satA* open reading frame including DNA encoding Asp-116, which is proposed to form part of the active site (5). Southern analysis revealed that the genotypes of three tetracycline-sensitive recombinants for each strain were identical with respect to *satA* (examples are shown in Fig. 2). For all six isolates, the single wild-type *satA* gene had been replaced with the mutated  $\Delta$ *satA*::*kan* gene. Southern analysis also confirmed that the *kan* cassette was inserted only in the target *satA* gene. Phenotypic analysis confirmed loss of GCAT (data not shown). No GCAT could be detected by immunoblotting, and no acyltransferase activity was observed in the spent-culture supernatants taken at several different stages of growth of *A. salmonicida* 644 $\Delta$ G and *A. salmonicida* MT1326 $\Delta$ G. Immunoblotting also excluded the accumulation of any truncated product of GCAT inside the cells. Serine protease production in the *satA* mutant strains was unaffected, as was the growth pattern, outer membrane profile, and presence of the A-layer (defined by Congo red binding together with the outer membrane profiles) (data not shown).

(ii) ***A. salmonicida*  $\Delta$ *aspA*::*kan* mutants.** The *aspA* gene was cloned as a 2-kb PCR product from *A. salmonicida* AS1102 (see Materials and Methods). Kanamycin replacement mutagenesis resulted in removal of the central 159-bp *Nco*I fragment encoding the active-site Ser-330 (9) (Fig. 1). Mutants of *A. salmonicida* MT1326 and 644Rb were created by allelic replacement. The genotypes of three isolates of each strain were confirmed by Southern blotting (examples are given in Fig. 2).

The AspA serine protease-deficient phenotype of each mutant was confirmed by using azocasein as substrate. No activity could be detected in 60-h culture supernatants (Table 2), and no product was detected by immunoblotting of 24-, 40-, or 60-h

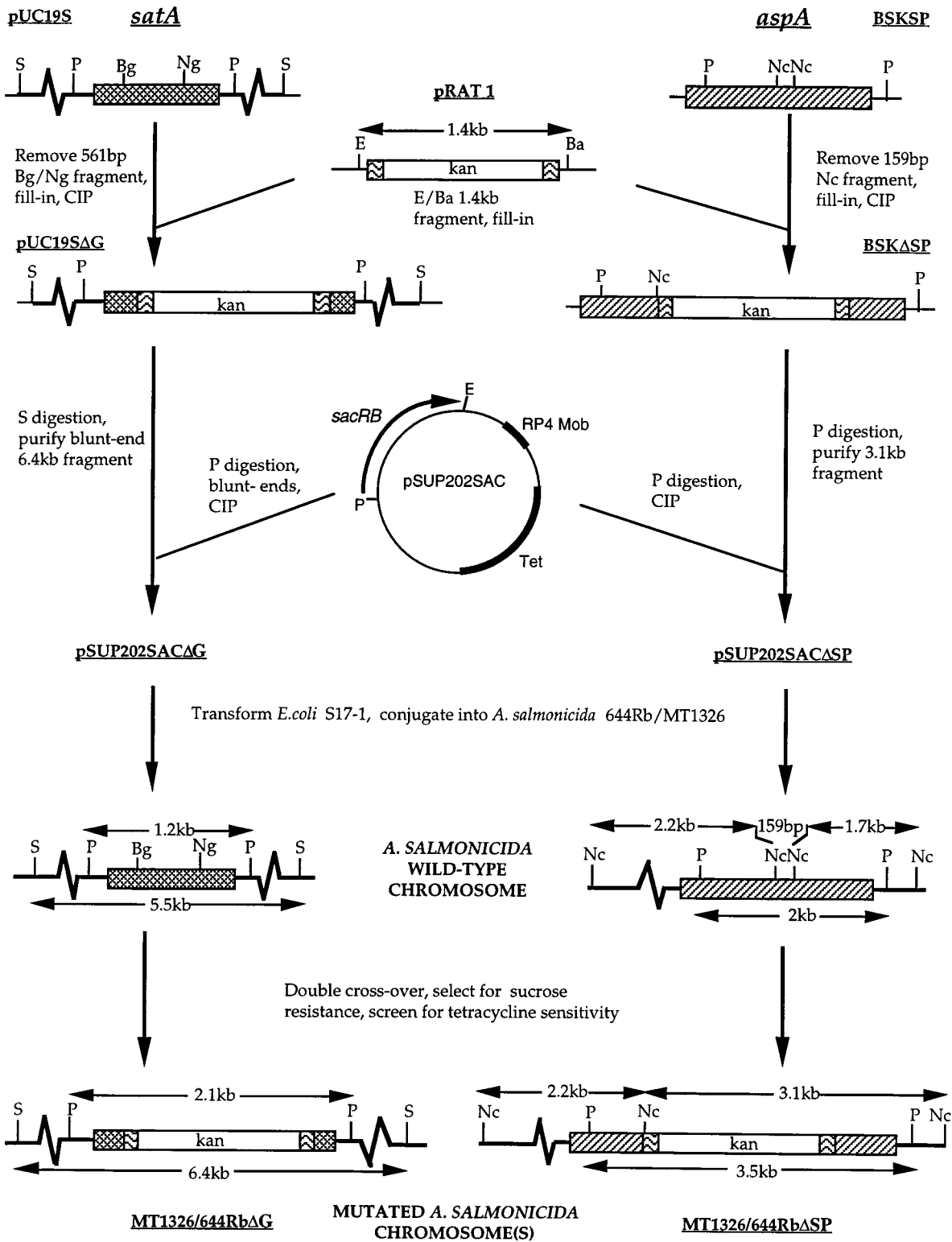


FIG. 1. Schematic diagram of the procedure used to create the *satA* and *aspA* deletion mutant strains of *A. salmonicida*. Thick line, *A. salmonicida* chromosomal DNA; thin line, vector DNA; cross-hatched box, *satA* gene encoding GCAT; hatched box, *aspA* gene encoding serine protease; wavy lines, 1,436-bp *Hae*II fragment originating from Tn903, encompassing the kanamycin resistance gene, *kan* (blank); *sacRB*, levansucrase gene conferring sucrose sensitivity. Ba, *Bam*HI; Bg, *Bgl*III; E, *Eco*RI; Nc, *Nco*I; Ng, *Ngo*IV; P, *Pst*I; S, *Sac*I; CIP, calf intestinal phosphatase.



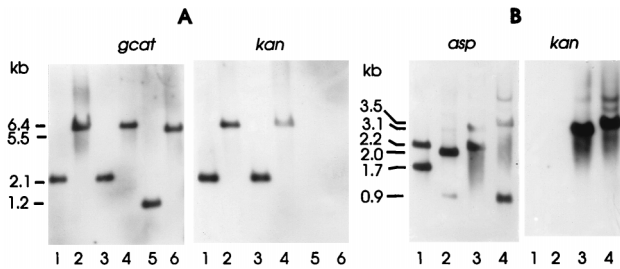


FIG. 2. Southern blot analysis of chromosomal deletion mutants. (A) Chromosomal DNA from wild-type *A. salmonicida* 644Rb (lanes 5 and 6), *A. salmonicida* 644Rb $\Delta$ G (lanes 1 and 2) and *A. salmonicida* MT1326 $\Delta$ G (lanes 3 and 4) digested with *Pst*I (lanes 1, 3, and 5) and *Sac*I (lanes 2, 4, and 6) and hybridized with a *satA* or kanamycin cassette probe (*kan*) as indicated. Wild-type *satA* fragments were 1.2 kb (*Pst*I) and 5.5 kb (*Sac*I); *satA*:*kan*-derived fragments were 2.1 kb (*Pst*I) and 6.4 kb (*Sac*I). (B) Wild-type MT1326 (lanes 1 and 2) and MT1326 $\Delta$ ASP (lanes 3 and 4) digested with *Nco*I (lanes 1 and 3) or *Pst*I (lanes 2 and 4) and detected with an *aspA* or kanamycin cassette probe (*kan*) as indicated. Relevant wild-type *aspA* fragments were 1.7 kb (*Nco*I) and 2 kb (*Pst*I); *aspA*:*kan*-derived fragments were 3.1 kb (*Nco*I) and 3.5 kb (*Pst*I). Three independent  $\Delta$ *satA*:*kan* isolates for each strain were identical, as were three independent  $\Delta$ *aspA*:*kan* isolates.

culture supernatants (data not shown) from any of the  $\Delta$ *aspA*:*kan* mutants (referred to as  $\Delta$ SP). It was noted from both the enzyme assays and the immunoblots that the parent *A. salmonicida* MT1326 produced considerably more of this enzyme (5.33 U) than did the wild-type *A. salmonicida* 644Rb (0.18 U). While the azocasein substrate is apparently specific to serine protease, Azocoll is degraded by a wider range of proteases. The use of Azocoll as the substrate also reflected loss of AspA in the mutants (compare the activities without inhibitor and in the presence of EDTA [Table 2]). For MT1326 $\Delta$ SP, the remaining activity could be attributed largely to the secreted metalloprotease. However, low levels of a second serine protease (PMSF sensitive) were detectable in samples from all mutant strains. With Azocoll substrate and EDTA and PMSF inhibitors, synergistic activity between the metalloenzyme(s) and serine protease(s) was also evident in all samples. A large increase in metalloprotease activity was evident in the MT1326 $\Delta$ SP strains. Since the mutant and parent strains grew at similar rates (all in absence of kanamycin), the

TABLE 2. Proteolytic activity of 60-h culture supernatants from wild-type and *aspA* mutant *A. salmonicida*

Strain <sup>a</sup>	Activity (units <sup>b</sup> ) in supernatant with substrate <sup>c</sup> :			
	Azocasein (no inhibitor)	Azocoll		
		No inhibitor	10 mM EDTA	6.25 mM PMSF
MT1326 (wt)	5.33	1.98 <sup>1</sup>	0.84	0.21 <sup>d</sup>
MT1326 $\Delta$ SP-1	<0.015	1.46 <sup>1</sup>	0.21	0.86
MT1326 $\Delta$ SP-2	<0.015	1.37 <sup>1</sup>	0.24	0.83
644Rb (wt)	0.18	0.49 <sup>2</sup>	0.27 <sup>3</sup>	0.07
644Rb $\Delta$ SP-1	<0.015	0.22 <sup>2</sup>	0.12 <sup>3</sup>	0.05
644Rb $\Delta$ SP-2	<0.015	0.25 <sup>2</sup>	0.17 <sup>3</sup>	0.1

<sup>a</sup> wt, wild type. 1 and 2 indicate isolate 1 and isolate 2, respectively.

<sup>b</sup> One unit of protease activity = 1 OD unit change/ml of culture supernatant/h/OD<sub>650</sub> culture density.

<sup>c</sup> <0.015, below the limit of detection, which was 0.015 unit. Superscript numbers indicate that the mutants were significantly different from the respective parental wild type as calculated by Student's *t* test with 4 degrees of freedom: 1, *P* = 0.0026; 2, *P* = 0.0013; 3, *P* = 0.0067.

<sup>d</sup> In the presence of EDTA plus PMSF, the activity of the MT1326 wild-type supernatant against Azocoll was reduced to 0.05 unit.

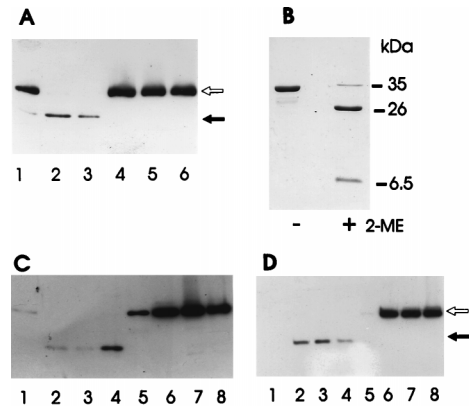


FIG. 3. Importance of the 64-kDa serine protease in pro-GCAT processing. (A) Processing of pro-GCAT by MT1326 $\Delta$ G culture supernatant in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of 6.25 mM PMSF. Samples were incubated for 10, 20, or 60 min (lanes 1 and 4, 2 and 5, and 3 and 6, respectively) before being analyzed by SDS-PAGE and immunoblotting. (B) Coomassie blue-stained SDS-polyacrylamide gel of purified and processed GCAT solubilized in the absence (-) or presence (+) of 5%  $\beta$ -mercaptoethanol (2-ME). The approximate sizes of intact GCAT and processed fragments are indicated. (C and D) Absence of in vivo processing of pro-GCAT in *A. salmonicida* 644Rb $\Delta$ SP (C) and *A. salmonicida* MT1326 $\Delta$ SP (D) mutants. Culture supernatant samples were taken after growth for 24, 40, 50, or 65 h (lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8, respectively). Lanes: 1 to 4, parent strains; 5 to 8,  $\Delta$ SP mutants. An immunoblot of an SDS-polyacrylamide gel of 10-fold-concentrated samples is shown. In all cases, the open arrow indicates pro-GCAT (35.1 kDa) and the solid arrow indicates the N-terminal fragment of processed GCAT (approximately 26 kDa).

most likely explanation for this is the absence of serine protease-mediated degradation of metalloprotease in the mutant strains.

**Serine protease-mediated processing of pro-GCAT.** In vitro studies had previously indicated that the AspA serine protease was responsible for pro-GCAT processing in *A. salmonicida* (13). Culture supernatants from *A. salmonicida* MT1326 also mediated PMSF-sensitive processing of pro-GCAT (Fig. 3A). Confirmation of the role of the *aspA*-encoded serine protease in pro-GCAT processing was obtained by analysis of GCAT in culture supernatants from the *A. salmonicida*  $\Delta$ *aspA*:*kan* mutants. No processed GCAT could be detected in samples from either *A. salmonicida* 644 $\Delta$ SP or *A. salmonicida* MT1326 $\Delta$ SP, while only the mature form of GCAT was seen in samples taken at the same time points from the parent strains (Fig. 3C and D). Lower levels of processed GCAT than of pro-GCAT were consistently observed in immunoblots. This could be attributed to the lower reactivity of the N-terminal fragment of GCAT with the antibody and possibly also to further proteolytic degradation of processed GCAT.

Previous estimates of the size of processed AsGCAT vary from 24 to 26 kDa (7, 13, 24). However, in this study, the use of nonreducing conditions in SDS-PAGE and overproduced pro-GCAT demonstrated that serine protease-processed GCAT was approximately 33 kDa. The C-terminal fragment (approximately 6.5 kDa) was not degraded but remained associated with the N-terminal fragment via a disulfide bond between Cys-225 (N-terminal fragment) and Cys-281 (C-terminal fragment) (Fig. 3B). Clearly, on processing, only a small fragment of pro-GCAT was lost. Maintenance of the C-terminal fragment is consistent with the similar structure of trypsin-processed AhGCAT (19) and the proposal that Ser-16, Asp-116, and His-291 constitute the catalytic triad of the active site of this lipase (5).

The possibility that conservation of the double Gly sequence

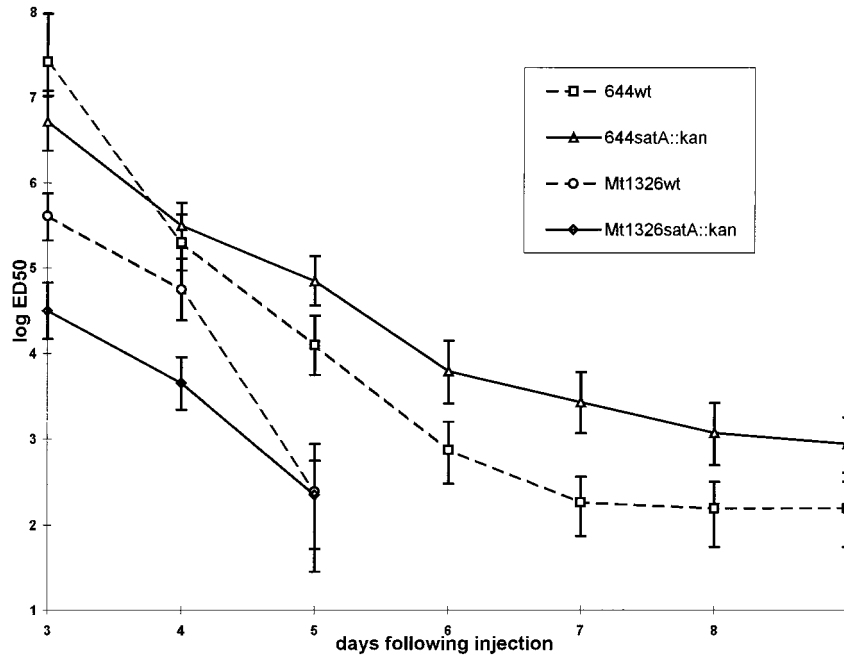


FIG. 4. Mortality rate in Atlantic salmon parr following i.p. injection with wild-type or *satA* mutant strains. Groups of 20 fish were injected with one dose, ranging in a 10-fold series from  $10^9$  to  $10^3$  CFU of the respective parent or *satA* mutant of *A. salmonicida* or PBS, as described in Materials and Methods, and monitored for 9 days. From day 6 onward, the estimated ED<sub>50</sub> of both the parent and *satA* mutant of strain MT1326 was lower than the lowest dose tested (100 CFU). Values for the PBS control (number of dead fish out of 20 fish injected/tank): MT1326, 0, MT1326ΔG, 1 (day 5); 644Rb, 1 (day 7); 644ΔG, 0.

(see above) is important to proteolytic processing of GCAT was considered. However, replacement of Gly-228 with Ala-228 in AsGCAT did not inhibit the processing of proGCAT (data not shown).

**Importance of GCAT and serine protease to pathogenesis.**

(i) **i.p. injection.** The virulence of the *satA* deletion mutants was compared to that of their respective parent strains, *A. salmonicida* MT1326 and *A. salmonicida* 644Rb, by i.p. challenge of Atlantic salmon (parr, trial 1 [Fig. 4], smolts, trial 2 [Table 3]). In this model system, GCAT was clearly not a major virulence factor, as might be predicted from the earlier toxicity studies (24). Following injection of Atlantic salmon parr with the highly virulent strain *A. salmonicida* MT1326, one could only conclude that the LD<sub>50</sub> of both the parent and *satA* mutant was  $<10^2$ . Even at the lowest injected dose of  $10^2$ , the mortality was higher than 80% for both strains. At the end of trial 1 (9 days), the LD<sub>50</sub> of the *A. salmonicida* 644Rb parent strain was estimated to be  $1.55 \times 10^2$  (95% confidence inter-

vals =  $3.16 \times 10^2$  and  $5.48 \times 10^1$ ) compared to an LD<sub>50</sub> of *A. salmonicida* 644RbΔG of  $8.7 \times 10^2$  (95% confidence intervals =  $1.77 \times 10^3$  and  $4 \times 10^2$ ) (Fig. 4). The fish injected with the parent or the respective isogenic *satA* mutant died at very similar rates. The estimated ED<sub>50</sub> was already below the smallest number of bacteria injected (100 CFU) on day 6 for both parent and mutant strains of *A. salmonicida* MT1326. Also, very similar patterns of time to death were followed for *A. salmonicida* 644Rb parent and *A. salmonicida* 644Rb *satA*. In this analysis, a slightly higher but statistically different ED<sub>50</sub> was maintained from days 5 to 9 for the 644Rb *satA* mutant compared to its parent (Fig. 4).

Examination of mortalities revealed no discernible difference in the disease pathology caused by wild-type and *sat* mutant strains. The characteristic pathologic findings of the acute form of furunculosis were observed in both cases, namely, darkened skin; hemorrhages at the base of the fins, along the belly, and internally; and a swollen and hemorrhaging vent. Moribund fish displayed lethargy, increased respiration rate, and loss of equilibrium.

Atlantic salmon smolts were also challenged with *A. salmonicida* MT1326 and a comparison was made with the condition of the fish infected with the isogenic *satA* and *aspA* mutants. This also reflected the retention of very high virulence of both mutants tested. No statistically significant difference in virulence was detected at either 8 or 15 days (in the latter case, most fish had died) (Table 3). As above, no obvious difference in disease pathology between the parent and mutant strains was evident.

Analysis of bacteria isolated from dead fish in both trials confirmed that only kanamycin-resistant *A. salmonicida* strains were recovered from fish injected with either of the *satA* or *aspA* deletion mutants. Southern analysis with *satA* and *kan* or *aspA* and *kan* probes resulted in blots identical to those shown in Fig. 2A and B for MT1326ΔG and MT1326ΔSP, respec-

TABLE 3. Challenge of Atlantic salmon smolts by i.p. injection (trial 2)<sup>a</sup>

<i>A. salmonicida</i> strain	Day 8			LD <sub>50</sub> (CFU) on day 15 <sup>b</sup>
	ED <sub>50</sub> (CFU)	+95% CI (CFU)	-95% CI (CFU)	
MT1326	$2.63 \times 10^3$	$2.01 \times 10^4$	$1.42 \times 10^2$	$<10^2$
MT1326ΔG	$1.08 \times 10^3$	$8.18 \times 10^3$	$2.64 \times 10^1$	$<10^2$
MT1326ΔSP	$4.55 \times 10^3$	$7.13 \times 10^4$	$3.49 \times 10^1$	$6.25 \times 10^2$

<sup>a</sup> Groups of six fish were injected with each dose (ranging from  $10^8$  to  $10^2$  CFU) of the parent or respective mutants of *A. salmonicida* MT1326 or PBS, as described in Materials and Methods, and monitored for 15 days. There were no deaths among PBS controls.

<sup>b</sup>  $<10^2$ , LD<sub>50</sub> lower than the lowest dose tested ( $10^2$  CFU). The lower estimated 95% confidence interval (CI) for MT1326ΔSP was below zero; therefore, no valid statistical comparison to the parent strain can be made.

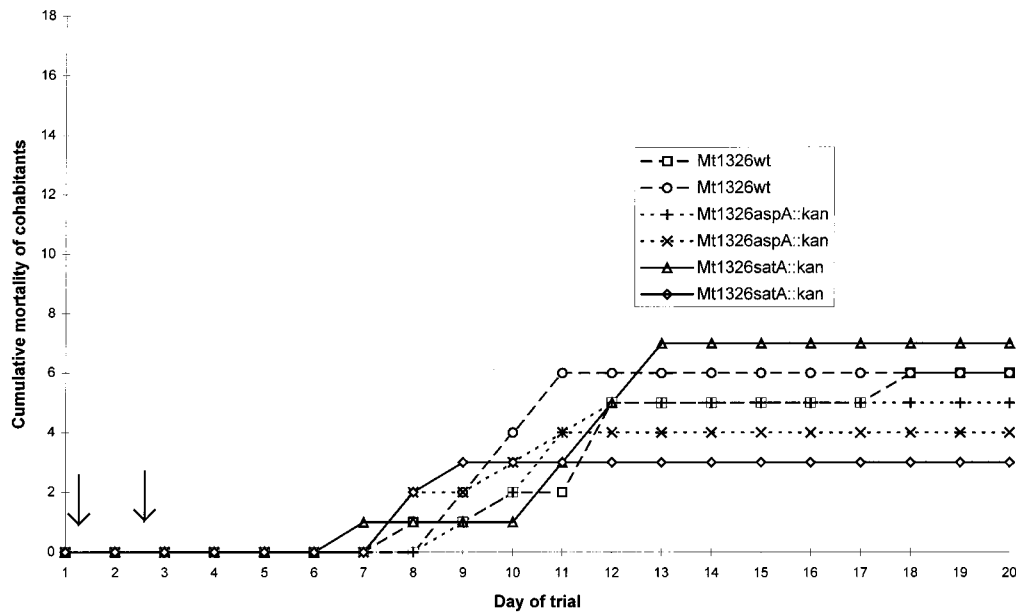


FIG. 5. Mortality rate in Atlantic salmon following cohabitation with fish infected with parent or mutant *A. salmonicida* MT1326 (trial 3). The cohabitation challenge was performed with duplicate tanks for each strain, as indicated, and 18 test fish plus 2 injected fish per tank (see Materials and Methods for details). Arrows indicate the death of injected fish within 2 or 3 days of injection. A consequence of the nature of this trial is the significant variation between duplicate tanks. Mean (and relative) mortalities were 33% (100%), 27.7% (83%), and 25% (75%), for MT1326 parent, MT1326 *satA*, and MT1326 *aspA*.

tively. This confirmed that the deletion in the *satA* and *aspA* open reading frames was stably maintained during the course of the trial and excluded any possibility of cross-contamination.

**(ii) Cohabitation challenge.** The evidence from the i.p. challenge trials contradicted the widely accepted role of GCAT in virulence. There was some concern that the i.p. challenge model of infection used in the trial was bypassing a critical stage in the natural infection process during which GCAT was required. For this reason, the virulence of both deletion mutants was further assessed by using a cohabitation model, which more closely replicates a natural outbreak of the disease (3, 26). Figure 5 shows the results of the cohabitation challenge for wild-type MT1326 and the isogenic *satA* and *aspA* deletion mutants. The average cumulative mortalities of fish infected with MT1326 $\Delta$ G (27%) and MT1326 $\Delta$ SP (25%) were only marginally lower than that (33%) of fish infected with the wild-type strain. The difference between these strains was not statistically significant (for independence of tank and bacterial strain,  $\chi^2 = 0.95$  with 2 degrees of freedom). This is in comparison to results of previous studies, where less virulent strains (*A. salmonicida* MT046 and MT004, with LD<sub>50</sub>s of 10<sup>6</sup> by i.p. injection) failed to induce any mortality in a cohabitation trial (2a). Thus, the cohabitation trial also indicated that both mutant strains retained virulence. It is noteworthy that the mortality rates due to both mutant strains were again indistinguishable from that due to the wild type. Fish began to die between days 6 and 8, and, except for one much later death, all the fish had died by days 11 to 13 (Fig. 5). Screening of the isolates recovered, as above, confirmed the stability of the mutations and absence of cross-contaminants.

## DISCUSSION

The absence of any significant effect of *satA* deletion on the virulence of two different isolates of *A. salmonicida* demonstrates that the lipase, GCAT, is not, as might be predicted from the toxicity studies (24), a major virulence factor of this

bacterium. (For comparison, loss of the A-layer is generally associated with an increase in the LD<sub>50</sub> from approximately 10<sup>2</sup> or less to >10<sup>6</sup> [34].) The possibility that the nonessential role of GCAT is a reflection of the challenge model is highly unlikely. A cohabitation model (3, 26), which closely approximates the natural route of infection and which would reflect requirements for the early establishment of infection, was tested in addition to the more invasive i.p. injection model.

The earlier toxicity studies (24) identified a high-molecular-weight complex of GCAT and LPS as the major lethal toxic product released from plate cultures of this bacterium. More recent studies suggest that much of the polysaccharide in this complex may be derived from the capsule rather than from LPS (4). The purified GCAT-LPS-polysaccharide complex had an LD<sub>50</sub> of 45 ng of protein/g Atlantic salmon body weight, whereas the soluble form of GCAT was less toxic, with an LD<sub>50</sub> of 338 ng of protein/g of Atlantic salmon body weight. It was concluded that GCAT itself was the major toxin, since up to 0.714 mg of *A. salmonicida* LPS/g of salmonid fish lacked toxicity (24). Also, toxicity was lost on heating the GCAT complex at 60°C for 30 min, providing additional evidence that the enzyme GCAT, and not any form of LPS or carbohydrate associated with the high-molecular-weight complex, mediated toxicity (24). The LPS or carbohydrate may simply increase the stability of the protein toxin in vivo and possibly enhance the targeting of GCAT to the eucaryotic cell membrane. The toxicity studies and pathogenicity trials are, of course, totally different models, with differences in the levels of GCAT, sites of delivery, and presentation of the toxin. For example, the extent to which GCAT is complexed with LPS following in vivo production in fish is unknown. Certainly, in vitro the ratio of soluble GCAT to GCAT-LPS complex is evidently dependent on the culture method (7, 24). Regardless of the factors that determine the degree of toxicity of GCAT, it is clear from the results presented here that this unusual lipase is not essential to the establishment of acute furunculosis and death in Atlantic salmon.



The results with the defined *A. salmonicida* MT1326  $\Delta$ aspA::kan mutant confirm the earlier findings of Drinan and Smith (12) that a mutant obtained by random chemical mutagenesis and deficient in serine protease had a maximum 10-fold increase in LD<sub>50</sub>. The sequence downstream of *aspA* has not been determined. However, if there had been any polar effect in the *aspA*::kan mutation, the results would not have been affected, since this mutation had no dramatic *in vivo* effect. Like GCAT, AspA serine protease is evidently not essential for acute *A. salmonicida* subsp. *salmonicida*-induced furunculosis in salmon, even though one of the primary roles of AspA serine protease might be in activating not only pro-GCAT but also precursors of other secreted enzymes or toxins. In agreement with studies with purified serine protease (13), AspA was shown here to be essential for pro-GCAT processing in broth cultures. Despite the existence of metalloprotease(s) and a second serine protease in spent growth medium from strain MT1326 $\Delta$ aspA::kan, none of the pro-GCAT was processed. The extent to which host proteases might replace serine protease in precursor processing remains to be seen. Although the larger decrease in virulence reported by Sakai (29) for a protease-deficient mutant could reflect real strain variation, the possibility that this chemically mutagenized mutant possessed multiple mutations cannot be excluded.

A marginal increase was observed in the LD<sub>50</sub> of the *A. salmonicida* 644Rb *satA* mutant, compared to its respective parent strain (Fig. 4). Whether this reflects a real marginal decrease in virulence of the *satA* mutant may be tested most effectively in competition trials. Should this be the case, it may reflect a role of GCAT, like serine protease or any of the other secreted enzymes and toxins, in the maximization of bacterial growth during infection. GCAT and serine protease may represent only two of a battery of extracellular aggressors that individually are not essential but together aid the progression of disease, e.g., by enhancing nutrient acquisition, tissue destruction, and attack on components of the host defense system. Loss of one may be compensated by another. For example, preparations of both GCAT and serine protease lead to the development of thrombosis and circulatory failure in Atlantic salmon (30). It is possible that no single potent toxin is produced by *A. salmonicida* and that maximization of bacterial growth is of prime importance to its pathogenesis. Proliferation and dissemination of the organism might then lead to mortality through acute bacteremia. The requirement for possession of a surface A-layer for the virulence of most strains of *A. salmonicida* (34) is certainly consistent with this notion.

However, although GCAT in combination with LPS is by far the most toxic product thus far identified in cultures of *A. salmonicida*, the possibility that this bacterium produces a more potent unidentified toxin(s) should not be excluded. Candidates for this might be the potent cytolytic aerolysin (21) or acetylcholinesterase (28), both of which have been identified in *A. hydrophila*. The *aerA* gene has been identified in *A. salmonicida*, but no active hemolysin is produced, possibly due to lack of correct *in vitro* processing (20, 21). Confirmation of acetylcholinesterase production still awaits the identification of a gene encoding this enzyme in any *Aeromonas* spp. In addition, it would not be surprising if *A. salmonicida* targeted toxins directly into eucaryotic cells via a "contact" (type III) secretion pathway (10). The widespread nature and the importance of this mechanism of toxin delivery are now evident from the recent identification of genes for this system in several different pathogens of the closely related enterobacterial family.

Finally, while secreted products in concert may enhance growth during infection, the importance of the extracellular enzymes to the bacterium may actually be in the final stages of

infection, i.e., dissemination, rather than in the disease process itself. We have recently shown that production of AspA protease is affected by the quorum-sensing *asaI/R* regulatory system and occurs only at high cell density *in vitro* (33). Serine protease is detectable at high levels *in vivo* in furuncles (15), but production at earlier stages of infection has not been analyzed. Nutrient limitation is likely to be most severe at high cell density. Thus, should AspA protease also be produced only at high cell density *in vivo*, its importance would be in maintaining bacterial viability and multiplication prior to release from the fish into the water, where nutrients are limiting and the viability of *A. salmonicida* is greatly decreased. This would be of particular importance to bacterial infection in the presence of very low densities of fish, as is found in the natural environment. Clearly, understanding of the relative importance of the secreted products of *A. salmonicida* to the pathogenesis of furunculosis is still in its infancy.

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