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Characterization of an ADP-Ribosyltransferase Toxin (AexT) from *Aeromonas salmonicida* subsp. *salmonicida*

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An ADP-ribosylating toxin named *Aeromonas salmonicida* exoenzyme T (AexT) in *A. salmonicida* subsp. *salmonicida*, the etiological agent of furunculosis in fish, was characterized. Gene *aexT*, encoding toxin AexT, was cloned and characterized by sequence analysis. AexT shows significant sequence similarity to the ExoS and ExoT exotoxins of *Pseudomonas aeruginosa* and to the YopE cytotoxin of different *Yersinia* species. The *aexT* gene was detected in all of the 12 *A. salmonicida* subsp. *salmonicida* strains tested but was absent from all other *Aeromonas* species. Recombinant AexT produced in *Escherichia coli* possesses enzymatic ADP-ribosyltransferase activity. Monospecific polyclonal antibodies directed against purified recombinant AexT detected the toxin produced by *A. salmonicida* subsp. *salmonicida* and cross-reacted with ExoS and ExoT of *P. aeruginosa*. AexT toxin could be detected in a wild type (wt) strain of *A. salmonicida* subsp. *salmonicida* freshly isolated from a fish with furunculosis; however, its expression required contact with RTG-2 rainbow trout gonad cells. Under these conditions, the AexT protein was found to be intracellular or tightly cell associated. No AexT was found when *A. salmonicida* subsp. *salmonicida* was incubated in cell culture medium in the absence of RTG-2 cells. Upon infection with wt *A. salmonicida* subsp. *salmonicida*, the fish gonad RTG-2 cells rapidly underwent significant morphological changes. These changes were demonstrated to constitute cell rounding, which accompanied induction of production of AexT and which led to cell lysis after extended incubation. An *aexT* mutant which was constructed from the wt strain with an insertionally inactivated *aexT* gene by allelic exchange had no toxic effect on RTG-2 cells and was devoid of AexT production. Hence AexT is directly involved in the toxicity of *A. salmonicida* subsp. *salmonicida* for RTG-2 fish cells.

Aeromonas salmonicida subsp. *salmonicida* is the etiological agent of furunculosis of *Salmonidae*. This fish disease causes most severe losses in production farms of salmon and trout and leads to the use of large amounts of antibiotics in closed and open waters for prevention and therapy of furunculosis. To develop efficient strategies to prevent outbreaks of *A. salmonicida* subsp. *salmonicida*, it is essential to know the main mechanisms of pathogenicity of this pathogen. Several potential virulence factors of *A. salmonicida* subsp. *salmonicida* have been described thus far. They include the surface array layer protein (7); hemolysins ASH1, ASH3, and ASH4 (12); H-lysin (29); salmolysin (19); serine protease AspA (32); and the glycerophospholipid:cholesterol acyltransferase (GCAT) complexed with lipopolysaccharide (18). Recent reports demonstrate the role of the S layer in adhesion (11) of *A. salmonicida* subsp. *salmonicida*. The other potential virulence factors of *A. salmonicida* subsp. *salmonicida* that are currently known do not seem to play a primary role in pathogenesis. GCAT and *aspA* gene deletion mutants showed that neither GCAT nor *aspA* is essential for acute *A. salmonicida* subsp. *salmonicida*-induced furunculosis (30). However, AspA is essential for pro-GCAT processing in broth cultures and might also be involved in activation of other secreted enzymes or toxins.

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Several pathogenic bacteria use ADP ribosylation as a key mechanism to modify the properties of host cell proteins and thus to modulate their function and induce disease. Hence ADP ribosylation of eukaryotic regulatory proteins is the underlying pathogenic mechanism of a heterogeneous family of bacterial protein toxins. ADP-ribosylating toxins are broadly distributed among highly pathogenic bacteria and are the primary cause of various severe human diseases such as diphtheria, cholera, and pertussis. Among this family of toxins, the ADP-ribosyltransferase toxin called exoenzyme S (ExoS) of *Pseudomonas aeruginosa* is one of the most prominent representatives. It is secreted via a type III-dependent secretion mechanism (33, 34). Recently it was shown that ExoS is a bifunctional toxin (23) containing an N-terminal part, which resembles that of the *Yersinia* YopE toxin and which catalyzes *rho*-dependent actin depolymerization, and a C-terminal ADP-ribosylating domain. Unlike most bacterial toxins, ADP-ribosylating toxin ExoS does not have a rigid target protein specificity. Intracellular expression of the amino-terminal domain of ExoS elicits the disruption of actin, while expression of the carboxyl-terminal domain of ExoS produces factor-activating exoenzyme S (FAS)-dependent ADP-ribosyltransferase activity and is highly cytotoxic to eukaryotic cells (20).

Here we characterize an ADP-ribosylating protein derived from *A. salmonicida* subsp. *salmonicida* which has a significant sequence similarity to the ExoS and the related ExoT toxins of *P. aeruginosa* and we demonstrate its role in toxicity to fish cells.

TABLE 1. *Aeromonas* strains used

Species	Strain	No. of *aexT*-positive strains[a]/ no. of strains tested
A. salmonicida subsp. *salmonicida*	ATCC 33658^T	1/1
A. salmonicida subsp. *salmonicida*	JF2267[b]	1/1
A. salmonicida subsp. *salmonicida*	JF2580[c]	
A. salmonicida subsp. *salmonicida*	Field isolates	10/10
A. bestiarum	CDC9533-76	0/1
A. bestiarum	Field isolates	0/2
A. caviae	ATCC 15468^T	0/1
A. caviae	Field isolates	0/3
A. encheleia	DSM 11577^T	0/1
A. eucrenophila	NCMB74^T	0/1
A. eucrenophila	Field isolate	0/1
A. hydrophila	ATCC 7966^T	0/1
A. hydrophila	Field isolates	0/15
A. jandaei	ATCC 49568^T	0/1
A. media	ATCC 33907^T	0/1
A. schubertii	ATCC 43700^T	0/1
A. schubertii	Field isolate	0/1
A. sobria	CIP 74-33^T	0/1
A. trota	ATCC 49657^T	0/1
A. trota	Field isolate	0/1
A. veronii	ATCC 35624^T	0/1
A. veronii	Field isolates	0/4

[a] Determined by Southern blotting using DIG-labeled RAEXT as the probe.
[b] Isolated freshly from an arctic char with typical symptoms of furunculosis. Identification was done phenotypically and by 16S rRNA gene sequencing.
[c] *aexT* mutant derived from JF2267 by allelic exchange with an *aexT*::Km^r construct.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmid cloning vectors. *Aeromonas* sp. strains (Table 1) were routinely cultured on blood agar plates (Trypticase soy agar supplemented with 0.1% $CaCl_2$ and 5% sheep blood) at 37°C except for *A. salmonicida* subsp. *salmonicida*, which was grown at 19°C. *A. salmonicida* subsp. *salmonicida* strain JF2267 was freshly isolated from an arctic char (*Salvelinus alpinus*) with typical furunculosis symptoms. The strain was identified as *A. salmonicida* subsp. *salmonicida* by a routine diagnostic agglutination test using rabbit anti-*A. salmonicida* subsp. *salmonicida*-specific antiserum and by sequence analysis of the *rrs* (16S rRNA) genes as described by Kuhnert et al. (15). For genetic modifications, *A. salmonicida* subsp. *salmonicida* was grown on Luria-Bertani (LB) agar plates (2) supplemented as necessary with 50 μg of kanamycin/ml or with 50 μg of chloramphenicol/ml. Liquid cultures of *A. salmonicida* subsp. *salmonicida* were made in TSB (2.75 g of Trypticase soy broth/100 ml, 1% glycerol, 0.1 M L-glutamic acid, pH 7.3). Ca^{2+}-depleted medium was made by addition of 1 mM nitrilotriacetic acid (NTA; Titriplex I; pH 7.3) to TSB.

Escherichia coli strains XL1-blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI^{q}ZΔM15* Tn *10* (Tet^r)][c]) (5) and BL21(DE3) (F′ *dcm ompT hsdS* [r_{B^-} m_{B^-}] *gal* λ[DE3]) (27) were used for cloning and expression of cloned genes respectively. Plasmids pBluescript II SK(−) and pBC-KS(−) (Stratagene, La Jolla, Calif.) were used as cloning vectors. Plasmid pETHIS-1 is a T7 promoter-based expression vector and allows addition of polyhistidine tails at the N-terminal end or at both the N- and C-terminal ends of proteins (26). The source of the aminoglycoside 3′-phosphotransferase gene *aph*(*3′*)-*Ia* conferring resistance to kanamycin (Km^r) was plasmid pSSVI186-IN (31). *E. coli* strains were grown at 37°C in LB broth supplemented when necessary with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (25 μg/ml) for selection and maintenance of recombinant plasmids. For blue-white differentiation of recombinant clones with pBluescript II SK(−), 125 μM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were added.

P. aeruginosa strain ATCC 27853 was grown for 8 h on LB plates at 20°C. To induce ExoS and ExoT secretion, cells were then incubated for 18 h at 20°C in 20 ml of TSB supplemented with 10 mM NTA (Titriplex I; pH 7.3) for chelation of Ca^{2+} ions.

PCR, cloning, preparation of gene probes, and genetic methods. PCR was carried out with a DNA thermal cycler (GeneAmp 9600; PE Biosystems, Norwalk, Conn.) in 50-μl reaction mixtures containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 350 μM (each) deoxynucleoside triphosphate, 0.25 μM forward and reverse primers, 0.5 U of *Taq* polymerase, and 5 ng of template DNA. The DNAs were amplified for 35 cycles with 30 s of denaturation at 94°C, 30 s of annealing at corresponding temperatures (Table 2), and 1 min of extension at 72°C. For fragments above 1 kb, the extension time was extended by 1 min per kb. When DNA fragments were produced by PCR for subsequent cloning and expression, the Expand-Long-Template PCR kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) containing polymerase with proofreading capacity was used instead of *Taq* polymerase. In addition, an extension step of 7 min at 72°C was added at the end of the last cycle in order to ensure full-length synthesis. For the production of digoxigenin (DIG)-labeled probes, PCR mixtures were supplemented with 40 μM DIG-11-dUTP (Roche Molecular Biochemicals).

A DNA fragment (called REXOS) corresponding to the catalytic portion of the *P. aeruginosa exoS* gene (14, 16) was amplified with primer pair REXOS-L and REXOS-R (Table 2), both containing *Eco*RI restriction site linkers. When genomic DNA (100 μg) of *P. aeruginosa* ATCC 27853 was used as template for PCR, 10% dimethyl sulfoxide was added. PCR fragments were purified with the QIAquick PCR purification kit (Qiagen, Basel, Switzerland). Plasmid pBluescript II SK(−) and purified PCR fragments were digested with *Eco*RI and ligated for 2 h at room temperature before transformation of *E. coli* K-12 strain XL1-blue. Plasmid constructs were sequenced to exclude artifacts.

To obtain pure, plasmid contaminant-free probes, the cloned *exoS*-derived fragment (REXOS) was excised with *Eco*RI and purified twice over agarose gels with the Jet-Sorb kit (Genomed GmbH, Bad Oeynhausen, Germany). This fragment was then used as the template for PCR with primers REXOS-L and REXOS-R (Table 2) for production of DIG-labeled probe REXOS.

A DNA fragment (called RAEXT) corresponding to the putative catalytic portion of the *A. salmonicida* subsp. *salmonicida aexT* gene was amplified with primer pair RASEXOS-L and RASEXOS-R (Table 2) and labeled with DIG. Genomic DNA derived from *A. salmonicida* subsp. *salmonicida* ATCC 33658^T served as the template.

All cloning procedures and genetic methods such as Southern blot analysis were performed essentially in accordance with standard protocols (2). DNA was extracted by the method of Pitcher et al. (22) and manipulated by conventional methods (2). The $CaCl_2$ method was used for preparation of competent cells (25). Sequencing reactions were performed with a *Taq* Dye Deoxy Terminator cycle sequencing kit (PE Biosystems), and reaction products were analyzed on an ABI Prism 310 genetic analyzer (PE Biosystems).

TABLE 2. Oligonucleotide primers

Primer	Sequence[a]	Position	Annealing temp (°C)
EXOS-L	cgcgaattcACTGGCTGGGCAAACTG	1128–1144[b]	52
EXOS-R	cgcgaattCCCGCTGACATCGATTC	2034–2019[b]	52
RASEXOS-L	GGCGCTTGGGCTCTACAC	1537–1554[c]	60
RASEXOS-R	GAGCCCGCGCATCTTCAG	2089–2072[c]	60
BASEXOSH8L	cgcgaattCGGCGAAACATCACAAGA	645–662[c]	59
BASEXOSH8R	ggactagTCCCGCCAGCATAAAAAAC	2165–2147[c]	59
AEXTDWN1	CCTGCACTGAGCACCCTCT	2191–2173[c]	56
KNTN903RINV1	GAGTTTTTCTAATCAGAATTGGT		56

[a] Lowercase letters indicate nucleotides added to create restriction enzyme recognition sites (underlined) for cloning.
[b] Based on nucleotide sequence L27629 of *P. aeruginosa exoS*.
[c] Based on nucleotide sequence of *aexT* (AF288366) of *A. salmonicida* subsp. *salmonicida*.

Transformation of *A. salmonicida* subsp. *salmonicida* with plasmid DNA was accomplished by electroporation using the Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.; settings: *U* (voltage) = 1.25 V, R_{par} (parallel resistance) = 400 Ω, *C* (condenser capacity) = 25 μFD) and the 0.1-cm Gene Pulser cuvette (Bio-Rad). *A. salmonicida* subsp. *salmonicida* grown on solid LB agar medium was resuspended in LB broth, washed three times with 15% glycerol (sterile), and finally suspended in 15% glycerol at 10^{10} cells/ml. For each electroporation 125 μl of bacterial suspension and 5 μl of plasmid DNA (500 μg/ml) were used. The pulse length was 8 ms.

Construction of phage λ gene library from *A. salmonicida* subsp. *salmonicida*. Genomic DNA (0.1 μg) from *A. salmonicida* subsp. *salmonicida* ATCC 33658^T was partially digested with restriction enzyme *Sau*3A to generate fragments in the 3- to 4-kb range, which were then ligated to λ ZapExpress digested with *Bam*HI (Stratagene). The ligated DNA was packed into prophage λ with the Gigapack III packaging extract (Stratagene). *E. coli* XL1-blue MRF′ (Stratagene) was used as a host. Phage plaques were lifted onto nylon filters and screened with DIG-labeled probes. Positive plaques were isolated and stored overnight at 4°C in 0.5 ml of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris [pH 7.5], 0.01% gelatin) containing 20 μl of chloroform. The in vivo excision of plasmids from selected phagemid plaques was done according to the instructions with the λ ZapExpress kit (Stratagene).

Sequence data analyses. Sequence alignment and editing were done with Sequencher software (Gene Codes Corporation, Ann Arbor, Mich.). Sequence comparisons were performed with BLAST (1), and sequences were aligned with the Wisconsin Package (Genetics Computer Group, Inc. [GCG], Madison, Wis.). The theoretical isoelectric pH (pI) and molecular masses of proteins were calculated with the GCG software.

Expression of recombinant AexT. To characterize the AexT protein and to produce polyclonal, monospecific antibodies directed against AexT, we expressed a polyhistidine-tailed AexT peptide named AexT-His in recombinant *E. coli* strain BL21(DE3). The entire coding region inclusive of the stop codon of the *aexT* gene was amplified by PCR using primers BASEXOSH8L and BASEXOSH8R (Table 2) and genomic DNA of *A. salmonicida* subsp. *salmonicida* as the template. The purified PCR product was digested with restriction enzymes *Eco*RI and *Spe*I and cloned into *Eco*RI- and *Spe*I-digested vector pETHIS-1 to obtain plasmid pJFFASAexT-His, encoding N-terminally polyhistidine-tailed AexT (AexT-His) under the control of the T7 promoter. For the expression of the *aexT*-His gene, plasmid pJFFASAexT-His was transformed into *E. coli* strain BL21(DE3).

E. coli BL21(DE3) cells harboring plasmid pJFFASAexT-His were inoculated in 50 ml of LB broth with ampicillin at 37°C to an optical density at 600 nm of 0.3 and induced by addition of 0.2 mM IPTG (final concentration). Cells were then grown for an additional 3 h. After this time, the cells were sedimented by centrifugation at 5,000 × *g* for 10 min, resuspended in 5 ml of buffer, pH 7.9, containing 10 mM Tris-HCl, 1 M urea, 250 mM NaCl, 2.5 mM imidazole, 3 M guanidinium HCl, 0.2 mM phenylmethylsulfonyl fluoride, and sonicated with a microtip for 20 min at 50% output interval in a Sonifier 250 (Branson Ultrasonics, Danbury, Conn.). This sonicated fraction was directly loaded onto a prewashed 1.25-ml-bed-volume Ni-NTA column (Qiagen) and was washed twice with 5 ml of binding buffer (2 M urea, 20 mM Tris, 500 mM NaCl, 5 mM imidazole, 60 mM guanidinium HCl, pH 7.9). Elution of polyhistidine-tailed protein AexT-His was performed with 40 ml of a gradient elution buffer of 5 to 500 mM imidazole in 2 M urea–20 mM Tris–500 mM NaCl–60 mM guanidinium HCl, pH 7.9. The gradient had a flow rate of 0.25 ml/min, and fractions of 1 ml were collected with a HiLoad system (Pharmacia LKB). The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17). Those containing purified fusion protein AexT-His were pooled and dialyzed overnight against 5 liters of 0.85% NaCl–10 mM Tris-HCl, pH 7.5.

Immunization of rabbits with purified proteins. Monospecific polyclonal antibodies against AexT were obtained by immunization of a rabbit with the purified AexT-His protein as described for other polyhistidine-tailed proteins (4). Purified and dialyzed recombinant protein solution (100 μg/ml) was mixed 1:1 with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and 2 ml of the emulsion was then injected subcutaneously into a rabbit. The rabbit was given a booster immunization with the same amount of protein emulsified with Freund's incomplete adjuvant 21 days later. On day 45 after the first immunization, the rabbit was bled, and serum was prepared and stored at −20°C.

Immunoblot analyses. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). For immunoblotting, Western blots were blocked with 1% milk buffer for 30 min and then incubated with the rabbit antiserum (1:1,500) in milk buffer overnight at 4°C. After a thorough wash with water, phosphatase-labeled conjugate (goat anti-rabbit immunoglobulin G heavy plus light chains [catalog no. 075-1506; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.]) diluted 1:2,000 in milk buffer was added, and the reaction was visualized 90 min later by incubation with BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium.

Construction of an AexT-deficient *A. salmonicida* subsp. *salmonicida* JF2267 mutant. An AexT-deficient *A. salmonicida* subsp. *salmonicida* JF2267 mutant was constructed by allelic gene replacement with an insertionally inactivated *aexT* gene. Plasmid pJFFBAS211, derived from the phage bank and containing the entire *aexT* gene and sequences downstream of *aexT*, was used as a source for *aexT*. From this plasmid, *aexT* was subcloned as a 1.5-kb *Hin*dIII-*Spe*I fragment onto vector pBC-KS, a plasmid derived from the gene for ColE1, which confers chloramphenicol resistance. Inactivation of the *aexT* gene was obtained by insertion of the *aph*(*3′*)-*Ia* kanamycin resistance gene on a 1.3-kb *Bgl*II-*Bam*HI fragment from plasmid pSSVI186-IN into the *Bam*HI site located on the middle of the *aexT* gene (Fig. 1). The resulting plasmid, pJFFaexT::Km4, was then used for electroporation of *A. salmonicida* subsp. *salmonicida* JF2267. Selection for allelic gene replacement of *aexT* by the *aexT*::Km^r mutation was obtained by selection of kanamycin-resistant chloramphenicol-sensitive *A. salmonicida* subsp. *salmonicida* on LB agar plates containing kanamycin. Kanamycin-resistant colonies were selected and passaged twice on LB agar plates containing kanamycin. Four single colonies, which were shown to be sensitive to chloramphenicol, were retained, and the correct insertion of the *aexT*::Km^r allele and the absence of the wild-type (wt) *aexT* gene was verified by PCR using primer pairs RASEXOS-L and RASEXOS-R and AEXTDWN and KNTN903RINV1 (Fig. 1). Note that primer AEXTDWN matched a DNA sequence downstream the *aexT* gene (Fig. 1) which was not carried by plasmid pJFFaexT::Km4, used for the construction. Primer KNTN903RINV1 matched the *aph*(*3′*)-*Ia* gene of pJFFaexT::Km4. Strain JF2580 was shown to contain the *aexT*::Km^r allele at the locus of *aexT* and was retained as the *aexT* mutant. Strain JF2580 contains no functional *aexT* gene, as evidenced by PCR.

Infection of fish cell cultures with *A. salmonicida* subsp. *salmonicida*. Rainbow trout (*Oncorhynchus mykiss*) gonad cells (RTG-2; ATCC CCL-55) were grown in 75-cm^2 tissue culture flasks (Techno Plastic Products AG, Trasadingen, Switzerland) at 22°C in minimum essential medium (GibcoBRL Life Technologies, Basel, Switzerland) supplemented with 2 mM L-glutamine (GibcoBRL Life Technologies), 1× nonessential amino acids (GibcoBRL Life Technologies), 3 g of sodium bicarbonate/liter, and 10% fetal bovine serum. Three days before infection, the cells were trypsinized and subsequently seeded into 24-well culture plates (Techno Plastic Products AG) at 6 × 10^5 cells per 2-cm^2 well in 1 ml of supplemented medium. Monolayered RTG-2 cells were then infected at multiplicities of infection of 2:1 and 20:1 (ratio of bacteria to fish cells) with the different *A. salmonicida* subsp. *salmonicida* cultures resuspended in phosphate-buffered saline (PBS), pH 7.4. In four control wells, 100 μl of PBS, pH 7.4, was added. As a further control, 1.2 × 10^6 bacteria of *A. salmonicida* subsp. *salmonicida* JF2267 were added to 1 ml of supplemented cell culture medium. After 2, 8, and 24 h of infection at 19°C, the fish cells were inspected and photographed under a green-filtered phase-contrast microscope (Axiovert 100; Zeiss, Jena, Germany). Detachment of the cells from the flask was achieved by vigorous shaking and scraping off of the cells from the wells. The suspended cells were centrifuged for 10 min at 5,000 × *g*. The pellet was then resuspended in 50 μl of SDS sample loading buffer (2) for SDS-PAGE and immunoblot analysis. Supernatant was mixed with 1/2 volume of SDS sample loading buffer. Equivalent amounts of pellets and supernatants were applied to SDS-PAGE gels. As a control, *A. salmonicida* subsp. *salmonicida* JF2267 and JF2580 were incubated in cell culture medium without cells for the same periods.

ADP-ribosyltransferase assays. ADP-ribosyltransferase assay mixtures contained 100 μM [^{14}C]NAD (specific activity: 6 Ci/mol) and 0.2 M sodium acetate, pH 6, in a total of 20 μl. As a source of FAS, 4 μl (approximately 200,000 cells) of noninfected RTG-2 fish cells were used in the reaction mixture. The reaction was started by adding 4 μl of a solution containing approximately 0.5 μg of recombinant AexT-His protein or 4-μl aliquots of supernatants from either *P. aeruginosa* ATCC 27853 or *A. salmonicida* subsp. *salmonicida* cultures. An aliquot of pure growth medium was used for background determination. The reaction was performed at 20°C for 1 h and stopped by the addition of 500 μl of 10% trichloroacetic acid (TCA). The mixtures were blotted onto filters (GS; 0.22-μm pore size; Millipore, Bedford, Mass.) with a vacuum pump and washed five times with 0.75 ml of 10% TCA. The filters were air dried, and scintillation liquid (Emulsifier Scintillator Plus; Packard Instrument Company, Meriden, Conn.) was added. Scintillation was detected as counts per minute on a liquid scintillation counter (Wallac 1410; Pharmacia, Dübendorf, Switzerland). Experiments were performed in triplicate, and scintillation was counted three times per experiment. Background counts were subtracted, and results with their standard deviations are given in counts per minute. Due to the high background of ADP-ribosyltransferase activity of the fish cells themselves, the activity of AexT

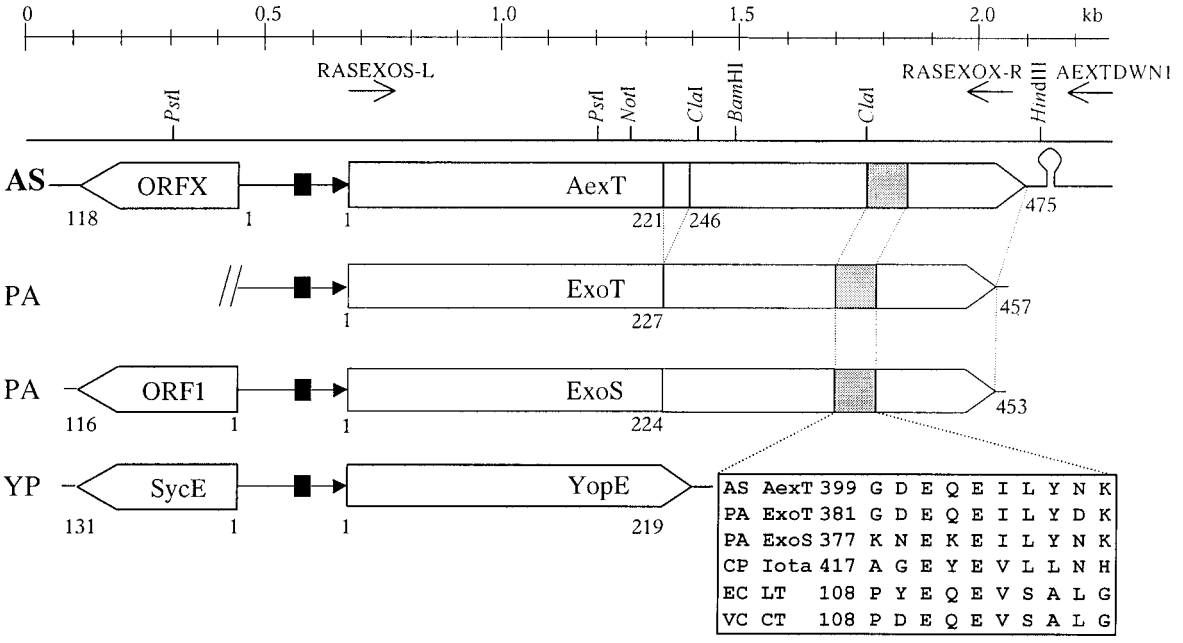

FIG. 1. Genetic map of the gene encoding AexT and ORFX of *A. salmonicida* subsp. *salmonicida* in alignment with the corresponding genes of *P. aeruginosa* and *Y. pestis*. Maps were constructed from the current sequence data and from EMBL/GenBank accession no. AF288366 for *A. salmonicida* subsp. *salmonicida* AexT, L27629 for *P. aeruginosa* ExoS, L46800 for *P. aeruginosa* ExoT, and AF053946 for *Y. pestis* YopE. The last is also representative of the analogous genes of *Y. pseudotuberculosis* and *Y. enterocolitica* (8). Top line, scale in kilobase pairs; line below, physical map of the locus showing a few sites for restriction enzymes (arrows, locations of the oligonucleotide primers that were used for verification of the insertion of the Km^r cassette in the *aexT* mutant. Boxes with arrowheads, ORFs. Numbers indicate corresponding amino acid positions. The putative biglutamic acid active sites (grey boxes) are shown in detail. Black boxes, transcription activator (ExsA) binding site; black triangles, consensus sequences for the transcription promoter. Abbreviations: AS, *A. salmonicida* subsp. *salmonicida*; PA, *P. aeruginosa*; YP, *Y. pestis*; CP, *Clostridium perfringens*; EC, *E. coli*; VC, *V. cholerae*; Iota, iota toxin; LT, heat-labile toxin; CT, cholera toxin.

in fish cells infected with *A. salmonicida* subsp. *salmonicida* could not be measured.

Nucleotide sequence accession numbers. The sequence of the *aexT* gene and its neighboring ORFX-containing gene was submitted to the GenBank/EMBL database and was given accession no. AF288366. The sequence of the *rrs* (16S rRNA) of *A. salmonicida* subsp. *salmonicida* strain JF2267 has accession no. AF200329.

RESULTS

Cloning and characterization of the *aexT* gene. Analyses of different *Aeromonas* sp. with broad-range ADP-ribosylating toxin probes revealed a signal for a potential ADP-ribosyltransferase gene in *A. salmonicida* subsp. *salmonicida*. Specifically, this signal was obtained with probe REXOS, which is derived from the catalytic domain of ExoS. This probe was then used to screen a λ phage gene library of *A. salmonicida* subsp. *salmonicida* ATCC 33658^T. Three positive overlapping clones were found and were joined together to form a continuous DNA fragment of 2,260 bp. The derived DNA sequence of this fragment revealed a complete open reading frame (ORF) of 1,428 bp showing high similarity with ADP-ribosylating toxin ExoT of *P. aeruginosa*. In analogy to ExoT, the protein encoded by this ORF was called *Aeromonas* exoenzyme T (AexT), and its corresponding gene was called *aexT*. The cloned fragment contains an additional ORF, named ORFX, which shows similarity to the *sycE* gene of *Yersinia* sp. and to ORF1, which precedes *exoS* of *P. aeruginosa* (Fig. 1). ORFX is preceded by a ribosomal binding site (RBS) and followed by a putative *rho*-independent transcription termination site. The sequenced DNA fragment encoding AexT and ORFX revealed a high G+C content of 60%, which is above the average G+C content of *A. salmonicida* subsp. *salmonicida*, 55% (3). The ORF of *aexT* contains an ATG initiation codon and a TGA stop codon. The 87 bp preceding ATG show 71% identical nucleotide positions to the sequence preceding *exoS* and *exoT* in *P. aeruginosa*. The putative RBS, AGAAG, is positioned 10 bp upstream of the ATG. The putative promoter sequence −10 box (TAGACT) and the canonical −35 box (CCGATA) of *aexT* are located at the same positions as those for *exoS* and *exoT*. Upstream of the promoter −10 and −35 box sequences, there is a consensus binding site (TACAAA AA) similar to the one found upstream of *exoS* and *exoT*, which in *P. aeruginosa* is known to be bound by transcriptional regulator ExsA (9, 13). An inverted repeat is located 25 bp downstream of the stop codon of the *aexT* gene, representing the putative transcription termination sites similar to those of *exoS* and *exoT* (Fig. 1). PCR amplification and DNA sequence analysis were used to find the same genes and regulatory elements with identical nucleotide sequences in strain JF2267, a virulent strain of *A. salmonicida* subsp. *salmonicida*, which was freshly isolated from an arctic char (*S. alpinus*) with typical furunculosis symptoms (Table 1). Strain JF2267, in contrast to strain ATCC 33658^T, was shown to be virulent and was used for further studies. Southern blot analyses of genomic DNA of various *Aeromonas* spp. (Table 1) with a DIG-labeled probe for *aexT* (RAEXT) revealed a single copy of *aexT* in all *A. salmonicida* subsp. *salmonicida* strains tested (Table 1).

None of the other *Aeromonas* strains analyzed showed hybridization signals with the *aexT* probe.

Analyses of the AexT sequence. The amino acid sequence for AexT was deduced from the nucleotide sequences using the universal genetic code. AexT has a calculated pI of 5.13 and a molecular mass of 50.1 kDa. BLAST searches (1) revealed the similarity of AexT to ExoT and ExoS over the whole length. In addition, similarity to the YopE cytotoxin of *Yersinia pseudotuberculosis* (EMBL/GenBank accession no. P08008), *Yersinia pestis* (accession no. P31493), and *Yersinia enterocolitica* (accession no. M34280) was found within the N-terminal 210 amino acids (aa) of AexT (Fig. 1). Comparisons of the amino acid sequence of AexT with those of ExoT and ExoS revealed AexT to be 62.8% identical to ExoT (57.9% identical to ExoS) and 67.5% similar to ExoT (62.8% similar to ExoS). A segment of 25 aa in the middle of AexT is not found in the other ADP-ribosylating toxins. Gap comparisons of ExoT with ExoS showed them to be identical in 75.1% of the positions and similar in 77.7%. The N-terminal domain amino acids of AexT were 33.5% identical and 37.4% similar to those of cytotoxin YopE of *Y. pseudotuberculosis* and 26.8% identical and 32.8% similar to those of YopE of *Y. pestis* (Fig. 1). The biglutamic acid active site (GDEQEILYNK) found for various ADP-ribosylating toxins (23) is also conserved within the C-terminal domain of AexT (Fig. 1).

Toxicity of *A. salmonicida* subsp. *salmonicida* strains and expression of AexT. The toxicity of *A. salmonicida* subsp. *salmonicida* strain JF2267 and that of its *aexT* mutant derivative JF2580 were assessed by infecting cultured RTG-2 rainbow trout gonad cells. Infection with *A. salmonicida* subsp. *salmonicida* strain JF2267 caused a toxic effect resulting in characteristic cell rounding and detachment of cells within 2 h (Fig. 2). In contrast, RTG-2 cells infected with the *aexT* mutant showed no morphological changes at all, like the control cells incubated with PBS (Fig. 2). The same results were obtained after 8 h of incubation. After 24 h of incubation, the cells incubated with strain JF2267 had lysed, while those incubated with *aexT* mutant JF2580 or with PBS still remained unchanged. The results were the same with a multiplicity of infection of 2:1 or 20:1 (ratio of bacteria to fish cells). To assess the production of AexT by *A. salmonicida* subsp. *salmonicida* JF2267 and JF2580 after 2 h of incubation with RTG-2 cells (multiplicity of infection, 2:1) or after 2 h of incubation in cell culture medium alone, pellets as well as supernatants of the cells infected with the bacteria or the bacteria in cell culture medium alone were analyzed on immunoblots with monospecific polyclonal anti-AexT antibodies. The immunoblot analysis showed a strong reaction of a band at 58 kDa, corresponding to the native AexT protein, for the pellet of the RTG-2 cells infected with *A. salmonicida* subsp. *salmonicida* JF2267. No reaction was found in pellets of bacteria incubated in cell culture medium alone or in samples containing the pellets of RTG-2 cells infected with *aexT* mutant JF2580 or of RTG-2 cells alone (Fig. 3A). When the supernatants of the samples were analyzed, a weak reaction with anti-AexT antibodies was found in the sample of RTG-2 cells infected with JF2267 but not in the sample of RTG-2 cells incubated with *aexT* mutant JF2580 or in RTG-2 cells alone or in strain JF2267 in culture medium alone (Fig. 3B). These results indicate that *A. salmonicida* subsp. *salmonicida* JF2267 produced AexT after interaction with RTG-2 fish cells but that no detectable amounts of AexT were found after incubation with cell culture medium alone. Most of the AexT that was produced by *A. salmonicida* subsp. *salmonicida* JF2267 after contact with RTG-2 cells was found in pelleted cellular and bacterial material, showing that AexT was located inside the cells or was associated with the cells. The *aexT* mutant did not produce any AexT under any of the conditions tested.

When *A. salmonicida* subsp. *salmonicida* JF2267 was grown in standard liquid TSB, no AexT could be revealed by immunoblot analysis. To analyze whether depletion or repletion of TSB medium of iron or calcium ions could affect production of AexT, *A. salmonicida* subsp. *salmonicida* strain JF2267 was cultured under various conditions consisting of TSB supplemented with either 10 mM $CaCl_2$ or 0.01 to 1 mM EDTA or 1 mM EDTA plus 1 mM phenylmethylsulfonyl fluoride or 10 or 100 mM NTA or 0.1 mM $Fe^{III}Cl_3$ or 0.1 mM $Fe^{II}Cl_2$ or 0.1 mM EDDA (ethylenediamine-di[*o*-hydroxyphenylacetic acid]) or 20 mM sodium oxalate. Some AexT protein could be detected in concentrated culture supernatant when the medium was supplemented with 10 mM NTA to complex free Ca^{2+} (Fig. 4). No AexT was found in strain JF2267 in the other supplemented growth media or in *aexT* mutant JF2580 under any of these conditions. When supernatant from a culture of *P. aeruginosa* ATCC 27853^T grown in Ca^{2+}-depleted medium was analyzed on immunoblots with anti-AexT antibodies, distinct reactions could be detected with proteins of 49 and 53 kDa, representing the ExoS and ExoT proteins, respectively, as expected from the amino acid sequence similarities to AexT (Fig. 4). In spite of the presence of an intact copy of the *aexT* gene, *A. salmonicida* subsp. *salmonicida* strain ATCC 33658^T did not show expression of AexT under any of the above-mentioned conditions. Furthermore RTG-2 cells were inert to infection with ATCC 33658^T.

Biochemical activity of recombinant AexT. To characterize AexT biochemically, we determined the ADP-ribosyltransferase activity of a purified recombinant AexT-His protein that was obtained from *E. coli* strain BL21(DE3) transformed with plasmid pJFFASAexT-His. Purified and renatured AexT-His revealed ADP-ribosylating activity resulting in a signal of 123 ± 11 cpm for 0.5 μg of recombinant protein in our standard assay. In comparison, 4 μl of culture supernatant of *P. aeruginosa* strain ATCC 27853, grown under Ca^{2+}-depleted conditions and used as a positive control standard, produced 4,286 ± 125 cpm under the same experimental conditions. The ADP-ribosylating activity in supernatants *of A. salmonicida* subsp. *salmonicida* JF2267 cultures grown in Ca^{2+}-depleted medium could not be measured with accuracy in this test. The counts per minute were at the limit of sensitivity of the method of measurement, although the AexT protein could be detected by immunoblotting (Fig. 4). *aexT* mutant strain JF2580 and strain ATCC 33658^T showed no ADP-ribosyltransferase activity, as expected from immunoblot analyses. Moreover ADP-ribosyltransferase could not be determined in infected fish cells due to high background activity.

DISCUSSION

In this study we present genetic, biochemical, and biological evidence for a new toxin, AexT, of *A. salmonicida* subsp. *sal-*

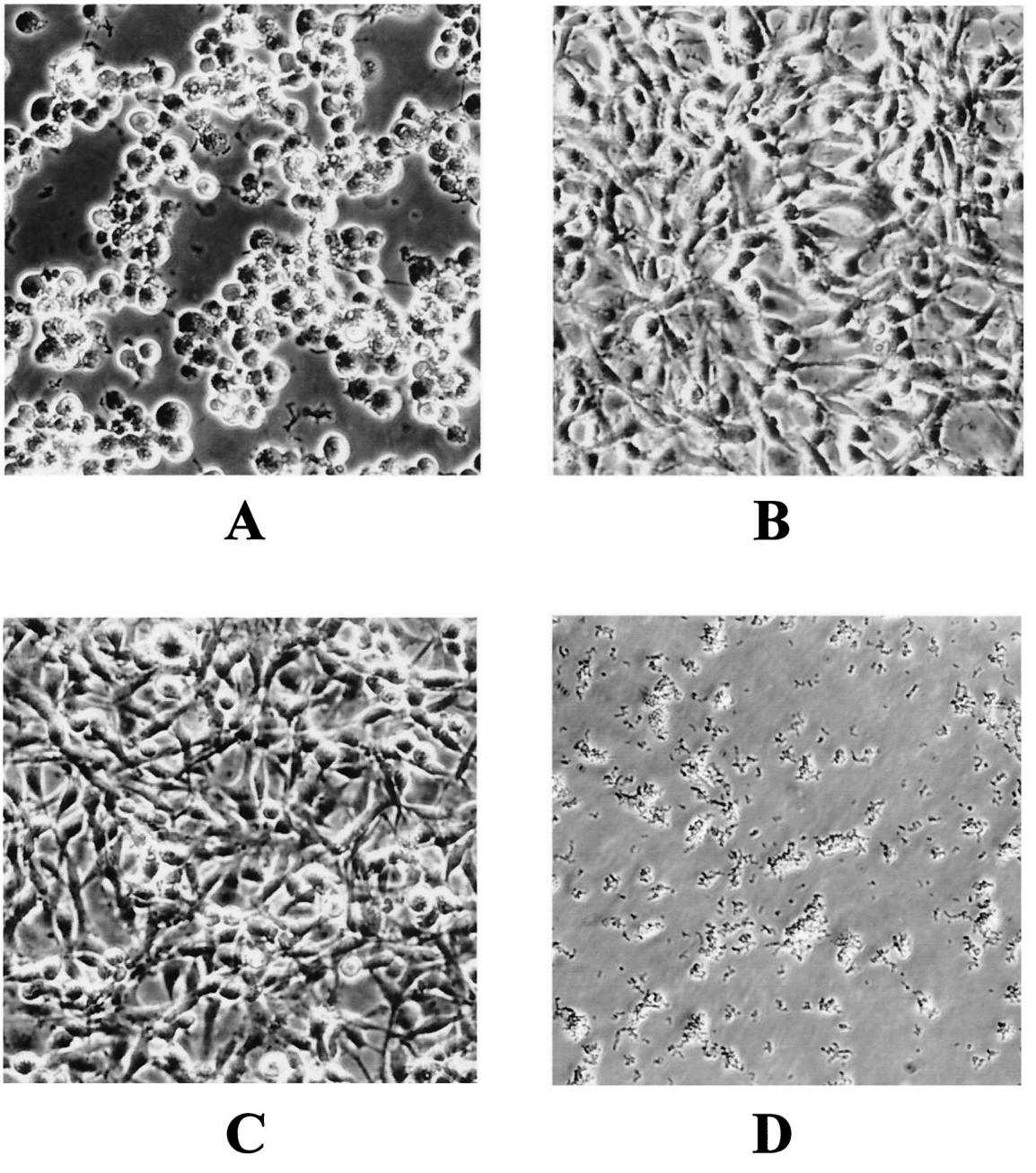

FIG. 2. Toxicity of AexT-producing *A. salmonicida* subsp. *salmonicida* to RTG-2 fish cells. The cells were photographed 2 h after inoculation. (A) RTG-2 cells inoculated with AexT-producing (wt) *A. salmonicida* subsp. *salmonicida* strain JF2267. (B) RTG-2 cells inoculated with isogenic *aexT* mutant JF2580. (C) RTG-2 cells inoculated with 100 μl of PBS buffer. (D) Strain JF2267 in culture medium without fish cells.

monicida, which belongs to the family of ADP-ribosylating toxins. The *aexT* gene was cloned by screening a gene library of *A. salmonicida* subsp. *salmonicida* with broad-range ADP-ribosylating toxin gene probes. The *aexT* gene was found to be specific for *A. salmonicida* subsp. *salmonicida*, where it was found in a single copy in virulent strain JF2267, in strain ATCC 33658^T, and in a further 10 field isolates. The *aexT* gene, in contrast, was not found in 18 other *Aeromonas* species. ADP-ribosylating toxins are generally very potent bacterial toxins, known to be primarily responsible for the high pathogenicity of *P. aeruginosa*, *Vibrio cholerae*, *Corynebacterium diphtheriae*, and *Bordetella pertussis.* The amino acid sequence of AexT of *A. salmonicida* subsp. *salmonicida* closely resembles those of ExoS and ExoT of *P. aeruginosa*, and antibodies directed against AexT cross-reacted with ExoS and ExoT. Additionally, AexT shows N-terminal similarity with YopE of *Yersinia* spp. ExoT, ExoS, and YopE are well-characterized cytotoxins, which catalyze *rho*-dependent actin depolymerization and which induce rounding of eukaryotic cells grown in culture (20, 24). AexT was shown to have ADP-ribosyltransferase activity, as measured for recombinant purified AexT-His. The ADP-ribosyltransferase activity of AexT in culture supernatants of

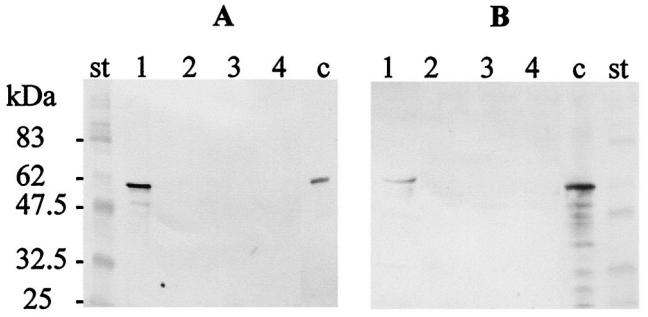

FIG. 3. Biosynthesis of AexT by *A. salmonicida* subsp. *salmonicida*. The immunoblots were reacted with anti-AexT antibodies and contained RTG-2 cells inoculated with JF2267 (lanes 1), RTG-2 cells inoculated with mutant JF2580 (lanes 2), RTG-2 cells with PBS (lanes 3), and *A. salmonicida* subsp. *salmonicida* JF2267 in culture medium (lanes 4). Lanes c, purified recombinant AexT-His as a control; lanes st, molecular mass standard. (A) Pellets containing cells and bacteria. (B) Supernatants.

virulent *A. salmonicida* subsp. *salmonicida* strain JF2267 was at the limits of detection and could not be measured accurately. This might be due to the low specific ADP-ribosyltransferase activity of AexT compared to that of ExoT in *P. aeruginosa* and to the small amount of AexT expressed under culture conditions as revealed by immunoblot analysis.

Infection of RTG-2 fish cell cultures with *A. salmonicida* strain JF2267 induced strong morphological changes of the cells (Fig. 2), which were accompanied by the production of AexT, which was found to be intracellular or tightly cell associated, as revealed on immunoblots with anti-AexT antibodies. These morphological changes of the cells were similar to those reported for cells infected with ExoS-producing *P. aeruginosa* (10) and YopE-producing *Yersinia* species (21). Prolonged incubation of RTG-2 cells with *A. salmonicida* subsp. *salmonicida* JF2267 led to cell lysis. In contrast, isogenic *aexT* mutant JF2580, which was derived from JF2267 by allelic exchange with an in vitro-mutated *aexT*::Km^r gene, had no effect at all on RTG-2 fish cells, even after prolonged incubation or infection with a 10-fold-greater amount of bacteria (multiplicity of infection, 20:1). Furthermore, no expression of AexT was detected when JF2580 was used for the infection of fish cells. Hence AexT is a main factor responsible for the toxic effect of A. *salmonicida* subsp. *salmonicida* strain JF2267 on RTG-2 fish cells.

The expression of *aexT* was shown to be induced by contact with fish cells or, to a minor extent, by low Ca^{2+} concentrations in the medium. Incubation of *A. salmonicida* subsp. *salmonicida* JF2267 in cell culture medium alone did not result in any production of AexT toxin. This indicated that AexT production in *A. salmonicida* subsp. *salmonicida* occurred specifically during infection of the host. Furthermore we have shown that, upon infection of RTG-2 cells with virulent A. *salmonicida* subsp. *salmonicida* JF2267, most of the AexT toxin was found in the cellular material and only minor amounts were found in the culture medium. This indicates that AexT is directly translocated to the fish cells, supposedly via a specific secretion mechanism, or tightly associates with the surfaces of the fish cells. The dependence of *A. salmonicida* subsp. *salmonicida* strain JF2267 on fish cells or on Ca^{2+}-restricted conditions for the expression and secretion of the AexT protein toxin suggests that regulation of expression of the *aexT* gene and secretion of AexT might be coupled to a type III secretion system. This assumption is further strengthened by ORFX, present upstream of *aexT*, which shows high similarity to gene *syc*E (specific Yop chaperone E) of *Y. pestis*. SycE serves as a secretion signal and is a part of the type III secretion pathway for secretion of YopE (6). Furthermore *aexT* was shown to be preceded by a consensus sequence for the binding of a transcriptional activator, known in *P. aeruginosa* as ExsA, which is involved in type III-dependent gene expression (9).

Interestingly, AexT was not produced by *A. salmonicida* subsp. *salmonicida* strain ATCC 33658^T, as shown by immunoblot analysis with anti-AexT antibodies, and did not affect the morphology of fish cells, in spite of the presence of the *aexT* gene and the sequences upstream of *aexT*. As the *aexT* gene and sequences upstream are the same in AexT-producing strain JF2267 and in strain ATCC 33658^T, we deduce that the alteration responsible for the loss of AexT production in strain ATCC 33658^T resides outside the *aexT* operon. In this respect, it has to be noted that *A. salmonicida* subsp. *salmonicida* strains ATCC 33658^T and JF2267 have the same hemolytic activity, as estimated on blood agar plates, implying that the toxic effect for RTG-2 cells is not due to the *A. salmonicida* subsp. *salmonicida* hemolysins. The loss of expression of *aexT*, as observed in *A. salmonicida* subsp. *salmonicida* strain ATCC 33658^T, probably caused by passages under in vitro cultivation, might be a frequent event in *A. salmonicida* subsp. *salmonicida* strains and could explain the currently observed variations in virulence as well as differences in efficacy of protection of whole-cell antigen vaccines (28).

In summary, the present data show that AexT is an ADP-ribosylating toxin of *A. salmonicida* subsp. *salmonicida* which has a toxic effect on fish cells upon infection. The *aexT* gene is induced upon interaction with a culture of fish cells, and the AexT protein causes cell damage.

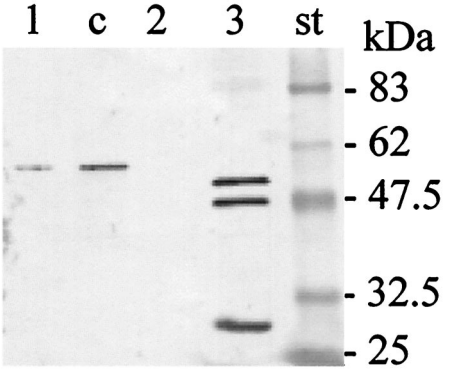

FIG. 4. Expression of AexT by *A. salmonicida* subsp. *salmonicida* in low-Ca^{2+} medium and serological cross-reactions with ExoS and ExoT. Bacterial cultures were grown in Ca^{2+}-depleted TSB medium. Lane 1, *A. salmonicida* subsp. *salmonicida* wt strain JF2267; lane 2, *A. salmonicida* subsp. *salmonicida aexT* mutant JF2580; lane 3, *P. aeruginosa* strain ATCC 27853. Culture supernatants were concentrated 20-fold and analyzed on immunoblots with anti-AexT antibodies. Lane c, purified recombinant AexT-His as a control; lane st, molecular mass standard. The identity of the band at 30 kDa reacting with *P. aeruginosa* ATCC 27853 (lane 3) is not determined.

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