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Characterization of *Aeromonas salmonicida* Mutants with Low-Level Resistance to Multiple Antibiotics

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Aeromonas salmonicida **mutants selected for low-level resistance to small-molecular-mass antibiotics occur at frequencies that suggest point mutations and exhibit pleiotropic effects such as a multiple low-level antibiotic resistance, changes in outer membrane protein profiles, and loss of major exoprotease activity. Multiple low-level resistance appeared as the result of decreased outer membrane permeability associated with a change from a 38.5- to a 37-kilodalton (kDa) outer membrane protein. This decreased outer membrane permeability was determined by rates of nitrocefin hydrolysis by periplasmic β-lactamase activity. The findings described above were supported by isolation of revertant strains selected for regained exoprotease activity, which also lost multiple low-level resistance and possessed outer membrane protein profiles indistinguishable from those of the original parent strains. Exoprotease from parent and revertant strains was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a major extracellular protein of approximately 69 kDa. No accumulation of a protein in this molecular mass range was observed in extracellular or periplasmic fractions from the mutants. The results suggested that exoprotease loss is not simply the result of an inability to export protease from the periplasm because of outer membrane protein changes, as has been reported for certain mutants of some other gram-negative bacteria. Also, several growth conditions were used, including some that have been reported to influence outer membrane protein expression and permeability in other enteric gram-negative bacteria. Although exoprotease expression in *A. salmonicida* was influenced by these conditions, no major outer membrane protein changes which would correspond to changes observed in the mutants were observed in parent strains.**

Aeromonas salmonicida is a gram-negative pathogen that causes furunculosis in fish. In addition to the envelope layers present in all gram-negative bacteria, this particular bacterium possesses a regularly structured surface array protein layer (A layer) associated with the virulence of the organism in salmonid fish (16, 33). The underlying outer membrane of *A. salmonicida* has not been studied extensively, but accumulated information from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles suggests that the major protein species and lipopolysaccharides (LPSs) are remarkably similar among different wild-type isolates (3, 4, 6, 7, 24). A 42-kilodalton (kDa) outer membrane protein has been isolated from *A. salmonicida* and was reported to display characteristics similar to those of porin proteins of other gram-negative enteric bacteria (5).

The outer membrane of gram-negative bacteria is believed to act as a molecular sieve containing porin proteins which facilitate the penetration of small hydrophilic molecules into the periplasmic compartment (for a review, see references 23 and 27). Some outer membrane proteins appear to be involved with the export of certain enzymes from the periplasm to the external milieu (8, 13, 15).

We previously reported that mutants exhibiting a low-level resistance to several smaller-molecular-mass antibiotics can be obtained from *A. salmonicida* at frequencies suggesting point mutations after exposure of wild-type strains to low inhibitory concentrations of any of several antibiotics (34). The SDS-PAGE profiles of outer membrane fractions from these mutants revealed the lack of a major protein of approximately 38.5 kDa (present in wild-type strains) and the presence of a major protein of approximately 37 kDa which is lacking in wild-type strains. Concomitant with the acquisition of multiple low-level antibiotic resistance and change in outer membrane proteins in these mutants was a loss of the major exoprotease (caseinase) activity.

In some respects, these *A. salmonicida* mutants resembled mutants isolated by similar methods from *Escherichia coli* and other gram-negative bacteria which exhibit multiple low-level resistance to a similar range of antibiotics and which show outer membrane protein changes (for a review, see references 11 and 27). For example, such mutants of *E. coli* K-12 were found to lack OmpF and strongly express the less-efficient porin OmpC (12, 26). The expression of these and other porin proteins in enteric bacteria has been shown to be influenced by environmental conditions, such as medium osmolarity (high osmolarity favoring OmpC expression and low osmolarity favoring OmpF expression), growth temperature (low temperature favoring OmpF expression), and, possibly, the presence or absence of cyclic AMP (for a review, see references 23 and 27).

In other respects, the *A. salmonicida* mutants resembled certain mutants of *Aeromonas hydrophila* (15) and *Pseudomonas aeruginosa* (8) in which loss of exoprotease activity was associated with changes in outer membrane proteins. In these cases, protease was determined to be trapped in the periplasm, suggesting some role for outer membrane proteins in the export of protease across the outer membrane.

The purpose of this study was to examine the relationships between the pleiotropic effects observed in the *A. salmonicida* mutants, in particular to determine whether the observed change in outer membrane proteins coincided with a change in outer membrane permeability and a restriction of protease export from the periplasm. Revertant strains were obtained to further investigate these relationships. Furthermore, the effects of some environmental changes on exopro-

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tease and outer membrane protein expression in *A. salmonicida* were examined.

MATERIALS AND METHODS

Bacterial strains. Sources of wild-type *A. salmonicida* strains, the A-layer-negative (A^-) mutants, and the protease-negative (Prt^-) mutants with low-level resistance to multiple antibiotics (*mlr*) derived from them were described previously (34). All strains were maintained on brain heart infusion (BHI) agar.

Mutants (e.g., $AsLA^R$) of the AsL wild-type strain selected for increased β-lactamase activity were isolated as colonies grown on BHI agar plates containing 4 μg of ampicillin ml^{-1} after incubation at 22°C. The plates were surface inoculated with approximately 10^8 cells as estimated by the optical density at 660 nm (OD_{660}) from an 18-h BHI broth culture (incubated at 22°C and 150 rpm on a shaker [model G-26; New Brunswick Scientific Co., Inc., New Brunswick, N.J.]). Colonies were checked for increased β-lactamase activity by observing color change after the addition of a small drop of nitrocefin solution (Oxoid Ltd., Basingstoke, England).

Prt^- *mlr* mutants (e.g., $AsLA^R$-N1) were isolated from the $AsLA^R$ mutant as colonies grown on BHI agar plates containing 0.4 μg of nalidixic acid ml^{-1} as described above and subsequently checked for antibiotic susceptibilities (34) and exoprotease (10).

To obtain protease-positive (Prt^+) revertant strains, single colonies of the Prt^- *mlr* mutants grown on BHI agar were inoculated into 250-ml Erlenmeyer flasks containing 50 ml of casein broth medium consisting of (liter^{-1}): K_2HPO_4, 21 g; KH_2PO_4, 9 g; Casamino Acids (Difco Laboratories, Detroit, Mich.), 1 g; L-tryptophan, 0.03 g; glucose, 10 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; and casein, 10 g. The flasks were incubated at 22°C and 150 rpm for ≥72 h. Samples of the cultures were plated on BHI agar containing a skim milk-agar overlay and incubated at 22°C to detect Prt^+ revertant colonies (10).

Outer membrane permeability. Cultures grown as described above in BHI broth for 18 h were used to inoculate 0.7 ml into 10 ml of BHI broth containing 10 μg of ampicillin ml^{-1} in a 25-ml Erlenmeyer flask. The flasks were incubated as indicated above until the OD_{660} was 0.5 to 0.6 (approximately 3 h). Culture samples were then harvested by centrifugation at 6,000 × *g* for 10 min. Cell pellets were suspended in an equal volume of 0.033 M Tris hydrochloride (pH 7.2) containing 20% sucrose, 12.5 mM EDTA, and 1.5 mg of lysozyme ml^{-1}. The suspensions were incubated with gentle stirring for 2 h at room temperature to generate spheroplasts. The suspensions were then centrifuged at 10,000 × *g* for 10 min at 4°C, and the pellets were suspended in an equal volume of 0.033 M Tris hydrochloride containing a few crystals of DNase. β-Lactamase activities were measured essentially as described by Nicas and Hancock (25) using samples of the culture and the culture supernatant to determine intact-cell activity. Broken- or disrupted-cell activity was the sum of the spheroplast supernatant and suspended lysed spheroplast fraction activities. Although the major portion of β-lactamase activity was found in the spheroplast supernatant fraction, the amount released was not consistent (50 to 90%), as has been observed by other workers who used a variety of shock release procedures for *P. aeruginosa* (2). The assays were performed with a Perkin-Elmer Lambda 3 double-beam spectrophotometer at room temperature, and the activity was recorded as nanomoles of nitrocefin hydrolyzed minute^{-1} milligram of cell protein^{-1} by using an extinction coefficient of 18,400 at 486 nm (Oxoid Ltd.). Calculation of the K_m and V_{max} enabled the approximation of the permeability coefficient (35).

SDS-PAGE of extracellular, outer membrane, and periplasmic fractions. Cultures were grown on BHI agar overlaid with cellulose dialysis tubing, and cells were harvested as previously described (34). Of the 10 ml of cell suspension washed off each 150- by 15-mm plate, 1 ml was used to extract periplasmic material by the method of Ames et al. (1), precipitated with trichloroacetic acid (10% [wt/vol], final concentration) at 4°C, and treated as previously reported for outer membrane protein extracts (34). This method was simple and extracted β-lactamase activity as efficiently as the spheroplast method described above. The remaining 9 ml of cell suspension was centrifuged, and aliquots of the supernatant containing extracellular products were precipitated with trichloroacetic acid and treated as described above. The cell pellets were used to extract outer membrane proteins as previously reported (34).

The SDS-PAGE gels were prepared and the samples were suspended, electrophoresed, and stained as described previously (34), except that the gel thickness was 1 mm and the running gel length was 11 cm.

For LPS SDS-PAGE analyses, the precipitated, washed, and dried samples described above were suspended (34) to give approximately 1 μg of protein μl^{-1}, as estimated by the protein assay of Lowry et al. (20). The suspended samples were digested for 1 h at 37°C after the addition of 1 μg of pronase in 1 μl for each 5 μl of sample. The digested samples were then electrophoresed as described above, and the gels were developed with silver stain as described by Hitchcock and Brown (14).

Antibiotic susceptibilities. The MICs of antibiotics for the *A. salmonicida* wild-type and mutant strains, as well as the zones of inhibition produced by antibiotic disks with these strains, were determined as previously reported (34).

Electron microscopy. Cells were cultivated on BHI agar with cellophane overlays and removed by washing as described above. Samples of the resulting cell suspension were applied to grids, washed, stained, and examined by transmission electron microscopy (22).

Protease assay. Cultures were grown in BHI broth or the synthetic amino acid broth of Shieh and Reddy (30) with additions when specified, and protease activity was assayed as previously reported (34) by using azocasein and the method of Jensen et al. (17).

Chemicals. BHI agar, BHI broth, antibiotic disks, and nitrocefin were from Oxoid Ltd. Antibiotics and azocasein were from Sigma Chemical Co., St. Louis, Mo. DNase I and lysozyme were purchased from Boehringer Mannheim Biochemicals, Dorval, Quebec, Canada, and pronase was obtained from Calbiochem-Behring, La Jolla, Calif. The SDS-PAGE reagents were from Bio-Rad Laboratories, Richmond, Calif., except for SDS and glycine Analar R, which were purchased from BDH, Poole, England. The low-molecular-weight electrophoresis calibration kit was from P-L Biochemicals, Inc., Dorval, Quebec, Canada.

RESULTS

Outer membrane protein profiles and permeability. The Prt^{-1} *mlr* mutants of *A. salmonicida* were obtained in two ways. Exposure of wild-type strains to low inhibitory concentrations of any one of several low-molecular-mass antibiotics (e.g., β-lactams, fluoroquinolones, and chloramphen-

TABLE 1. Properties and MICs of antibiotics for *A. salmonicida* AsL parent and mutant strains

Organism		Exoprotease	MIC (μg of antibiotic ml^{-1})				
Strain	Description		Ampicillin	Chloramphenicol	Nalidixic acid	Novobiocin	Tetracycline
AsL	Parent	+	0.2	1	0.2	8	15
AsL-P1[a]	*mlr*, from AsL	−	0.8	4	1.0	48	60
AsL-P1R1[b]	Prt^+ revertant, from AsL-P1	+	0.2	1	0.2	8	15
$AsLA^{Rc}$	Ampicillin resistant, from AsL	+	64	1	0.2	8	15
$AsLA^R$-N1[a]	*mlr*, from $AsLA^R$	−	128	4	1.0	48	60
$AsLA^R$-N2[a]	*mlr*, from $AsLA^R$	−	128	4	1.0	48	60

[a] Mutants with multiple low-level resistance (*mlr*) selected in the presence of low inhibitory concentrations of penicillin (4 μg ml^{-1}, AsL-P1) or nalidixic acid (0.4 μg ml^{-1}, $AsLA^R$-N1 and $AsLA^R$-N2).
[b] Revertant selected for regained exoprotease activity.
[c] Mutant selected in the presence of high inhibitory concentrations of ampicillin (4 μg ml^{-1}).

icol) allowed the isolation of mutants with the *mlr* phenotype at frequencies suggesting a point mutation (0.7×10^{-7} to 7×10^{-7}) (34). These mutants all lacked exoprotease activity and were cross-resistant. Also, Prt^- clones were found to occur at a high frequency after subculture of some lyophilized cultures of wild-type strains (10). These Prt^- strains also exhibited the *mlr* phenotype. The Prt^- *mlr* mutants (12 examined from 6 wild-type isolates) all demonstrated the same outer membrane protein changes when compared with the wild-type strains (34). These findings suggested that the outer membrane protein changes in the Prt^- *mlr* mutants might affect the permeability of the outer membrane to the low-molecular-mass antibiotics and release of protease.

To demonstrate a change in outer membrane permeability, the rates of hydrolysis of the β-lactam compound nitrocefin were compared between a strain possessing the wild-type outer membrane protein profile and one possessing the Prt^- *mlr* mutant outer membrane protein profile. This technique has been used with other gram-negative bacteria to measure outer membrane permeability through periplasmic β-lactamase activity (2, 25, 35).

The wild-type strains examined were susceptible to β-lactam antibiotics and possessed very little detectable β-lactamase activity. To increase this activity, mutants resistant to high levels of β-lactam antibiotics (4 μg of ampicillin ml^{-1}) were isolated and found to contain an active, inducible β-lactamase activity. These mutants (e.g., $AsLA^R$) retained exoprotease activity, and their susceptibilities to antibiotics other than β-lactam compounds were unaltered (Table 1). Also, SDS-PAGE profiles of their outer membrane proteins and LPS were indistinguishable from those of the parent wild-type strains (Fig. 1). From one such β-lactam-resistant mutant ($AsLA^R$), mutants exhibiting the Prt^- *mlr* phenotype were isolated after exposure to low inhibitory concentrations of nalidixic acid (0.4 μg ml^{-1}). Several of these doubly selected mutants were examined (e.g., $AsLA^R$-N1 and $AsLA^R$-N2) and were found to exhibit the Prt^- *mlr* phenotype (Table 1). They had the outer membrane protein profile typical of singly selected Prt^- *mlr* mutants (Fig. 1), and they retained an active, inducible β-lactamase activity.

To compare the outer membrane permeabilities of the $AsLA^R$ strain and the Prt^- *mlr* $AsLA^R$-N1 strain derived from it, a procedure similar to that of Nicas and Hancock (25) was used. For both strains, the periplasmic β-lactamase activity was in excess of the rate of nitrocefin hydrolysis by whole cells, ranging from 40- to 60-fold higher in $AsLA^R$ and from 180- to 300-fold higher in $AsLA^R$-N1 under the conditions used (Table 2). The rate of nitrocefin hydrolysis by whole cells was directly proportional to the concentration of nitrocefin used from 10 to 100 μM. Although the standard deviation was quite high, as has been observed by other workers (25), the results show that the outer membrane of the Prt^- *mlr* $AsLA^R$-N1 strain is significantly less permeable to nitrocefin than that of the $AsLA^R$ strain as determined by the calculation of permeability coefficients (25, 35). The results obtained were similar to those reported for *P. aeruginosa* and its porin F-deficient mutant (25).

Isolation and characterization of phenotypic revertants. Selection of revertant strains from Prt^- *mlr* mutants was based on recovery of exoprotease activity. These Prt^+ revertants were then examined for antibiotic susceptibilities and outer membrane protein changes to further establish a connection between the pleiotropic effects observed in the Prt^- *mlr* mutant phenotype.

Since NH_4^+ present in media significantly reduces exoprotease production (28, 31; see below) and the exoprotease is active against casein, adding a nitrogen source in the form

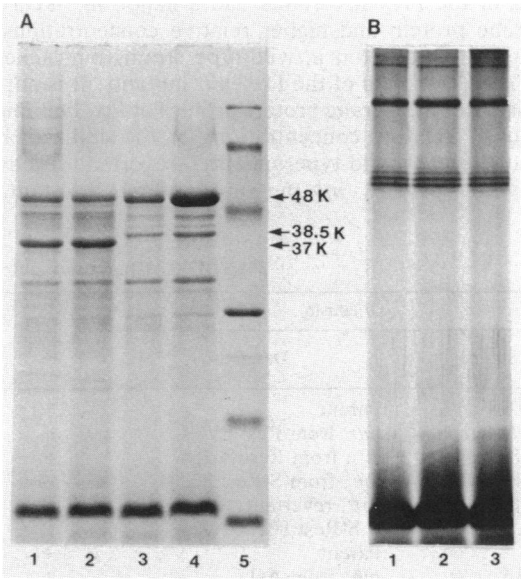

FIG. 1. SDS-PAGE of outer membrane proteins (A) and LPSs (B) of *A. salmonicida* AsL wild-type and mutant strains. (A) Lanes: 4, AsL (wild type); 3, $AsLA^R$ (ampicillin resistant); 2, $AsLA^R$-N1 (ampicillin resistant, Prt^- *mlr*); 1, $AsLA^R$-N2 (ampicillin resistant, Prt^- *mlr*); 5, molecular mass standards, including phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and β-lactalbumin (14,400). (B) Lanes: 1, AsL; 2, $AsLA^R$; 3, $AsLA^R$-N1. The sources of the strains are given in Table 1. The arrows indicate proteins discussed in the text (K, kDa).

TABLE 2. Nitrocefin hydrolysis by *A. salmonicida* AsLA^R (Prt$^+$) and AsLA^R-N1 (Prt$^-$ *mlr*) mutant strains

Strain	Mean rate of nitrocefin hydrolysis (V) (±SD [n = 6])[a]			Outer membrane permeability coefficient C (10^4)[b]
	Intact cells	Disrupted cells	Disrupted cells/intact cells	
AsLA^R	80 (±40)	3,730 (±1,430)	49 (±10)	6.5
AsLA^R-N1	13.5 (±6.5)	3,100 (±1,380)	243 (±54)	1.3

[a] Nanomoles minute^{-1} milligram of cell protein^{-1} at a final concentration of 50 μM nitrocefin.
[b] Minute^{-1} milligram of cell protein^{-1}, calculated by the method of Zimmermann and Rosselet (35).

of casein might provide a selective procedure for isolating Prt$^+$ revertants. However, *A. salmonicida* has amino acid requirements for growth which are not fully met by casein alone. Attempts to isolate Prt$^+$ revertants directly from Prt$^-$ *mlr* mutants on solidified medium were unsuccessful, since neither wild-type nor mutant strains grew well on casein medium without added amino acids and both grew well when amino acids were included. Therefore, a liquid medium was prepared, containing amino acids and glucose to provide carbon excess with respect to nitrogen available in the amino acids and a high concentration of casein. Growth of Prt$^-$ *mlr* mutants in this medium was biphasic, with phase 2 (after approximately 72 h of incubation) providing the Prt$^+$ revertant clones.

These Prt$^+$ revertants (six isolates from three Prt$^-$ *mlr* mutants examined) had lost the *mlr* phenotype and displayed antibiotic susceptibilities (Tables 1 and 3) and outer membrane protein profiles (Fig. 2A) indistinguishable from those of the original parent strains. Outer membrane protein profiles of the Prt$^+$ revertants had a major 38.5-kDa outer membrane protein and higher relative concentrations of a 48-kDa protein as seen in wild-type strains but lacked the major 37-kDa protein of the Prt$^-$ *mlr* mutants. It is not clear why the outer membrane protein profiles of Prt$^-$ *mlr* mutants have lower relative concentrations of the 48-kDa protein than do those of wild-type and Prt$^+$ revertant strains. All three, wild-type, Prt$^-$ *mlr* mutant, and Prt$^+$ revertant, possessed an A layer as determined by electron microscopy (Fig. 3). The 48-kDa protein in outer membrane profiles appears to represent the surface array A-layer protein because of its similarity to the reported molecular mass (16, 18, 19) and the absence of a major protein band at 48 kDa in outer membrane profiles of A$^-$ mutants (Fig. 2B).

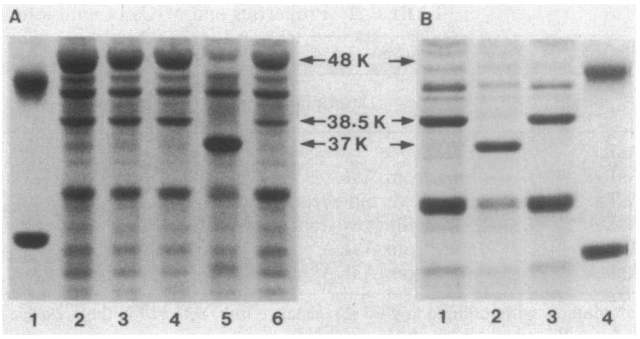

FIG. 2. SDS-PAGE of outer membrane proteins of *A. salmonicida* strains. (A) Lanes: 6, AsL (wild type); 5, AsL-P1 (Prt$^-$ *mlr*); 4, AsL-P1R1 (Prt$^+$ revertant); 3, AsL-P1R2 (Prt$^+$ revertant); 2, AsL-P1R3 (Prt$^+$ revertant). (B) Lanes: 1, S-Rest.80204 (A$^-$); 2, S-Rest.80204-A1 (A$^-$, Prt$^-$ *mlr*); 3, S-Rest.80204-A1R1 (A$^-$, Prt$^+$ revertant). Molecular mass standards, ovalabumin (43,000) and carbonic anhydrase (30,000), are shown in lane 1 of panel A and lane 4 of panel B. The sources of the strains are given in Tables 1 and 3. The arrows indicate proteins discussed in the text (K, kDa).

A$^-$ mutants were isolated from wild-type strains and used to derive A$^-$ Prt$^-$ *mlr* mutants and then A$^-$ Prt$^+$ revertants. These A$^-$ strains behaved similarly to their A$^+$ counterpart strains except for the lack of a major 48-kDa protein in outer membrane profiles (Table 3; Fig. 2B). No A layer was seen on cells of the A$^-$ strains by transmission electron microscopy (Fig. 3). No differences were observed between outer membrane LPS SDS-PAGE profiles of wild-type strains, Prt$^-$ *mlr* mutants, or Prt$^+$ revertants or the A$^-$ counterpart strains of these three types (Fig. 1B). They were essentially as reported for other *A. salmonicida* strains (4, 6, 7).

SDS-PAGE protein profiles of extracellular and periplasmic fractions. In an attempt to elucidate the fate of protease in Prt$^-$ *mlr* mutants, SDS-PAGE profiles of extracellular and

TABLE 3. Characteristics of *A. salmonicida* parent and mutant strains

Organism		A layer[a]	Exoprotease[b]	Antibiotic disk zone of inhibition (mm diam)[c]				
Strain	Description			Ampicillin (10 μg)	Chloramphenicol (30 μg)	Nalidixic acid (30 μg)	Novobiocin (30 μg)	Tetracycline (30 μg)
Rest.80204	Parent	+	+	31	29	39	18	32
Rest.80204-A1[d]	*mlr*, from Rest.80204	+	−	23	20	33	9	26
S-Rest.80204[e]	A$^-$, from Rest.80204	−	+	30	28	38	17	32
S-Rest.80204-A1[d]	*mlr*, from S-Rest.80204	−	−	23	20	31	9	26
S-Rest.80204-A1R1[f]	Prt$^+$ revertant, from S-Rest.80204-A1	−	+	30	28	39	17	32
AsL	Parent	+	+	30	29	39	17	12
AsL-A1[d]	*mlr*, from AsL	+	−	22	20	32	10	6.5
AsL-A1R2[f]	Prt$^+$ revertant, from AsL-A1	+	+	31	30	40	19	12
AsL-P1[d]	*mlr*, from AsL	+	−	22	18	32	8	6.5
AsL-P1R2[f]	Prt$^+$ revertant, from AsL-P1	+	+	31	30	40	19	12

[a] Determined by transmission electron microscopy.
[b] Determined by plating on skim milk-agar overlay plates.
[c] Antibiotic disk diameter was 6.5 mm. Variation was a maximum of 2 mm between replicates.
[d] Mutants with multiple low-level resistance selected in the presence of low inhibitory concentrations of ampicillin (0.4 μg ml^{-1}, Rest.80204-A1, S-Rest.80204-A1, and AsL-A1) or penicillin (4 μg ml^{-1}, AsL-P1).
[e] A-layer-negative mutant isolated after incubation at elevated temperature (16).
[f] Revertants selected for regained exoprotease activity.

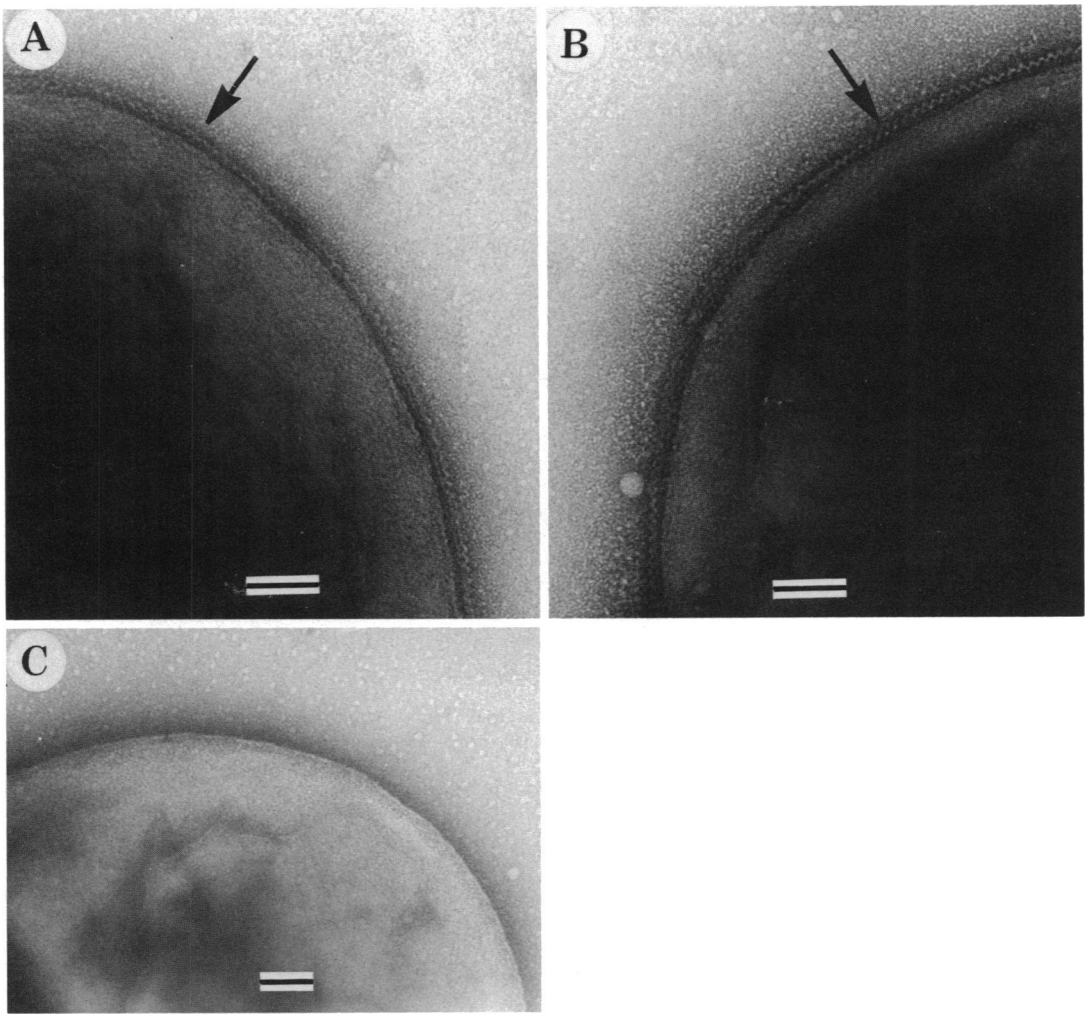

FIG. 3. Electron micrographs of *A. salmonicida* AsL (wild type) (A), AsL-P1 (Prt$^-$ *mlr*) mutant (B), and S-AsL (A-layer-negative) mutant (C). The arrows indicate the A layer. Bar = 0.1 μm.

periplasmic fractions were compared for wild-type, Prt$^-$ *mlr*, and Prt$^+$ revertant strains.

The profiles of extracellular proteins (ECPs) from wild-type strains (five examined) grown under conditions conducive to exoprotease production (e.g., BHI) contained a major protein band at approximately 69 kDa (Fig. 4). No major protein band was seen at this location in ECPs from Prt$^-$ *mlr* mutants, but a major protein band at 69 kDa was observed from ECPs of Prt$^+$ revertants. This protein band appears to represent the major exoprotease (caseinase) activity, since other workers have purified exoprotease from other *A. salmonicida* strains and determined it to be a major ECP of similar molecular mass (70 to 71 kDa) (9).

To confirm that our strains were similar, exoprotease was isolated from one wild-type strain (Rest.80204) by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography, and gel filtration and produced a single protein band of approximately 69 kDa by SDS-PAGE (data not shown). Also, the 69-kDa protein band was significantly reduced in ECPs of Prt$^+$ strains grown under conditions in which exoprotease activity was significantly reduced or not detected (e.g., BHI containing 1% NH_4Cl). The minor protein band at 69 kDa in ECPs of Prt$^-$ *mlr* mutants may represent small amounts of exoprotease not detected by the assay conditions used or another minor protein band which is masked when large amounts of exoprotease are present.

The other major difference in ECPs released by the strains examined was the amount of liberated LPS. However, this appeared to be a function of the absence of the A layer, since significantly higher amounts of LPS were observed only in the extracellular fractions from A$^-$ strains (data not shown). This may have been the result of the production of *A. salmonicida* outer membrane vesicles (21) which are trapped or restricted in their release by the surface A layer in A$^+$ strains but are readily released into the surrounding milieu by A$^-$ strains (22).

To investigate the possibility that the protease becomes trapped and accumulates in the periplasm of Prt$^-$ *mlr* mutants, SDS-PAGE profiles of periplasmic fractions were obtained for three wild-type strains, their Prt$^-$ *mlr* mutants, and Prt$^+$ revertants (Fig. 4). No major 69-kDa protein band was detected in periplasmic fractions from the Prt$^-$ *mlr* mutants. A minor protein band at this location was observed in samples from wild-type and Prt$^+$ revertant strains, although it may represent some contamination of periplasmic fractions with ECPs. Some other differences were noted, including the presence of a protein band at approximately 86 kDa in Prt$^-$ *mlr* mutant samples that was weakly represented

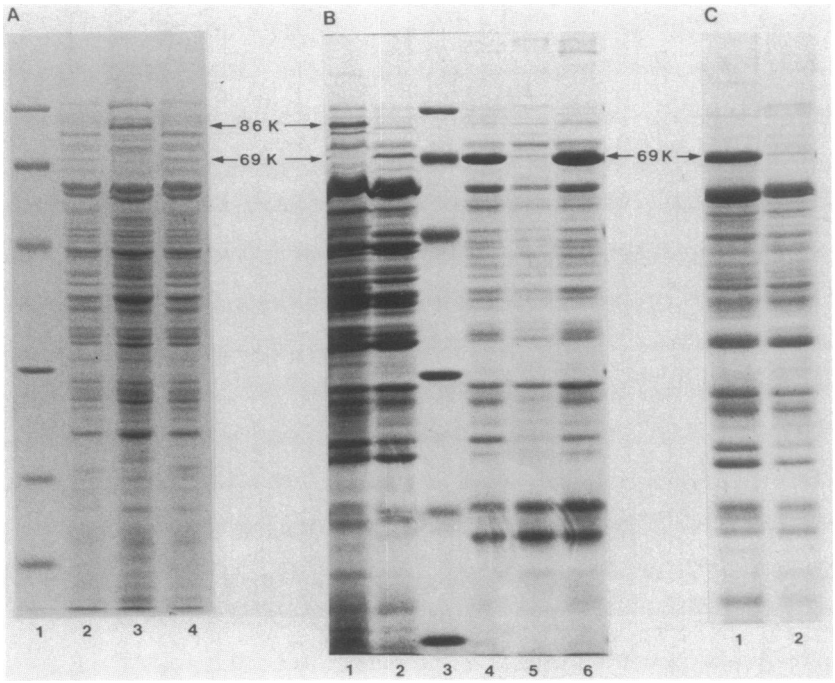

FIG. 4. SDS-PAGE protein profiles of periplasmic and extracellular fractions from *A. salmonicida* strains. (A) Periplasmic fraction lanes: 2, AsL (wild type); 3, AsL-P1 (Prt$^-$ *mlr*); 4, AsL-P1R1 (Prt$^+$ revertant). (B) Periplasmic fraction lanes: 1, AsL-P1; 2, AsL (heavily applied for emphasis). Extracellular fraction lanes: 4, AsL; 5, AsL-P1; 6, AsL-P1R1. (C) Extracellular fraction lanes: 1, Rest.80204 (wild type); 2, Rest.80204-A1 (Prt$^-$ *mlr*). Molecular mass standards are shown in lane 1 of panel A and lane 3 of panel B. The sources of strains are given in Tables 1 and 3. The arrows indicate proteins discussed in the text (K, kDa).

or lacking in samples from Prt$^+$ strains. However, the 86-kDa protein band was subsequently observed in Prt$^+$ strain samples from cultures grown under conditions in which exoprotease was weakly expressed or not detected (e.g., BHI containing 1% NH_4Cl).

Effects of some environmental conditions on outer membrane protein expression and exoprotease activity. *A. salmonicida* S-Rest.80204 (A$^-$) was grown under a variety of conditions and examined to see whether changes in outer membrane protein expression similar to those observed for OmpF and OmpC in *E. coli* K-12 could be initiated which might then assist in explaining the consistent outer membrane protein alterations in the Prt$^-$ *mlr* mutants. Exoprotease activity was also monitored. The A$^-$ S-Rest.80204 strain was used because of its lack of autoagglutination (16, 33) and ease of extraction of outer membrane fractions (34). Some results were also obtained by using the AsL wild-type strain. The conditions used included growth at different temperatures in BHI broth (12, 22, and 30°C), variations in medium osmolarity in BHI broth and Luria broth (up to 0.6 M NaCl and 0.73 M sucrose), growth in BHI broth containing subinhibitory concentrations of novobiocin or 1% NH_4Cl, and growth in a basal amino acid medium broth with and without cyclic AMP.

The outer membrane protein profiles of the S-Rest.80204 and AsL strains were essentially the same as those presented in Fig. 2, with no detectable changes in major outer membrane proteins under any growth conditions tested. However, the exoprotease activity in culture fluids was affected by many of these conditions, and some of these results are shown in Table 4. The addition of 1% NH_4Cl to BHI broth significantly reduced the production of detectable exoprotease activity without affecting the antibiotic susceptibilities of the Prt$^+$ strains or growth greatly. High concentrations of sucrose or NaCl, novobiocin concentrations close to the MIC, and low growth temperature severely affected growth and exoprotease activity. However, exoprotease activity was significantly reduced at concentrations of these agents which did not appear to greatly affect growth. Growth in basal amino acid medium was slower and exoprotease activity was very low but increased when cyclic AMP was included in the medium. The effects of ammonium ion and low growth temperature on the production of exoprotease were reported previously (29, 31).

DISCUSSION

The Prt$^-$ *mlr* mutants of *A. salmonicida* obtained at frequencies suggesting a point mutation showed pleiotropic

TABLE 4. Exoprotease activity of *A. salmonicida* S-Rest.80204 grown in BHI broth cultures with indicated additions

Addition	OD_{660}[a]	Exoprotease activity[b]
None	3.9	1.3
Novobiocin		
2 μg ml^{-1}	3.8	1.1
4 μg ml^{-1}	3.8	0.6
NH_4Cl, 0.19 M	3.0	≤0.03
NaCl		
0.26 M	3.0	1.0
0.43 M	2.2	0.2
0.52 M	0.7	≤0.03
Sucrose, 0.56 M	1.8	≤0.03

[a] OD_{660} of cultures after 24 h of incubation.
[b] Change in OD_{370} of acid-soluble azocasein minute^{-1} milliliter of extracellular culture fluid^{-1}.

effects (34). These included changes in outer membrane protein profiles, reduced susceptibilities to many smaller-molecular-mass antibiotics, and loss of exoprotease activity.

The decreased antibiotic susceptibilities of the Prt$^-$ *mlr* mutants appears to be the result of the changes in outer membrane proteins observed. This suggestion is supported by the significantly lower outer membrane permeability to nitrocefin in a strain with the Prt$^-$ *mlr* outer membrane protein profile than in a strain with the wild-type outer membrane protein profile. Further, it is supported by the isolation of Prt$^+$ revertant strains from the Prt$^-$ *mlr* mutants which have antibiotic susceptibilities and outer membrane protein profiles indistinguishable from those of the original wild-type strains. The isolation of these phenotypic revertant strains also supported the suggestion that the pleiotropic effects observed were the result of a single-point mutation.

The decreased outer membrane permeability of the Prt$^-$ *mlr* mutants appears to be connected to the 38.5- to 37-kDa outer membrane protein change and not to the relative decrease in concentration of the 48-kDa protein as determined by band intensity in SDS-PAGE. The 48-kDa protein corresponds to the surface array A protein (16, 18, 19) isolated with the outer membrane fractions. Mutants (A$^-$, A$^-$ Prt$^-$ *mlr*, and A$^-$ Prt$^+$ revertant strains) which lack the 48-kDa protein have no detectable A layer as determined by transmission electron microscopy but otherwise display the same antibiotic susceptibilities, outer membrane protein profiles, and exoprotease activities as their A$^+$ counterpart strains. The reason for the decreased relative concentrations of the A protein in outer membrane protein profiles of the A$^+$ Prt$^-$ *mlr* strains is not known, since electron micrographs of these mutants clearly show the presence of the A layer. The Prt$^-$ *mlr* phenotype may have some influence on the retention of the A protein in the outer membrane fractions during isolation.

The loss of exoprotease activity in the Prt$^-$ *mlr* mutants corresponded to the lack of a major ECP of approximately 69 kDa. The identification of the 69-kDa ECP as the major exoprotease was indicated by purification of exoprotease activity from a wild-type strain to a single protein band of approximately 69 kDa and the presence of a major 69-kDa protein in ECPs of Prt$^+$ revertant strains. Also, other workers have reported a similar molecular mass for purified exoprotease from other *A. salmonicida* strains (9).

The relationship between loss of exoprotease activity and outer membrane protein changes in Prt$^-$ *mlr* mutants is unknown. One explanation might be that the 38.5-kDa outer membrane protein of the Prt$^+$ strains is involved in protease export across the outer membrane. Mutants of other gram-negative bacteria that show outer membrane protein changes and reduced exoprotease activity have been reported to accumulate protease in the periplasm. The protease so accumulated was in the form of an active enzyme in *A. hydrophila* mutants (15) but was in an inactive form in *P. aeruginosa* mutants (8). In the latter case, the accumulated inactive form was indistinguishable in molecular mass from the active exoprotease. The Prt$^-$ *mlr* mutants of *A. salmonicida* appeared to behave differently, since no accumulation of detectable periplasmic exoprotease activity or of a protein band in the 69-kDa range was detected by SDS-PAGE of mutant periplasmic fractions. Small amounts of a 69-kDa protein were frequently observed in periplasmic protein profiles of samples from wild-type and Prt$^+$ revertant strains. However, this latter observation may have been the result of small amounts of ECPs extracted with the periplasmic fractions of Prt$^+$ strains.

A protein band at approximately 86 kDa observed in profiles of periplasmic fractions from Prt$^-$ *mlr* mutants was weakly detected or not seen in periplasmic protein profiles of Prt$^+$ strains grown on BHI. However, the accumulation of an 86-kDa protein in the periplasm of Prt$^+$ strains could be initiated by growth of the strains under conditions that prevent the production of detectable exoprotease activity. Further investigation of the 86-kDa protein may prove to be interesting, since the protein may represent a high-molecular-weight inactive precursor of the protease or some other protein which may require the protease for processing (32).

Another possible explanation for the concomitant loss of exoprotease with the outer membrane protein changes in Prt$^-$ *mlr* mutants is that the mutation may involve some mechanism of coordinated control over the expression of these proteins. Therefore, a Prt$^+$ *A. salmonicida* strain was examined under a variety of growth conditions, including some that have been reported to affect exoprotease production by *A. salmonicida* (29, 31) and others that have been reported to affect outer membrane protein expression in other gram-negative bacteria (23, 27). Although exoprotease expression in *A. salmonicida* was altered by a variety of these growth conditions, no changes in the expression of major outer membrane proteins were observed. The results suggested that the expression of outer membrane proteins and production of exoprotease in *A. salmonicida* at least are not tightly coupled and further that the expression of outer membrane proteins in *A. salmonicida* is stable to such environmental influences as osmolarity (sucrose or NaCl) and temperature under the conditions used.

Although the results of this investigation have established a relationship between exoprotease production and outer membrane protein changes that affect permeability in *A. salmonicida* mutants, the reason for this relationship has not been elucidated. It does not appear to be the result simply of a periplasmic accumulation of protease resulting from outer membrane protein changes, as has been reported for mutants of certain other gram-negative bacteria (8, 15). Further studies, for example using immunoprecipitation techniques, might provide insight into the fate of protease in the *A. salmonicida* mutants. Also, continued experimentation with the 38.5- and 37-kDa outer membrane proteins may prove to be useful in studying outer membrane permeability in *A. salmonicida*. We are investigating the relationships between these proteins.

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