Cloning of the Gene for the Surface Array Protein of *Aeromonas salmonicida* and Evidence Linking Loss of Expression with Genetic Deletion

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Received 3 March 1987/Accepted 8 June 1987

A gene bank of DNA from the fish pathogenic bacterium *Aeromonas salmonicida* was constructed in the bacteriophage λgt11. Phage λgt11/10G, a recombinant carrying a 4.0-kilobase fragment of *A. salmonicida* DNA, was found to express the surface array protein (A protein) in *Escherichia coli*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the protein expressed from the cloned gene had a subunit molecular weight of 49,000, which was identical to that of subunits in the native assembled A layer. Genomic Southern analysis showed that the gene coding for this predominant cellular protein was in a single copy on the chromosome and was conserved among a wide range of *A. salmonicida* strains with different phenotypic characteristics and isolated from diverse geographic locations, fish species, and means of pathogenesis. Results of genomic blotting experiments also showed that loss of expression of the A layer resulting from growth at 30°C was accompanied by genetic rearrangement in which N-terminal sequences of the gene for A protein were lost by deletion.

Regular surface protein arrays and less well characterized additional surface layers have been reported on a number of eubacterial and archael bacterial species (4, 9, 25). Even though structural and chemical studies of several eubacterial surface arrays have been extensive (25, 26), little has been done to link the structural arrangements to specific phenotypic functions. It is generally thought that S layers contribute to the restriction of access of particular solutes to the cell and the maintenance of cell shape; however, except in the case of the shape-determining features of archael bacterial S layers (19, 20), little evidence has been presented to corroborate this. Probably the best studied surface protein array in terms of functional characterization is the A layer of *Aeromonas salmonicida*.

The paracrystalline surface protein array A layer consists of a single protein species (A protein) with a subunit molecular weight (Mr) of 49,000. Possession of the A layer confers virulence on both typical and atypical strains (13, 35) of *A. salmonicida* infecting salmonid and nonsalmonid fish species. Mutants lacking only the A layer generated by growth at elevated temperatures showed a drop in virulence by a factor in excess of 105 (13). The A layer helps to protect the cell against the bactericidal activity of serum (21) and specifically binds heme and protoporphyrin IX (15). Interactions between the cell and macrophages also appear to depend on the possession of the A layer (34). Computer image analyses of electron micrographs of the *A. salmonicida* A layer indicate two distinct tetragonal patterns showing P4 symmetry but with different apparent porosities which may reflect a functional difference in the permeability of the layer (29). Because biochemical and genetic evidence indicates that the layer is composed of a single protein species, the molecular arrangements which allow this protein to perform such a variety of functions are intriguing.

In a more general sense, S-layer proteins, such as A protein, represent an important class of cell surface molecules. These proteins must be exported across two membranes; but unlike secretory proteins such as protease and hemolysin, they then need to be assembled into a complex structure on the cell surface. The magnitude of the array is such that the protein must be produced in very large amounts, and this is the predominant protein species produced by the cell. The questions posed by the biochemistry of S-layer synthesis, assembly, and structure and the genetics of expression and control are important. The genetic and biochemical systems used in S-layer production are also potentially valuable. A problem commonly encountered in the application of recombinant DNA technology is the transfer of protein products out of bacterial cells. Use of genes for exporting a high-copy-number protein such as A protein may overcome this problem.

Using transposon mutagenesis, we have previously described mutations which helped elucidate the functions that are necessary for the synthesis, export, and assembly of the A layer (2). Specifically, this involves the movement of A protein through the periplasm and its translocation across the outer membrane, as well as the dependence of layer assembly on the production of homologous chain length O-polysaccharide-substituted lipopolysaccharide (2). To analyze further the expression of the A layer and to define the primary structure of A protein, we found it imperative to clone the structural gene for the surface layer subunit. Here we report the cloning of the A-protein gene and provide evidence that loss of expression of the gene in a variety of *A. salmonicida* strains is accompanied by genetic deletion.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The *A. salmonicida* strains used in this study are listed in Table 1. *Escherichia coli* strains used as recipients for the λgt11, pUC18, and pLG338 (31) cloning vectors are also listed in Table 1. All strains were maintained as stock cultures at −70°C in tryptic soy broth (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% (vol/vol) glycerol. *Aeromonas* strains were grown aerobically on tryptic soy agar (GIBCO) at 20°C. Avirulent *Aeromonas* strains gener-

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ated by growth at elevated temperatures were prepared as described by Ishiguro et al. (13). Cultures grown at 20°C in tryptic soy broth were divided and diluted in tryptic soy broth. Samples of each strain were grown at 20 and 30°C for 36 h under static conditions. Following incubation appropriate dilutions were plated onto tryptic soy agar containing 0.003% (wt/vol) Congo red to distinguish the isolates lacking the A layer (12), and the strains were reisolated. E. coli strains were grown on yeast-tryptone (Difco Laboratories, Detroit, Mich.) at 37°C, while lysogenic λgt11 strains were grown at 32°C and induced at 43°C.

**Isolation of A. salmonicida DNA and cloning in λgt11.** Whole-cell DNA from *A. salmonicida* strains was purified by the method described by Stern et al. (28). Approximately 0.5 g of wet packed cells was suspended in 10 ml of 20 mM Tris hydrochloride (pH 7.5) containing 100 mM NaCl and 1 mM EDTA, and the solution was made to 10 μg/ml with lysozyme (Sigma Chemical Co., St. Louis, Mo.). Triton X-100 was added to a final concentration of 2% (vol/vol), and the suspension was incubated at 50°C for 1 h. Proteinase K (5 μg/ml; E. Merck AG, Darmstadt, Federal Republic of Germany) was then added, and incubation was continued at 50°C for an additional 2 h. CsCl (10 g) and ethidium bromide (0.5 ml of a 10-μg/ml solution) were then added, and the solution was centrifuged to equilibrate in a fixed-angle rotor (type 70.1 Ti; Beckman Instruments, Inc., Palo Alto, Calif.). Linear and covalently closed DNAs were recovered separately. Ethidium bromide was removed by repeated extraction with isooamyl alcohol, and the samples were then dialyzed against 10 mM Tris hydrochloride (pH 7.5) containing 1 mM EDTA. Whole-cell DNA samples were made by pooling fractions of chromosomal and plasmid DNA.

Purified whole-cell DNA from *A. salmonicida* A449 (a virulent A-layer-producing strain) was digested with EcoRI in a buffer consisting of Tris hydrochloride (25 mM, pH 8.5), MgCl₂ (2 mM), and glycerol (7%; vol/vol), which are conditions that are known to give rise to EcoRI "star" activity (18, 23). Digestion times were adjusted to optimize the proportion of restriction fragments in the 2- to 8-kilobase (kb) range. Digested DNA was extracted once with phenol (equilibrated with digestion buffer), once with phenol-chloroform, and once with chloroform; and then it was ethanol precipitated. Digested DNA (1.5 μg) suspended in 10 mM Tris hydrochloride containing 1 mM EDTA was ligated to EcoRI-digested, dephosphorylated λgt11 DNA (Promega Biotech, Mississauga, Ontario, Canada) at a ratio of 3:1 (insert:vector) with T4 DNA ligase (New England Biolabs, Inc., Beverly, Mass.). Portions of the ligation mixture were packaged in vitro by using lysates and protocols from Promega Biotech. The gene library was amplified by the procedure described by Huynh et al. (11).

**Immunoscreening of the λgt11 library.** Recombinant phage were plated at a density of approximately 2,500 plaques per plate in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (final concentration, 0.02%) at 43°C for 3 h to ensure induction. Plates were then overlayed with precut nitrocellulose membranes (Millipore Corp., Bedford, Mass.), which had previously been soaked in 10 mM isopropyl-β-D-thiogalactopyranoside and dried, and incubated at 37°C for an additional 6 h. Membranes were marked to align them with corresponding plates and then removed and dried. Plates were stored at 4°C. Dried nitrocellulose membranes were then blocked in a solution of Tris-buffered saline (TBS) containing gelatin (Tris hydrochloride [20 mM, pH 7.4], NaCl [500 mM], gelatin [1%; vol/vol]). Membranes were then incubated in a fresh solution of TBS-gelatin containing rabbit polyclonal antiseraum raised against purified A protein (22) at a dilution of 1:500. After washing in TBS, membranes were incubated with *Staphylococcus aureus* protein A which had been radiolabeled with 125I by the lactoperoxidase technique (10). Following extensive washing with TBS the membranes were dried and exposed to film (X-AR5; Eastman Kodak Co., Rochester, N.Y.) for autoradiography. The plaques that gave positive signals were purified and rescreened by the same procedure.

**Isolation of recombinant phage DNA, restriction mapping, and subcloning.** Recombinant phage that gave a positive reaction with anti-A-protein antiserum were amplified by growth on strain Y1090 by a plate lysate technique (17). These lysates were then used to infect strain Y1089, which has a high frequency of lysogenization, at multiplicities of infection ranging from 1 to 20. Lysogenic strains that had the temperature-sensitive phenotype and produced phage and that on induction reacted with antiserum were used to purify DNA. DNA preparation was done essentially as described by Silhavy et al. (24). Restriction maps of the insert were made by comparing digests of recombinant phage DNA with λgt11 DNA purified in the same manner by agarose gel
electrophoresis. Fragments of the insert DNA were subcloned using the plasmid vectors pUC18 and pLG338 essentially as described by Maniatis et al. (17).

**Expression of A. salmonicida DNA in phage lysates.** Phage lysates were prepared by infecting rapidly growing cultures of strain Y1090 with recombinant or Agt11 phage at a multiplicity of infection of 10. Following induction at 43°C for 20 min the cultures were transferred to 37°C for an additional 60 min. Samples of the phage lysate were then solubilized in loading buffer (Tris hydrochloride [60 mM, pH 6.8], glycerol [1.0%], 2-mercaptoethanol [5%], sodium dodecyl sulfate [3%], bromophenol blue [0.01%]) at 100°C for 10 min and subjected to polyacrylamide gel electrophoresis by the method described by Laemmli (6, 16). Gels were either stained with Coomassie brilliant blue R-250 (Serva, Heidelberg, Federal Republic of Germany) or Western blotted to nitrocellulose membranes (pore size, 0.45 μm) by the methanol-Tris glycin system described by Towbin et al. (33). Immunoblots were developed by using either rabbit polyclonal anti-A-protein or monoclonal antibody (MAb) to the A-protein molecule. Visualization was done with 125I-radiolabeled S. aureus protein A, and autoradiography was done as previously described (2).

**Oligonucleotide probes.** Mixed-sequence oligonucleotides based on different regions of the A-protein amino acid sequence were synthesized on an automated synthesis instrument (8600; Biosearch, San Rafael, Calif.). Oligonucleotides were radiolabeled with 32P with T4 polymerase kinase (New England Biolabs) as described by Maniatis et al. (17).

**Southern blotting.** A. salmonicida DNA (2 μg) was digested with restriction endonucleases as specified by the manufacturers. Digested DNA was electrophoretically separated on 1% agarose gels and transferred to nitrocellulose membranes essentially as described by Maniatis et al. (17). Approximately 5 × 10^6 cpm of pRJ3, pRJ5, or Agt11/10G (containing the region 5’ to the gene, the N-terminal region, or the entire A-protein gene, respectively) was added to the prehybridized reaction and hybridized at 68°C for 18 h. After stringent washing by procedures described previously (17), the dried membranes were exposed to film (X-AR5; Eastman Kodak) for various lengths of time. A-HindIII fragments were end-labeled and included on gels as size standards.

**RESULTS**

**Agt11 library of A. salmonicida DNA.** Whole-cell DNA of A. salmonicida A449 was partially digested with EcoRI by using conditions which resulted in “star” activity. Fragments of approximately 2 to 8 kb were ligated into EcoRI-cut, dephosphorylated Agt11 DNA and packaged in vitro. Approximately 4 × 10^8 recombinant phage were obtained from a packaging experiment with 0.5 μg of total DNA (3:1 insert:vector ratio). Recombinant phage made up 96% of the total number of viable phage particles. Estimation of the insert size from random clones showed that inserts varied from about 1 to 6 kb (unpublished observation). By assuming a mean insert size of 2 kb, a single packaging experiment with 0.5 μg of total DNA represented >200 copies of the A. salmonicida A449 genome (ca. 2.4 × 10^9 daltons; unpublished data). A portion of the initial library (338) used to generate the recombinant phage titer to 2.5 × 10^8 PFU/ml.

**Immunoscreening of the Agt11 library and expression of the A-protein gene in phage lysates.** Recombinant phage were screened by using a combination of polyclonal antibodies and MAbs to purified A protein. By using precut nitrocellulose membranes, plaques were lifted from agar plates (diameter, 90 mm) at a density of approximately 2,500 per filter. Approximately 10^9 plaques were screened in total, giving rise to 12 immunoreactive plaques. Subsequent attempts to repurify the plaques were unsuccessful in all but a single case, indicating an instability in recombinant phage containing the A-protein gene. The lone stable clone (Agt11/10G) grew very poorly, giving only very small plaques, but it could be propagated and repurified.

Expression of the A-protein gene was initially examined by using phage lysates. Lysates of the Agt11/10G clone contained significant quantities of a protein of M, 49,000, which reacted with polyclonal antibody to A protein (Fig. 1), and MAbs AA1 (14), IA10, and IXA7, which bound epitopes on different portions of the A protein. The recombinant protein comigrated on polyacrylamide gels with mature A protein purified from the surface of the cell.

**Localization of the A-protein gene.** Much difficulty was encountered in subcloning the A-protein gene in a single piece, despite the use of a wide variety of subcloning vectors. This made attempts to delineate the gene by immunologic techniques unfeasible. The gene was localized by using two oligonucleotide probes generated from amino acid sequences (14) at the N terminus of the A protein (i.e., 17-mer TTT-1 [residues 7 to 12, Asn-Asp-Asn-Thr-Phe-Val] and 14-mer TTT-2 [residues 20 to 24, Thr-Lys-Gln-Pro-Val]). The restriction map in Fig. 2 indicates that the gene (ca. 1.4 kb) is located on a 4.0-kb piece of A. salmonicida DNA. Several fragments within the 4.0-kb insert were subcloned into pUC18 (Fig. 2), but despite numerous attempts the 0.8-kb BamHI-KpnI fragment that was reactive with the N-terminal oligonucleotide probes could not be subcloned by using the high-copy-number pUC18 vector. The 0.8-kb BamHI-KpnI fragment was successfully subcloned, however, by using the low-copy-number vector pLG338 (pRJS); and preliminary nucleotide sequencing in this 0.8-kb BamHI-KpnI insert allowed us to locate the N-terminal region of the
A-protein gene and determine the orientation of the gene in the restriction map (Fig. 2). Results of this sequencing also indicate that the insert likely contains the promoter region as well; and we speculate that the presence of the promoter region (and, possibly, the N-terminal region of the A protein), if in a high copy number, renders this fragment of DNA lethal in E. coli. This 0.8-kb BamHI-KpnI-containing plasmid was used as a probe for the A-protein gene in subsequent Southern blotting experiments, in conjunction with a combination of restriction enzyme digestions that cleaved the parental DNA to give the entire gene (and 5′ region) on a single restriction fragment (i.e., HindIII-PstI).

Southern blotting. Whole-cell DNA was purified from a number of A. salmonicida strains (Table 1), including both virulent (i.e., A-layer-producing [A⁺]) and avirulent (i.e., non-A-layer producing [A⁻]) isolates. DNA samples (2 μg) were digested with restriction enzymes and separated on agarose gels prior to Southern blotting onto membranes (GeneScreen Plus). When probed with the plasmid pRJ5 (containing the 0.8-kb BamHI-KpnI insert in pLG338 with the N-terminal portion of the A-protein gene), the structural gene for A protein in the parental strain (A449) was shown to be on a 3.5-kb PstI-HindIII fragment (Fig. 3, lane 1a). Other virulent strains producing the A layer, including A450, A451, and A488 (Fig. 3, lanes 2a, 3a, and 4a, respectively), were shown to react similarly. All the so-called atypical isolates tested also had a 3.5-kb PstI-HindIII probe-reactive fragment. Type strains A440 (ATCC 14174) and A251 (NCMB 1102), which do not produce the A layer, differed from the A⁺ strains in that their DNA did not react with the pRJ5 probe (data not shown).

When probed with pRJ3 (containing sequences immediately 5′ to the A-protein gene), all A⁺ strains showed the same reactive 3.5-kb HindIII-PstI fragment (Fig. 4). In strain A440 (ATCC 14174) the probe reacted with the 4.0- and 5.0-kb fragments, and in the A⁻ strain NCMB 1102 the probe reacted with a 6.0-kb HindIII-PstI fragment. Confirmatory experiments with the entire A-protein gene in λgt11/10G as a probe against BamHI-digested whole-cell DNA verified this result, i.e., all isolates producing the A layer showed identical hybridization patterns, while the two A⁻ strains showed different hybridization patterns (data not shown). Plasmid DNAs isolated from strain A449 showed no reactivity with any of the probes used (data not shown).

To confirm that the different hybridization patterns shown by A⁺ and A⁻ strains of A. salmonicida resulted from a genomic rearrangement which accompanied the switching off of A-layer production, A⁻ derivatives of four strains were isolated by growth at 30°C. When DNA from the respective A⁺ parents and A⁻ derivatives was subjected to Southern analysis with plasmid pRJ5, a deletion of the 5′ region of the A-protein gene was seen with each A⁻ derivative because no

FIG. 2. Restriction map of a 4.0-kb fragment of A. salmonicida A449 DNA cloned into the EcoRI site of λgt11. The shaded segment corresponds to the restriction fragments carrying A-protein gene sequences (determined with oligonucleotide probes and protein Mr), with the sequence reading from left to right (determined from nucleotide sequencing). pRJ1 to pRJ5 represent plasmid constructs containing fragments of insert DNA (pRJ1 to pRJ4 in pUC18 and pRJ5 in pLG338). Restriction endonuclease abbreviations: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; S, SstI.

FIG. 3. Southern blot analysis of A. salmonicida chromosomal DNA from matched isolates phenotypically producing (A⁺) or not producing (A⁻) the A layer. A⁺ strains were isolated following growth at 30°C, as described in the text. Lane 1a, A449; lane 1b, A449/30; lane 2a, A450; lane 2b, A450/30; lane 3a, A451; lane 3b, A451/30; lane 4a, A488; lane 4b, A488/30. Strains represented in lanes 1a, 2a, 3a, and 4a were A⁺ and had a 3.5-kilobase-pair probe-reactive PstI-HindIII fragment (indicated by an arrow) when probed with pRJ5, while A⁻ strains in lanes 1b, 2b, 3b, and 4b showed no reactivity.

FIG. 4. Southern blot analysis of A. salmonicida chromosomal DNA probed with pRJ3. DNA samples (2 μg) were digested with PstI-HindIII, electrophoresed on 1% agarose gels, transferred to membranes (GeneScreen Plus), probed with radiolabeled pRJ3, and washed under stringent conditions (17). Lane 1, A449 (parental strain); lane 2, A488; lane 3, A492; lane 4, A450; lane 5, A251 (NCMB 1102); lane 6, A440 (ATCC 14174); lane 7, A451; lane 8, A400; lane 9, A419; lane 10, A452; lane 11, A475; lane 12, A491. Strains used in lanes 1 to 8 were typical A. salmonicida isolates, while strains in lanes 9 to 12 were so-called atypical isolates. A 3.5-kb probe-reactive fragment was seen in all strains that produced the A layer (indicated by an arrow).
FIG. 5. Southern blot analysis of *A. salmonicida* chromosomal DNA digested with HindIII and PstI from matched isolates phenotypically producing (A+) or not producing (A-) the A layer. A- strains were isolated following growth at 30°C, as described in the text. Lane 1a, A449; lane 1b, A449/30; lane 2a, A450; lane 2b, A450/30; lane 3a, A451; lane 3b, A451/30; lane 4a, A488; lane 4b, A488/30. Strains represented in lanes 1a, 2a, 3a, and 4a were A+ and had a 3.5-kilobase-pair probe-reactive PstI-HindIII fragment (indicated by an arrow) when probed with pRJ3, while A- strains in lanes 1b, 2b, 3b, and 4b showed signs of genetic rearrangement.

Probe reactive fragment was apparent (Fig. 3, lanes 1b, 2b, 3b, and 4b). When probed with pRJ3 (adjacent 5′ sequences), the A- derivatives no longer showed reactivity with a 3.5-kb HindIII-PstI fragment (Fig. 5). In two of the A- derivatives (A449/30 and A450/30), the probe-reactive fragment was a smaller 2-kb fragment; and in another derivative (A488/30), the new reactive fragment was a 3.0-kb fragment. In the case of strain A451, the reactive fragment was shifted to a 6-kb fragment corresponding exactly to the size of the reactive fragment in the A- strain A251 (NCMB 1102).

**DISCUSSION**

We cloned the structural gene for the A-protein subunit of the surface array of the fish pathogen *A. salmonicida* and by using stringent hybridization conditions, we have shown that the gene for this important virulence factor is strongly conserved within the species. Indeed, the gene was present in all the strains tested, including strains belonging to both the typical and so-called atypical phenotypic groups, strains isolated from a variety of freshwater and marine fish, strains isolated from diverse geographic locations, as well as strains producing markedly different pathogenesis in fish. This underlines the importance of this gene and its gene product in the ability of *A. salmonicida* to infect fish. Surprisingly, little or no homology was seen with the 5-layer gene of the closely related fish pathogen *Aeromonas hydrophila* (unpublished data), even though this species also produces a tetragonal array containing protein subunits of *M. 52,000* (7).

As suggested by the results of our previous transposon mutagenesis study (2), in which the single insertion of Tn5 resulted in the loss of ability to produce A protein, genomic Southern analysis of A-layer-producing strains of both typical and atypical phenotypes showed that the gene appears to be present as a single genomic copy located on a HindIII-PstI fragment of approximately 3.5 kb. Southern analysis of plasmid DNA failed to detect the gene in the *A. salmonicida* strains tested, indicating that the A-protein gene is chromosomal.

The intact A layer is the predominant surface antigen of the cell, and for the array to cover the entire cell surface it is estimated that the cell must produce approximately 5 × 10^5 copies of the A-protein subunit (22). This indicates that the A-protein gene must have an extremely efficient promoter. The efficiency of this promoter may also explain the difficulties we encountered in attempting to clone the A-protein gene using a variety of other expression vectors, including plasmid pBR322 and cosmid pH79, and in subcloning the entire A-protein gene to pUC18. So, while we were able to clone the gene using Agt11 and mapped the gene to the right end of the 4-kb insert of *A. salmonicida* DNA, we were unable to subclone the entire gene. Interestingly, *Caulobacter crescentus* also appears to have only a single gene copy for its surface array subunit protein (27), so it too must have an extremely efficient promoter.

This is the first tetragonal array-forming protein to be cloned. Although the Agt11 system can provide proteins fused with β-galactosidase, on this occasion it did not. The cloned protein appeared to be immunologically identical to the A protein produced by *A. salmonicida*, as judged by immunoblotting with both polyvalent antibodies and MAbs. The subunit *M* of the protein expressed by the cloned gene was also indistinguishable from the *M* 49,000 mature native protein found in the assembled array of the *A. salmonicida* strain from which the gene was cloned. As was the case with the hexagonal array-forming protein of *C. crescentus* (27), there was no indication that A protein is first produced as a larger precursor form. This is consistent with our previous observation that the *M* of periplasmically accumulated A protein in Tn5 insertion mutants was identical to that of the mature native A-layer subunit (2). This mutant is still capable of exporting other proteins such as hemolysin and protease (unpublished data), indicating that the export pathway for the A-protein gene product is distinct from these other exported proteins.

Importantly, the availability of the cloned A-protein gene allowed us to provide the first molecular information on the long-observed phenomenon of the loss of ability of typical strains to produce the A layer after growth at temperatures above 25 to 27°C. The loss, which can occur in a single subculture at growth temperatures above 35°C, is accompanied by the loss of virulence for fish and is readily detected by virtue of the loss of cell autoagglutination, the loss of ability to bind Congo red, and increased sensitivity to phage (13). This switching off is accompanied by genomic rearrangement, and results of Southern genomic analysis suggest that the mechanism of rearrangement is not incidental and involves a deletion of genetic material, including the 5' region of the gene. The rearrangement in Southern blot profiles of all strains examined can be explained by deletion of genetic material from the 3.5-kb fragment containing the A-protein gene. In cases in which the pRJ3 probe reacts with a smaller fragment (2 or 3 kb), the loss of internal sequences is postulated. In cases in which the reactive fragment increases in size or splits to give two reactive fragments, a larger deletion, incorporating the 3' restriction site, is proposed such that the new restriction fragment may be larger or contain probe-reactive sequences on two fragments. In this it is interesting that Smit and Agabian (27) have also suggested that elimination of the ability to produce a surface protein array during laboratory subculture of *C. crescentus* could result from genetic rearrangement.

A similar switching off of surface antigen expression resulting from subculture and accompanied by a loss of virulence has been seen in *Neisseria gonorrhoeae* fimbriae.
(37). In the case of the gonococcus this switching off of fimbiae expression is irreversible, and the inability to revert to fimbiae expression is correlated with deletions in promoter and 5′ regions of the fimbrin gene (3, 32). As yet we have been unable to switch the A-protein gene back on in A. salmonicida, so the phenomenon cannot be regarded as phase variation. Other pathogenic microorganisms show programmed gene rearrangements that result in surface antigen variation in vivo and that influence the pathogenesis of the organism concerned (1, 5, 8, 30, 36). Whether the expression of the A. salmonicida A layer is switched off during in vivo growth and what effect this might have on pathogenesis remains to be established. Clearly, however, in vitro manipulation of the A-protein switch dramatically alters the virulence of the organism.

ACKNOWLEDGMENTS

This study was funded, in part, by the Natural Sciences Research Council of Canada. R.J.B. was the recipient of a graduate student fellowship from the Natural Sciences and Engineering Research Council, Ottawa, Ontario, Canada.

We thank Josiane Feutrier for essential assistance, B. M. Phipps for antisera, E. E. Ishiguro for strains, and W. W. Kay for valuable advice.

LITERATURE CITED