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The physical and genetic map of the *A. salmonicida* A449 chromosome: Molecular characterization of *recA* and a novel *fla* operon

by

Elizabeth Rhoda Osondu Umelo
B.Sc., University of PortHarcourt, 1991

A Dissertation Submitted in Partial Fulfillment of
the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

We accept this dissertation as conforming
to the required standard

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ABSTRACT

Aeromonas salmonicida is a Gram negative pathogenic fish bacterium. To facilitate the construction of the chromosomal map of *A. salmonicida* A449, several previously uncharacterized genes, including *rec*A and four *fla* genes were identified. While the location of all the genes identified as a result of this study were mapped on the chromosome, the *rec*A and *fla* genes were further characterized at the molecular level.

The *A. salmonicida* A449 *recA* was cloned, sequenced and expressed *in vitro*. The 1059 bp *rec*A open reading frame encoded a 353 amino acid protein with predicted molecular weight (M_r) of 37,900. Southern blot analysis was performed to demonstrate the high degree of conservation between the A449 *rec*A and those of the other typical and atypical strains of *A. salmonicida* examined. The predicted amino acid sequence of *A. salmonicida* A449 RecA was found to possess a number of domains identical to those characterized in *Escherichia coli* RecA. These included domains for adenosine triphosphate binding, DNA binding and protein-protein interactions. The *A. salmonicida* A449 *recA* was mobilized into an *E. coli recA* strain and was shown to allow increased survival in the presence of the chemical mutagen methyl methane sulfonate and ultra violet (uv) irradiation. The rate of the *A. salmonicida* A449 *rec*A-mediated recombination in *E. coli* was increased by exposure to uv light, which suggested that SOS induction in *A. salmonicida* paralleled that of *E. coli*. The *A. salmonicida* A449 *recA* also possessed a potential regulatory SOS-box in the DNA 5' of the gene.

A novel flagellin operon was identified in *A. salmonicida* A449, characterization of which revealed the presence of two tandemly linked flagellin structural genes *fla*A and *fla*B. The *fla*A and *fla*B genes were in turn tandemly linked to *fla*G encoding a protein of unknown function, and *fla*H encoding a protein homologous to the Hook Associated Protein II of other bacteria. The *fla*A and *fla*B genes with 79% nucleotide sequence identity, were conserved in typical and atypical strains of *A. salmonicida*, and displayed significant divergence at the nucleotide level from the *fla* genes of the motile species *Aeromonas hydrophila* and *Aeromonas veronii* biotype *sobria*. *fla*A, *fla*B and *fla*G encode unprocessed proteins with predicted M_rs of 32,351, 32,056 and 15,965 respectively. When cloned under the control of the Ptac promoter, *fla*B was highly expressed when induced in *E. coli* DH5α, and FlaB protein was detectable even in the uninduced state. In *flaA* clones containing intact upstream sequence, FlaA was barely detectable when uninduced and poorly expressed on induction. The *A. salmonicida* flagellins are antigenically cross-reactive with *A. hydrophila* TF7 flagellin(s), and evolutionally closely related to the flagellins of *Pseudomonas aeruginosa* and *Vibrio anguillarum*. Electron microscopy showed that *A. salmonicida* A449 expresses unsheathed polar flagella at extremely low frequency.

Finally, the physical and genetic map of the chromosome of *A. salmonicida* A449 was constructed using pulsed-field gel electrophoresis and Southern blot analysis. The three restriction enzymes used in the map construction were *Ceu*I, *Pme*I and *Pac*I. The chromosome of *A. salmonicida* A449, with an estimated size of 4,658 ± 29.75 kb, was determined to be circular in structure. Several genes of *A. salmonicida*, including those which encoded proteins implicated in virulence, were localized on the chromosome map. The chromosomal locations of the *rec*A and *fla* genes were also identified.

The global genomic relationship between the typical and atypical strains of *A. salmonicida* was investigated by comparing the *Ceu*I cleavage fingerprint of the respective genomes. The results showed that the typical strains were indeed very homogenous as had been previously reported. The atypical strains expressed extensive variation both in the number of DNA fragments obtained with *Ceu*I and also in the digestion fingerprint. The comparison of the *Ceu*I digestion fingerprint of atypical strains revealed a clustering of some strains which suggested that this could be a powerful taxonomic tool for better classification of the atypical group.

The two *A. sobria* strains analyzed with *Ceu*I were also homogenous and showed significant similarities to the *A. salmonicida* typical strains *Ceu*I genomic fingerprints. In contrast, four *A. hydrophila* strains yielded *Ceu*I-derived fragments which like the atypical strain varied both in number and patterns. There was also minimal observed similarities between the genome of *A. hydrophila* strains and the *A. salmonicida* strains.

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LIST OF ABBREVIATIONS

A	Absorbance
A_{600}	Absorbance at 600 nm
ATP	Adenosine triphosphate
Ap	Ampicillin
bp	Base pair
BHI	Brain heart infusion
BSA	Bovine serum albumin
cfu	Colony forming units
Cm	Chloramphenicol
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanidine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
ECP	Extracellular proteins
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FUP	Forward universal primer
G	Gravitational force
g	Grams
h	Hour
Ig	Immunoglobulin
IPTG	Isopropyl β-D-thiogalactoside

J/m^2	Joules per meter squared
Km	Kanamycin
kb	Kilobase
kD	Kilo Dalton
l	Liter
LBB	Luria-Bertani broth
LBA	Luria-Bertani agar
LPS	Lipopolysacharide
LD_{50}	Lethal dose (50%)
M	Molar
mb	Megabase
MMS	Methyl methane sulfonate
mg	Milligram
μg	Microgram
min	Minute
ng	Nanogram
ml	Milliliter
μl	Microliter
mM	Millimolar
M_r	Molecular weight
nm	Nanometer
ORF	Open reading frame
PCR	Polymerase chain reaction
PFG	Pulsed field gel
PFGE	Pulsed field gel electrophoresis
PBS	Phosphate buffer saline
PEG	Polyethylene glycol

pI	Isoelectric point
PMSF	Phenyl methenesulfonyl fluoride
RE(s)	Restriction enzyme(s)
RNAse	Ribonuclease
RUP	Reverse universal primer
s	Second
sdH_20	Sterile distilled water
SD	Shine-Dalgano ribosomal binding site
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	Sodium citrate buffer
TSA	Tryptone soy agar
TSB	Tryptone soy broth
Tet	Tetracyclin
TE buffer	10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)
TBS	Tris-buffered saline (10 mM Tris-HCl (ph 7.5), 0.9% NaCl)
Tris	Tris (Hydroxymethyl) aminoethane
UPGMA	Unweighted Pair Group with Arithmetic mean
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-Bromo-4-Chloro-3-indoyl-β-D-galactopyranoside

LIST OF OLIGONUCLEOTIDES

GCAT1	5′-GCAGGCACCACCTGCTTCAC-3′
GCAT2	5′-CAGTCGGGCGTCAGTCTTCC-3′
exeDF	5′-CGACGTTTATGTCGGCTTGTC-3′
exeDR	5′-ACCACCTATGCTGGCATTGC-3′
MIPG1	5′-GCTGCAGGAGTAACCGCAGCC-3′
MIPG2	5′-CAGGACGTGCCCAGAAGCTGG-3′
pEU1	5′ CCCGCAACTGGATGCCAGCTGCGAC-3′
pEU2	5′-CAGTGGTGGCAGCGGTTCGTGGCTG-3′
pEU3	5′-TTGGAACCCATTTCTCGTG-3′
pEU4	5′-CGTGATCGTTGTAGGTGTCG-3′
pEU5	5′-CGATGTCGATTTGGAGGCTGTGTCC-3′
pEU6	5′-CCTCTGTACGCGCCATTGTAGCACG-3′
5SRNA	5′-GCCTGGCGGCCATAGCGCCGTGGAACCAC-3′
AP1	5′-GGCTGATCTCTTCATCCTCACCC-3′
AP2	5′-CAGAGTGAAATCTACCAGCGGTGC- 3′
Rec1	5′-GAA(G)AAA(G)CAA(G)TTC(T)GGA(C,G,T)AAA(G)GG-3′
Rec6	5′-GAGGGTTCAGTGGGTTCGCAG-3′
Rec7	5′-GCCTCAGTACGTCTGGACATC-3′
Rec9	5′-CAGTATACTTAGCCCAACTTC-3′
FL7	5′-GGCGTTAATGCGATGAAGCG-3′
FL8	5′-GTACCAAACATGTCGCTGATG-3′
FL10	5′-GCTTAGGAGAATGGTTATGGC-3′
FLB6	5′-GTTCTGCTTGGTCAGGTTGG-3′
PilAF	5′-AAAAACCCGGGAGCAGATGCCTGCGCC-3′
pilAR	5′-GGCAAAGCAGGAAGAGCAGATGCCTGCGCC-3′

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DEDICATION

I dedicate this dissertation to my Mum, Rosina Umelo, a truly remarkable woman and mother.

INTRODUCTION

The genus *Aeromonas*

The genus *Aeromonas* was proposed by Kluyver and van Niel in 1936 to describe a group of Gram-negative rod shaped bacteria associated with freshwater environments that were generally motile by means of polar flagella (179). The aeromonads were initially grouped under the family *Vibrionaceae,* with the genera *Vibrio, Photobacterium* and *Plesiomonas* (288). However, data obtained from ribosomal RNA sequences as well as DNA-DNA hybridization analysis suggested that the genus *Aeromonas* was sufficiently different from the other genera in the family *Vibrionaceae* to warrant its removal. Hence *Aeromonadaceae* was proposed as the new family for the genus *Aeromonas* (72).

Over the years, many different methods have been applied in the identification and classification of species within the genus *Aeromonas* resulting in constant modifications and also considerable confusion (54, 55, 374). Bergey's Manual of Systematic Bacteriology listed *A. hydrophila, A. caviea, A. sobria* and *A. salmonicida* as the four main species in the family *Aeromonadaceae* (287). However, comparison of the aeromonads at the genotypic level indicated that the family was more complex. Thus, using DNA-DNA reassociation kinetics, Janda *et al.* identified 13 genospecies or hybridization groups (HG) within the genus *Aeromonas* (Table 1)(164, 165). The number of HGs was increased to 14 by Carnahan *et al.* using their so called "Aerokey II" method of identification (54). These HGs were similar to those proposed by Janda *et al.* (Table 1), except that *A. trota* was moved to HG14, and a new HG13 represented by strain ATCC 43946 was created. The Aerokey II method was comprised of a combination of at least five different

Table 1. Taxonomic status of the genus *Aeromonas* as determined by Janda *et al.* (158).

DNA Hybridization Group	Genospecies	Phenospecies
1	*A. hydrophila*	*A. hydrophila*
2	Unnamed	*A. hydrophila*
3	*A. salmonicida*	*A. hydrophila*
4	*A. caviae*	*A. caviae*
5	*A. media*	*A. caviae*
6	*A. eucrenophila*	*A. caviae*
7	*A. sobria*	*A. sobria*
8/10	*A. veronii*	*A. sobria*
9	*A. jandaei*	*A. sobria*
10/8	*A. veronii*	*A. veronii*
11	Unnamed	*A. veronii*
12	*A. schubertii*	*A. sobria*
13	*A. trota*	*A. sobria*

biochemical screens and was reported to be 97% accurate in identifying 60 coded clinical isolates and 100% of reference strains to the species levels. Another study using a fragment of the *rrs* gene encoding the 16S rRNA as probe in Southern blot analysis, revealed a genospecies-specific restriction fragment polymorphism within the *Aeromonas rrs* genes (222). This study confirmed the presence of 13 HGs similar to those proposed by Janda *et al.*, although there were differences in the placement of phenotypic strains within some groups.

Comparison of 16S rDNA sequences has provided a powerful tool for the determination of evolutionary history of numerous organisms (223, 375). Using this approach, a phylogenetic tree showing the evolutionary

relationship between different species in the genus *Aeromonas* was deduced (Fig. 1). However Sneath has suggested that the use of 16S rDNA sequence as a means of determining evolutionary history may be more complicated than is currently appreciated (330). Sneath, on analysis of the published sequences of the 16S rDNA genes of several *Aeromonas* species, deduced that genetic recombination events had occurred within the genes (330). He postulated that the differences within these sequences could have arisen from the formation of hybrids during recombinational cross-over events, therefore different regions of the gene would have a different phylogenetic history.

For practical purposes, the aeromonads can be broadly classified into two major groups, the mesophilic motile and psychrophilic non motile aeromonads. The mesophilic aeromonads can cause diseases in both poikilothermic and homeothermic animals (79, 106, 166), and comprise at least seven species, including *A. hydrophila*, *A. caviae* and *A. veronii* biotypes *sobria* and *veronii* (53, 288). The psychrophilic aeromonads have only been implicated in diseases of poikilotherms (39, 228, 320, 356). *A. salmonicida* is the typical representative species of the psychrophilic aeromonads, although *A. media* is often placed in this group because of its apparent lack of flagellation and motility (6, 16).

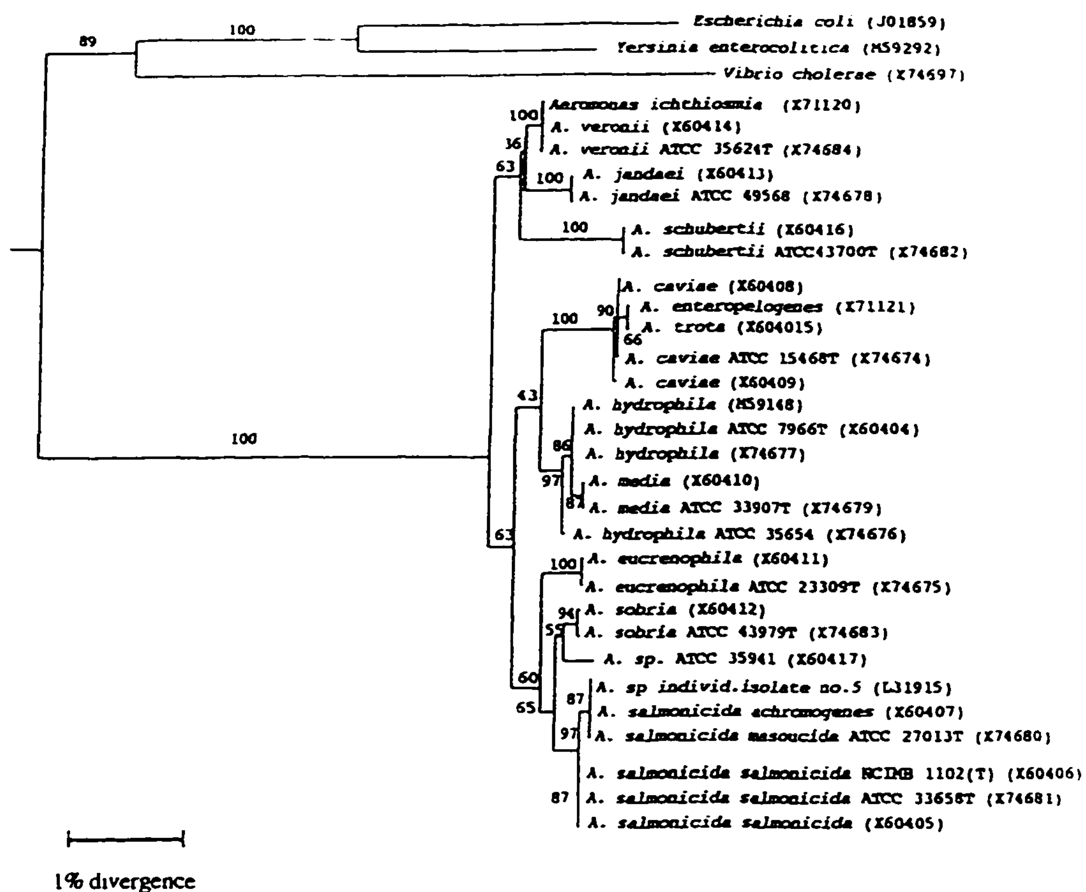

Figure 1. Phylogenetic tree of *Aeromonas* species based on 16S rRNA sequence comparisons.

The tree was derived using the neighbor-joining method (306). The horizontal branch lengths were drawn to scale while the vertical separation and order were for clarity. The GenBank accession numbers are in parentheses. The numbers at the forks represent bootstrap values (100), which indicate the frequency in which the group to the right occurred among 1000 replicates, and is reported to reflect the robustness of the tree. The scale bar represents 1% nucleotide sequence divergence (Adapted from (364)).

The species *A. salmonicida*

A. salmonicida was first isolated from brown trout in a German fish hatchery facility in 1894, and was originally called *Bacterium salmonicida* (97). In 1953, Griffin *et al.* proposed that *B. salmonicida* be included in the genus *Aeromonas* as *Aeromonas salmonicida* (129), and it has belonged to this genus ever since. Initially, the occurrence of *A. salmonicida* was thought to be restricted to hatchery facilities. Plehn, however, showed that *A. salmonicida* infection was prevalent in wild trout taken from twenty-five rivers and streams in Bavaria (284). Over the years the occurrence of *A. salmonicida* has been determined to be worldwide since the organism has been isolated from diseased fish all over Europe, America, Australia and Asia (17, 86, 107, 220, 237, 283).

Bergey's Manual of Systematic Bacteriology listed three subspecies of *A. salmonicida*, var. *salmonicida*, var. *achromogenes* and var. *masoucida* (288). However, the latest edition of Bergey's Manual of Determinative Bacteriology has included a new subspecies *smithia* (150), which was created based on a taxonomic study conducted by Austin *et al.* (18). The subspecies *salmonicida* was specified as the group that produce a brown pigment on media containing 0.1% tyrosine or phenylalanine, produce indole, ferment mannitol and hydrolyze esculin. The subspecies *achromogenes* does not produce the brown pigment, may produce indole, ferment mannitol and does not hydrolyze esculin, while subspecies *masoucida* did not produce the brown pigment, produce indole, ferment mannitol and hydrolyze esculin (288). The subspecies *smithia* does not readily produce the brown pigment, has a fermentative metabolism, positive for catalase and oxidase activities, negative for indole production, and does not hydrolyze esculin (18).

It is difficult to differentiate the species within each *Aeromonas* taxonomic group. In the *salmonicida* group, differentiation is based on a combination of phenotypic methods and molecular approaches. From a comprehensive genotypic and phenotypic study conducted by McCarthy (227), in which he compared 145 bacteria, mostly *A. salmonicida* strains and other motile aeromonads, he proposed three subspecies, *salmonicida, achromogenes* and *nova*. In 1988, Belland and Trust carried out a detailed genetic study of some *A. salmonicida* strains on sequence homologies using DNA-DNA reassociation and G+C content. Representatives from other groups such as *A. hydrophila, A. sobria, Aeromonas caviea, Haemophilus piscium, E. coli, Vibrio anguillarum* and *Campylobacter fetus* were also included (30). From their results, they agreed with McCarthy and Roberts (228) on the following four points:

(1) The three subspecies of *A. salmonicida* should be *salmonicida, achromogenes* and *nova*, (2) Subspecies *achromogenes* and *masoucida* should be combined into the same group *achromogenes*, (3) Subspecies *salmonicida* should be those strains isolated from salmonid fish. Subspecies *achromogenes* should be the strains of *A. salmonicida* also isolated from salmonid fish, but which do not quite fit into the *salmonicida* group. Subspecies *nova* should be those *A. salmonicida* strains isolated from different types of fish and diseases and which do not quite fit in the *salmonicida* group, (4) For working purposes, the subspecies *salmonicida* should be grouped as the "typical" strains while subspecies *achromogenes* and *nova* as the "atypical" strains. Unlike the typical strains which are quite a homogenous group, subspecies *nova* was very heterogeneous. Belland and Trust at this time did not have enough data from numerous strains to place

this subspecies into different groups but agreed that subspecies *nova* had a potential of being further subdivided (30).

However, following a taxonomic study of the *Aeromonas* spp. using a combination of phenotypic and DNA homology analysis, Austin *et al.* disagreed with the proposal to combine subspecies *achromogenes* and *masoucida* (18). From their work, they deduced that subspecies *achromogenes* was sufficiently different from *masoucida* (18). In addition, Austin *et al.* proposed the name *smithia* for a cluster of strains majority of which corresponded to the "*nova*" group initially proposed by McCarthy and Roberts (228) and supported by Belland and Trust (30).

The homogeneity of the typical strains have been confirmed using other studies such as restriction endonuclease fingerprinting (230), plasmid profile comparison (261), multilocus enzyme electrophoresis (41) and DNA hybridization using 16S and 23S rRNA probes (260). In the analysis using multilocus enzyme electrophoresis, Boyd *et al.* studied the electromorph profiles of nine different enzymes of 53 *A. salmonicida* strains isolated from diverse fish diseases and geographical origins. Their results suggested that the population structure of *A. salmonicida* was clonal, comprising two different clones for the species. Interestingly, the 42 typical strains examined belonged to electrophoretic type 1 (ET1) while the eleven atypical strains fell within ET2 (41). They deemed the low genetic diversity found within the test strains to be representative for the species given the wide geographic sources of the strains assayed and the limited ability of *A. salmonicida* to survive outside the host.

Both the typical and atypical *A. salmonicida* strains occur in freshwater, estuarine and marine fish (30, 356).

Pathogenesis

The typical strains of *A. salmonicida* cause furunculosis, a fulminant septicemic disease of salmonids. Phenotypically atypical *A. salmonicida* strains have been associated with chronic ulcerative diseases of other non salmonid fish such as minnows, goldfish, cod, and herring (39, 74, 275, 320). Latent infections in which the fish carries *A. salmonicida* asymptomatically have also been described (143). In the carrier state, infection appears to be established early in the fish's development and *A. salmonicida* appear to be predominantly localized in the kidney (233). It was postulated that disease could be induced by stress which the salmonid fish might encounter upon entering salt water. In hatchery facilities, stress due to overcrowding, low oxygen levels, and elevated water temperature could trigger outbreak of furunculosis in carrier fish (143). In overcrowded hatcheries, such outbreaks can lead to significant mortalities in the fish stock.

Recently, Mooney *et al.* used a PCR based assay to investigate the presence of *A. salmonicida* in the blood of wild Atlantic salmon obtained from three rivers in Ireland (248). Their results showed that 87% of the fish screened tested positive for the presence of *A. salmonicida* at a very low level suggesting they were latent carriers of the pathogen. The latent infection may not be detectable by culture methods, and a combination of corticosteroid injection and heat stress treatment at 18°C for 14 days has been reported to reproducibly trigger acute infection (48, 143). The latent state of *A. salmonicida* infection is not clearly understood, especially because strains isolated from carrier fish do not show reduced virulence. However McIntosh and Austin have speculated that L-forms of *A. salmonicida*, which are cells with defective cell walls, might play a role (233). In their experiments, McIntosh and Austin injected *A. salmonicida* L-forms, derived by either

treatment of cells with both lysozyme and antibiotics, or exposure to hyperimmune antiserum, into Atlantic salmon. Neither clinical symptoms were observed, nor were parental or L-forms recovered from the fish. Microscopic examination of some fish tissues revealed possible L-form cells present in some organs, particularly the kidney. However, fish challenged with *A. salmonicida* L-forms failed to develop disease even after stress was applied (233). Further studies are necessary to establish the role, if any, *A. salmonicida* L-forms may play in the carrier stage of infection or in the disease process.

Virulence factors are regarded as those determinants produced by a pathogen which contribute to its ability to cause disease in its host. Several factors produced by *A. salmonicida* have been implicated in virulence. A fraction called ECP (Extra-Cellular Products) has been isolated from *A. salmonicida,* either by concentration or precipitation of the constituents of the culture supernatants. The ECP, some constituents of which have been identified as proteases, hemolysins, a glycerophospholipid-cholesterol acyl transferase (GCAT) and lipopolysaccharide (LPS), has been linked to pathogenesis. The ECP of *A. salmonicida* injected either intramuscularly or intraperitonially has been shown to produce features identical to those observed with furunculosis (253, 307). The results discussed below, which have been obtained from examination of the contribution of individual constituents of the ECP to pathogenesis, suggest that mortality and morbidity associated with *A. salmonicida* infection may be largely dependent on the combined effects of the different factors rather than on individual effects alone, although some constituents of the ECP were clearly more toxic to fish than others. The surface virulence array protein (A-layer) also contributes significantly to the pathogenesis of *A. salmonicida.*

A-layer

A. salmonicida produces a paracrystalline tetragonal surface protein array known as A-layer which covers the entire cell surface. The A-layer is a polymer comprised of a single protein type, the A-protein, which has been purified and characterized biochemically (172, 278). The A-protein is encoded by a single copy gene (*vapA*) which has been cloned, sequenced and characterized (29, 65). DNA sequence analysis of *vapA* revealed an ORF of 1506 bp which translated into the A-protein or VapA protein with 502 amino acid residues and a predicted M_r of 50,778. The deduced amino acid sequence revealed the presence of a 21 residue signal sequence which is cleaved on the translocation of VapA across the cytoplasmic membrane. VapA had an observed M_r of approximately 50,000 kD, a predicted pI of 4.8 and a predicted 43.7% hydrophobic residues (65, 280). VapA is polymerized into the A-layer array in a P4 symmetry, composed of a major and minor tetragon, with a three-dimensional reconstruction showing a lattice constant of 12.5 nm (83). The major tetragon consisted of four major domains of the VapA subunits, and formed a large depression towards the inside of the layer. The minor tetragon was located towards the outside of the layer and has been proposed to provide connectivity within the layer.

The assembly of the A-layer onto the surface of the cell requires specific interaction with the O-polysaccharide portion of the LPS, because the absence of O-polysaccharide chains leads to inability to anchor the A-layer on the cell surface and accumulation of VapA as assembled sheets in the culture supernatant (28, 130). Studies using the bacteriophage 55R-1 which utilized O-polysaccharide chains as specific receptor (160), as well as immunofluorescence studies with O-chain-specific monoclonal antibody iiC5 revealed that numerous O-polysaccharide chains penetrated the A-layer and

were exposed on the cell surface (60). Ca^{2+} ions are also required for proper assembly of the A-layer because its absence resulted in the structural rearrangement of the A-layer (112).

The secretion of VapA from the cytoplasm to the cell surface occurs via a specific secretion pathway. Belland and Trust identified Tn5 mutants (TM mutants) in which either the export of VapA across the cytoplasm to the cell surface or its proper assembly on the cell surface was disrupted (28). In TM1, VapA protein accumulated in the periplasm and its secretion across the outer membrane was disrupted, while the secretion of other extracellular proteins such as proteases and hemolysins were not affected by the Tn5 insertion. This further supported the presence of a VapA specific secretion pathway. This mutant, TM1, suggested that VapA was translocated across the periplasmic space and that the protein normally encoded by the mutated gene in this strain played a significant role in the transport of VapA across the outer membrane. Recently, Noonan and Trust characterized the TM1 mutant and discovered that the Tn5 had inserted into a gene (*aps*E) encoding a protein with high sequence homology to a member of the general secretory system from *Klebsiella pneumoniae* (76, 265). Preliminary evidence such as the inability to complement the mutant phenotype with cloned *aps*E suggested that *aps*E is part of an operon, the other genes in this operon are likely to encode the rest of the proteins which make up the VapA specific export pathway. The deduced ApsE amino acid sequence suggested that this protein belonged to a family of ATP-binding secretion proteins although it was phylogenetically distinct from these proteins. The evolutionary deviation of ApsE from the other ATP-binding secretion proteins was probably due to selective pressures which optimized the protein for its role in the specific transport of VapA (265). The identification of the VapA-specific *aps*

secretion system was the second secretion pathway described for *A. salmonicida*. The *exe* secretion pathway was the first identified, and is utilized for the export of other extracellular proteins such as proteases (171).

Analysis of TM2 revealed two genes (*aso*A and *aso*B) which had nucleotide sequence homology with the *tdc*C gene of *E. coli* which encodes a protein with sequence homology to a threonine permease, an integral membrane protein (127, 266, 267). Mutation of *aso*A led to the disorganization of the cell surface, with membranous blebs consisting of VapA and LPS protruding from the cell surface (267). Although its exact function is unknown, the phenotype observed with the TM2 mutant suggested that AsoA is important for the proper structural organization of the *A. salmonicida* outer membrane.

The A-layer contributes to the ability of *A. salmonicida* to colonize fish and is considered to be an important virulence factor (162, 173, 251, 265, 361). The importance of the A-layer of *A. salmonicida* in infection was first identified by experiments conducted by Ishiguro *et al.* in which they showed that A-layer negative (A^-) strains or A^- high temperature mutants were avirulent in fish when compared to A-layer positive (A^+) strains (162). In other experiments, on injection of A^- and A^+ cells into fish, A^+ cells rapidly multiplied in tissues, especially the kidney, spleen and liver, leading to septicemia and death within 72 hours (173, 251). In contrast, A^- cells initially multiplied in the organs, but were rapidly cleared and the fish survived (173, 251). The *aps*E mutant of *A. salmonicida* described above which is unable to export VapA and hence unable to assemble the A-layer was also avirulent when utilized in fish challenge studies (265). This mutant is isogenic in A-layer, and provides the strongest proof of the role of A-layer in virulence.

The strategic location of the A-layer on the cell surface which must come into contact with host tissues suggests the layer must play some role in protecting *A. salmonicida* from the host defense mechanisms. Munn *et al.* showed that the A-layer protected the pathogen from the bactericidal activity of complement in both immune and non immune sera (250). This is likely to be crucial for *A. salmonicida* to establish a successful invasion.

The A-layer has also been shown to bind to fish cells including macrophages (112, 358), and porphyrins (174), immunoglobulins (279), and to the extracellular matrix proteins fibronectin and laminin (82) and collagen type IV (359). The A-layer also bound to other substances such as Congo red (161). The binding of Congo red to the A-layer provides a very convenient qualitative laboratory assay to differentiate A^+ and A^- *A. salmonicida*, since on binding Congo red, A^+ cells assume a deep red colouration while A^- cells which do not bind the dye remain colourless or assume a light orange colour. The A-layer inhibited hemagglutination (357) and protected *A. salmonicida* from bacteriophages (162) and predation from *Bdellovibrio bacteriovorus* (184). The A-layer also facilitated autoagglutination and adhesion to fish tissues (98). One or more of these binding properties may play an important role in the ability of *A. salmonicida* to evade the host's immune system, and allow for the pathogen to remain in a carrier state in the host.

LPS

A. salmonicida produces smooth LPS, some of the O-polysaccharides of which penetrate the A-layer and are exposed on the cell surface (84). Chart *et al.* examined the electrophoretic morphology of the LPS produced by various strains of *A. salmonicida* using intrinsic ^{32}P-radiolabeling and silver staining

(60). The higher molecular weight fraction of LPS was resolved into a small number of distinct bands, suggesting a high degree of homogeneity in the O-polysaccharide chain length. The O-polysaccharide chains of the LPS from different *A. salmonicida* strains had similar monosaccharide compositions and were also immunologically cross-reactive (60). Dooley *et al.* observed that *A. salmonicida* LPS had longer O-polysaccharide chains than the mesophilic aeromonads, and the two groups had common as well as species specific epitopes (84).

Recently, Thornton *et al.* reported the production of a unique form of LPS by *A. salmonicida* grown *in vivo* which is antigenically distinct from that normally produced by cells grown *in vitro* (345). Although antiserum raised against *in vivo* grown cells could detect both *in vivo* induced LPS, and that normally produced *in vitro*, antiserum produced against *in vitro* grown cells could not recognize the LPS induced *in vivo*. The possibility of there being two forms of LPS produced by *A. salmonicida* had been observed earlier (202). However, as with the novel LPS described by Thornton *et al.*, the new LPS described by Lee and Ellis were not well characterized nor compared with the previously described type (202, 345). Although some early reports had suggested that *A. salmonicida* LPS was not very toxic on injection of up to 0.714 g/kg of fish (276, 370), *in vitro* experiments studying the serum resistance properties of *A. salmonicida* suggest that LPS may play a role as a virulence factor (250). In their experiments, Munn *et al.* examined A^-O^+ (O-polysaccharide chain positive), A^-O^- (O-polysaccharide chain negative), A^+O^+ and A^-O^+ *A. salmonicida* strains in serum resistance experiments. Their results showed that the O-polysaccharide chains contributed to the serum resistance of *A. salmonicida*, particularly in non-immune sera. It was postulated that the O-polysaccharide chains could prevent access of the

membrane lytic complement compounds to their targets on the cell membrane. As previously mentioned, the A-layer was also shown to contribute to serum resistance in both normal and immune sera. Thus it would appear that the combined effect of both LPS and A-layer in protecting *A. salmonicida* against the lytic effects of both normal and immune sera are probably very important in allowing the bacterium to establish a successful infection.

Proteases

Fish suffering from furunculosis often exhibit elongated swellings along the musculature which are filled with a fluid substance. The fluid is regarded as liquefied muscle tissue, most likely produced as a result of proteolytic enzymes secreted by *A. salmonicida* (252, 307). Indeed several studies have reported the production of several proteases of different M_r by strains of *A. salmonicida* (110, 201, 291, 302, 307, 373). The presence of two different proteases have been confirmed, a metalloprotease (gelatinase) and a serine protease (also called caseinase).

Fyfe *et al.* compared the SDS-PAGE electrophoretic pattern of the extracellular proteins obtained from 10 different virulent strains of *A. salmonicida* grown under identical conditions (110). Two common components identified were a 70 and a 56 kD protein. These proteins were isolated and enzymatic assays revealed that the 70 kD protein was a serine protease while the 56 kD protein was a hemolysin which was hemolytic to trout erythrocytes. In a subsequent study, Fyfe *et al.* found that intramuscular injection of the purified 70 kD protease produced a lesion histologically similar to those observed in natural infections (109). However, they showed that the isolated protease had a significantly reduced damaging effect when

compared to the unfractionated ECP with an equal unit of proteolytic activity. The lesions produced following injection of the protease contained liquefied muscular tissues postulated to have resulted from the action of the protease. When Ellis *et al.* inhibited the protease found in the ECP with PMSF, there was not a major difference in the number of mortalities within fish groups administered with regular ECP or PMSF-treated ECP, although fish to which regular ECP was administered died a few hours sooner than fish injected with PMSF-treated ECP (94). When doses of ECP at 200 μg/g fish were administered, mortalities were practically identical in groups administered with regular ECP and PMSF-treated ECP. These results suggested that other constituents present in the ECP were very toxic to fish even without any lethal contribution from the serine protease, and that the virulence of *A. salmonicida* is not dependent on the serine protease alone.

Further evidence supporting a role for proteases in furunculosis was provided by Sakai (307). Sakai found that a protease deficient mutant NTG-1 obtained using the mutagen N-methyl-N'-nitro-N-nitrosoguanidine, remained auto-agglutinative, hemagglutinative, serum resistant, hemolysin positive, and leukocytolysin positive, however, its LD_{50} in Sockeye salmon was increased to over 10^8 compared to 10^4-10^5 in the wild type strain. In addition, the strain NTG-1 was also rapidly eliminated from rainbow trout when compared to the wild type strain. The protease(s) in question in this study was not identified or further characterized.

Perhaps the most important protease produced by *A. salmonicida*, and certainly the best characterized genetically, is the 70 kD serine protease; the gene (*asp*A) encoding this protease has been cloned and sequenced (373). The *asp*A gene, with an ORF of 1863 bp translated into a preprotein consisting of 621 amino acids, the first 24 amino acids constituting the signal sequence. The

predicted M_r of the mature 597- amino acid protein was 64,173 although SDS-PAGE analysis showed AspA migrated at an estimated 70 kD. The deduced amino acid sequence of AspA contained the consensus serine protease substrate binding site (NGTS). Biochemical analysis confirmed that AspA is a serine protease due to its sensitivity to PMSF and high optimum pH (pH 9.5) (373). The pathology observed on injecting purified AspA into fish suggests that this protease is important in the pathogenicity of *A. salmonicida*. The precise role played by AspA in furunculosis could have been unambiguously determined by studying an isogenic *asp*A mutant in fish challenge studies, but Whitby *et al.* did not proceed to this stage in their work.

The serine protease also appears to contribute indirectly to pathogenesis by activating other extracellular virulence factors produced by *A. salmonicida*. Eggset *et al.* reported that GCAT and H-lysin produced by *A. salmonicida* were secreted as inactive proforms which were weakly hemolytic. The serine protease cleaved the proenzymes into active forms which became highly hemolytic (90). It has also been suggested that the serine protease could be one means by which *A. salmonicida* scavenges for iron in the host since the protease was found to digest bovine transferrin releasing the bound iron (145)

GCAT

GCAT was isolated from the ECP of *A. salmonicida* and partially purified (46), and was reported to migrate at an estimated M_r of 23,600 on SDS-PAGE gels. Characterization of its enzymatic properties revealed that GCAT exhibited phospholipase, acyltransferase and lysophospholipase activities (46). Biochemical assays conducted by mixing partially purified GCAT with intact human erythrocytes suggested that the enzyme did not

penetrate the cell, rather it acted only on the external surface producing erythrocyte ghosts. However, other workers have since reported that GCAT is highly hemolytic to salmon erythrocytes (90, 201, 305).

From studying GCAT produced by a protease deficient strain, Eggset *et al.* reported that GCAT was secreted as a 38 kD inactive proform which was weakly hemolytic. The proform was activated on cleavage by exogenously added serine protease into a highly hemolytic 26 kD enzyme (90). The gene encoding the *A. salmonicida* GCAT has since been cloned and sequenced (257). The *GCAT* gene with an ORF of 1005 bp encoded a protein with 335 deduced amino acids and a predicted M_r of 37,357 (257). This deduced M_r is in agreement with that reported by Eggset *et al.* which corresponds to the inactive GCAT proform (90).

GCAT appears to exist in several dimeric forms in the ECP of *A. salmonicida* and also easily complexes with LPS (90, 201). Apart from its high hemolytic action against fish erythrocytes, other evidence has suggested that purified GCAT from *A. salmonicida* was potentially a major virulence factor, particularly in association with LPS (201). GCAT alone had an estimated LD_{50} of 340 μg/g fish and produced muscle necrosis. However an isolated GCAT/LPS toxin complex of M_r greater than 200 kD was lethal with an LD_{50} of 45 ng/g fish (201). The GCAT/LPS toxin complex was estimated to contain 65 mg carbohydrate and 2.5 mg total lipids per mg of protein. The toxin complex was reported to be highly hemolytic (T-lysin), leukolytic and cytotoxic. Antiserum prepared against the toxin complex neutralized the lethal toxicity of the crude ECP, suggesting that the toxin was one of the lethal factors produced by *A. salmonicida*. The toxin complex was found to be more resistant to proteolytic and heat inactivation than free GCAT. In addition, the specific hemolytic activity and lethal toxicity of GCAT was about 8-fold higher

in the complexed form (201), suggesting that the LPS moiety of the complex played an important role in toxicity.

It has been suggested that the high polyunsaturated fatty acid content of fish membranes compared to that of humans may be responsible for the specific lethality observed with the GCAT/LPS complex. The optimal substrate for GCAT has been reported to be phosphatidylcholine (PC) substituted with unsaturated fatty acids (46). Fish tissues had a high 58.6% content of PC in the membrane (200) compared to 29.5% in human tissues (369). Thus with such a high proportion of the fish membrane being susceptible to the enzymatic action of GCAT, the membrane could easily lose its integrity leading to lysis. In addition, it was reported that LPS penetrated phospholipid monolayers more efficiently when they are composed of polyunsaturated fatty acids (169). Therefore Lee and Ellis suggested that the increased toxicity of the GCAT/LPS toxin complex could be due to the fact that LPS aided the toxin to penetrate fish tissues more efficiently (201).

The nature of the hemolytic activity of GCAT/LPS has been investigated (305). Citrated salmon whole blood or washed erythrocytes were incubated with the GCAT/LPS complex and then the erythrocyte membranes were analyzed. They noticed a dose-dependent decrease in PC and an increase in lysophosphatidylcholine (LPC), and lysis occurred when the level of LPC rose above 10% of total lysophospholipids. Addition of soybean LPC to citrated salmon blood also resulted to hemolysis. Therefore they suggested that the accumulation of LPC as a result of the action of the GCAT/LPS toxin complex was responsible for lysis of salmon erythrocytes (305).

Hemolysins

A. salmonicida has been reported to produce several hemolysins with different M_r (110, 144, 262, 348, 349), some of which have been characterized. These hemolysins are grouped into two distinct types, H and T-lysins. H-lysins are broad spectrum hemolysins with maximum activity against horse erythrocytes, though they also affect trout leukocytes and gonad cells. T-lysins are apparently active only against trout erythrocytes. Some of the hemolysins described have been found to require extracellular protease to cause complete lysis.

Titball and Munn partially purified a protein of apparent M_r 25,900 that was hemolytic to horse erythrocytes which they called "H-lysin" (349). Injection of the partially purified H-lysin into fish did not produce any obvious pathological features although it was cytotoxic *in vitro*. The H-lysin was reported to copurify with a GCAT activity. The copurification of the two enzymatic properties could have been a result of similarity in M_r because GCAT had an estimated M_r of 23,600, and the two enzymes were purified and eluted using solutions of similar ionic strength. Titball reported that the observed activities were likely to be due to two different proteins since Buckley *et al.* had reported that the purified GCAT was stable to temperature fluctuations (46), while the H-lysin was very sensitive to temperature and enzymatic activity was easily lost (349).

Fyfe *et al.* described the isolation of a 56 kD T-lysin and a 70 kD serine protease from ten virulent strains of *A. salmonicida* (110). Intramuscular injection of a 0.20 ml protease-hemolysin mixture containing 20U and 5U of activity, respectively, into fish produced lesions filled with a fluid composed of lysed erythrocytes and liquefied muscle tissues, similar to those observed in natural furunculosis. Although the specific pathogenic effect of the

hemolysin could not be separated from that of the protease, the presence of lysed blood cells within the exudate is indicative of a hemolytic action from the hemolysin component of the injected mixture. The hemolysis of erythrocytes due to the action of hemolysins is bound to be detrimental to the health of the fish. Another T-lysin called Salmolysin with M_r 200 kD was purified to homogeneity from *A. salmonicida* (262). Salmolysin was reported to be a glycoprotein containing approximately 62% carbohydrates, the constituent sugars of which were not characterized. Salmolysin was found to have a potent hemolytic activity against salmon erythrocytes and was lethal to rainbow trout when injection intramuscularly. The LD_{50} was estimated at approximately 58 μg/kg of fish. However Lee and Ellis speculated that the carbohydrate content of the so called Salmolysin, its high M_r and observed LD_{50} may be explained by the aggregation of LPS and GCAT (93).

Recently, Hirono and Aoki reported the cloning and characterization of three genes *ASH*1, *ASH*3 and *ASH*4 encoding three different hemolysins from *A. salmonicida* (144). Southern blot analysis showed that *ASH*3 and *ASH*4 were present in 104 different *A. salmonicida* strains examined while *ASH*1 was only present in strain ATCC14174 from which it was cloned. The codon usage for the *ASH*1 was also different from that observed with other sequenced *A. salmonicida* genes. These two factors suggested that *ASH*1 may have been recently acquired from a different species. Protein data base searches did not reveal any similarity between the deduced amino acid sequence of ASH1 and previously characterized proteins. In contrast, there were high similarities between ASH3, ASH4 and hemolysins produced by both *A. hydrophila* and *A. sobria* (144). By maxicell analysis, the hemolysins ASH1, ASH3 and ASH4 had different molecular weights (60, 53, and 63 kD respectively) from those which had previously been reported for *A.*

salmonicida. However, ASH3 and ASH4 had molecular weights of 49 and 60 kD respectively by Western blot analysis. ASH1 and ASH3 were broad spectrum hemolysins while ASH4 lysed only rabbit and horse erythrocytes. The role of these three hemolysins in pathogenesis were not examined.

Capsule

Capsule production have been reported for the mesophilic aeromonads *A. hydrophila, A. caviae* and *A. veronii* biotypes *sobria*. Popoff *et al.* reported the inability to detect capsule production in 68 *A. salmonicida* strains (289), and indeed *A. salmonicida* is classified in the taxonomic reference books as non-capsulated (287). However, some recent studies have produced strong evidence supporting the fact that like the other aeromonads, *A. salmonicida* can indeed produce both cell-bound capsular polysaccharide (CPS) and other cell-detached exopolysaccharide (EPS) materials (38, 114, 115). These studies demonstrated that the nature of the growth environment influenced capsule production in *A. salmonicida*.

When grown *in vitro*, exopolysaccharide production was detected at the end of the logarithmic growth phase of *A. salmonicida*. Media rich in carbon sources such as glucose led to the production of capsules. In the study conducted by Bonet *et al.*, the yeast extract supply had a marked influence on the nature of polysaccharide formed i.e. CPS or EPS (38). While the total exopolysaccharides produced remained constant, alterations in the yeast extract concentration directed the exopolysaccharide production either towards CPS or EPS.

When grown *in vivo* confined within diffusion chambers implanted inside rainbow trout, *A. salmonicida* also produced a capsule which completely surrounded the cell surface (114). As with other capsulated

pathogenic bacteria (155, 249), the capsule produced by *A. salmonicida* grown *in vivo* was reported to contribute to increased resistance to phagocytosis and host lytic factors (114).

Furunculosis vaccine

The best kind of vaccine for fish is one that can be administered either orally, or by immersion, as opposed to injection of individual fish. In addition, because route of administration can affect the type of immune response obtained, vaccines should be probably best administered via the same route as normal infections are initiated.

Several attempts have been made over the years to produce a vaccine against furunculosis, with varying degrees of success. Initial attempts were centered on using either purified virulence factors or bacterins to stimulate the immune system against future infections (92, 95, 272, 361). However, none of these systems proved adequate. There was an observed absence of protective immunity in vaccinated fish which had high levels of agglutinating antibodies (274). *In vitro* studies by Munn *et al.* also showed that virulent A^+ *A. salmonicida* were resistant to serum killing, even in the presence of high levels of antibodies (250). In addition, *in vitro* studies showed that A^+ *A. salmonicida* were efficiently taken up by macrophages (111) and exerted a cytotoxic effect on macrophages (273).

The failures encountered while using purified total extracellular proteins of *A. salmonicida* or bacterins to protect fish against furunculosis, has subsequently prompted the search for disease control measures using attenuated live vaccine strains, because live cells stimulate cell-mediated immunity better than killed cells. Indeed in recent years, interference at the genetic level to produce attenuated strains appears to have produced more

desirable results in protecting fish against furunculosis. Several groups have described attenuated *A. salmonicida* vaccine strains.

In 1982, Cipriano and Stalipar described a laboratory passaged attenuated strain of *A. salmonicida* which protected fish from infection on subsequent challenge with a virulent strain, either by immersion or injection (68). However, the nature of the mutation(s) was unknown because the strain was a spontaneous mutant which had been selected during continuous laboratory passage in broth and agar over a twelve month period.

Thornton *et al.* in 1991 reported a unique spontaneous aminoglycoside-resistant, slow-growing mutant of *A. salmonicida*, and a rapidly growing apparent pseudorevertant strain, both of which gave protection to fish from infection following challenge with a virulent strain (346). Both mutants were found to produce a comparable normal array of extracellular proteins such as proteases and hemolysins, yet these mutants were avirulent. However, the A-layer was found to be disorganized in these strains and not uniformly layered as found on normal cells. To explain how these spontaneous mutants could protect fish against furunculosis, Thornton *et al.* suggested that the mutants may expose important cell surface antigens which were normally masked by the A-layer.

Vaughan *et al.*, in 1993 described an aromatic-dependent mutant (*aro*A) of *A. salmonicida* (365). The mutant was avirulent in fish and gave immunity and protection following subsequent challenge with virulent strains, compared to non-preimmunised fish (365). The enzyme 3-phosphoshikimate-1-carboxyl transferase encoded by the *aro*A gene is an enzyme important in the shikimate pathway for the biosynthesis of p-aminobenzoic acid which is required for folate biosynthesis. It was postulated

that the *aroA* mutants failed to grow in fish tissues due to absence of p-aminobenzoic acid (365).

The important fact which had been revealed by all three reports was that an attenuated live vaccine strain of *A. salmonicida* was a feasible means of immunization and protection of fish against furunculosis. However due to the high levels of regulation and tight controls implemented by the regulatory bodies on what can and cannot safely be released into the wild, attenuated live vaccine strains have to be highly characterized to be considered safe. A major problem with the first two cases of spontaneous mutants was that the mutation(s) were of an uncharacterized nature, potentially capable of reversion to wild type even if the chances might be low. With the *aro*A vaccine strain, although the mutation was well characterized and controlled, the metabolic deficiency could be circumvented by the availability of folic acid in the fish diet especially, in hatcheries where fish are fed a balanced and well regulated diet. In addition, the chances of correction of a single mutation are high, especially in a strain with a functional *recA*.

An ideal furunculosis vaccine would be one that has several well characterized attenuating mutations in the *A. salmonicida* genome. These mutations, preferably caused by deletions, should be widely separated on the chromosome. The *rec*A gene should also be mutated in the vaccine strain to prevent restoration of pathogenicity via homologous recombination. Such a vaccine should also provide strong protection in fish. Possession of these properties should likely facilitate licensing of the vaccine for commercial use.

Purpose of this dissertation

Fish is one of the world's major source of protein. As the world population continues to increase, the demand for fish also increases, with the

result that the ocean's natural supply of certain species of fish is rapidly being depleted. In an effort to keep up with demand of popular fish species, e.g. salmonids, intensive fish cultivation is carried out in fish farms. For example, there is an estimated 300,000 metric tons of salmonid fish farmed in Norway, 150,000 tons in Chile, 50,000 tons in Scotland and 30,000 tons in Canada (Dr. W. W. Kay, personal communications). Fish farming is the fastest growing sector of aquaculture, with a present estimated $35 billion market for seafood, including shellfish.

Fish disease has become a major problem worldwide, especially in fish farms where overcrowding and other stress factors make fish more susceptible to disease. In such crowded situations, outbreaks of disease including those caused by *A. salmonicida,* lead to massive fish death and loss of revenue to fish farmers. While several live vaccine strains of *A. salmonicida* have been described, none of these have been licensed for use commercially. Thus the need for a well characterized vaccine of *A. salmonicida* cannot be overstated.

Because of the importance of *A. salmonicida* as a fish pathogen, and because so little is known about the ability of this pathogen to produce disease in fish, the objectives of this research were to:

1. Provide the first chromosomal map of this organism and localize genes potentially involved in its pathogenicity.
2. Identify, characterize and mutate the *A. salmonicida recA*.

In so doing, information and tools would be provided which would facilitate the rational development of an attenuated live vaccine strain of *A. salmonicida*. To facilitate the *A. salmonicida* chromosomal map construction, some highly conserved genes from other bacteria were used as probes on *A. salmonicida* genomic digests. As a result we identified a novel flagellin

operon in *A. salmonicida* strain A449. Because *A. salmonicida* has been classified as nonmotile, and because flagella have been shown to be a major virulence factor in several bacterial pathogens suggesting the possibility that *A. salmonicida* could have a flagellated and motile phase of life which would contribute to pathogenesis, it was decided to expanded the thesis studies to:

3. Clone and characterize the flagellin genes identified in *A. salmonicida* strain A449.

MATERIALS AND METHODS

Bacterial strains, vectors and growth media

The *Escherichia* and *Aeromonas* strains used in this study are listed in Table 2. The cloning vectors and their relevant properties are listed in Table 3. All the strains were maintained in stock collections at -70°C in TSB (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 32% (v/v) glycerol. The strains were cultured using either TSB, TSA (BBL Microbiology Systems, Cockeysville, Md.), LBB or LBA, (242), Lactose MacConkey agar (GIBCO), or BHI (BBL, Microbiology Systems, Cockeysville, Md). The atypical strains of *A. salmonicida* were grown on TSA supplemented with 5% (v/v) horse blood or TSB supplemented with 10 µg/ml hemin (Sigma) (159). *A. salmonicida* strains were grown at 20°C for 24 to 48 h, while all other strains were grown at 37°C for 18 to 24 h. Antibiotics were used at the following concentrations; Ap 100 µg/ml, Km 50 µg/ml, Cm 180 µg/ml (Boehringer Mannheim, Germany) and Tet 50 µg/ml (Sigma Chemical Co., St Louis, MO).

Techniques used in the analysis of DNA

DNA Isolation

Chromosomal DNA

Two methods were utilized in the preparation of chromosomal DNA. A standard chromosomal DNA preparation was carried out on cells resuspended in buffer. This method resulted in DNA which was sheared at multiple locations during isolation. The DNA used in PFGE was prepared *in situ* from cells embedded in an agarose gel matrix and was designed to prevent any undesirable nicks in the DNA molecule.

Table 2. Bacterial strains used in this study

Species	Strain	Source (other strain designations)
A. salmonicida - typicals	A449	Brown trout, Eure, France
	A450	Brown trout, Tarn, France
	A488	Brook trout, U. S. A.
	A395	Scotland, U. K.
	A505	Japan
	A202	Japan
	A251	NCMB 1102, Atlantic salmon, U. K
	A440	Brook trout, U. S. A.
	A438	B. C. Canada
	A447	Weymouth, U. K.
- atypicals	A400	Goldfish, Boolara, Australia
	A401	Goldfish, Boolara, Australia
	A402	Goldfish, Boolara, Australia
	A404	Goldfish, Boolara, Australia
	A491	Goldfish, Maryland, U. S. A.
	A419	Goldfish, Arkansas, U. S. A.
	A460	Atlantic salmon, Nova Scotia, Canada
	A461	Atlantic salmon, Nova Scotia, Canada
	A462	Atlantic salmon, Nova Scotia, Canada
	A477	European carp, The Netherlands
	A480	European carp, The Netherlands
	A475	Shubunkin, Germany
	A522	Herring, B. C., Canada
	A523	Herring, B. C., Canada
	A588	Atlantic cod, Nova Scotia, Canada
	A600	Norway
	A601	Goldfish, Europe
	A602	
A. hydrophila	TF7	Trout lesion, Quebec, Canada

Cont'd

	Ah300	Human diarrheal feces
	Ah30	
	Ah55	
	Ah598	Human diarrheal feces
A. sobria biotype *veronii*	As701	Human septicemia, U. S. A.
	As702	Human septicemia, U. S. A.
E. coli	DH5α	(138)
	S17-1	(321)
	JM107	(377)
	JM109	(377)
	JC14604	(231)
	HB101	(42)

Table 3. Vectors used in this study.

Vector name	Relevant details	Reference
pUC18 and pUC19	High-copy-number plasmid	(377)
pBluescript (KS and SK)	High-copy-number plasmid	Stratagene
pNEB193	High-copy-number plasmid	New England Biolabs Inc.
pSUP202	Mobilizable vector plasmid	(321)
pK18*mobsac*B	Mobilizable vector plasmid	(312)
pUC4KISS	Source of Km^R cassette	(24)
pUC4KIXX	Source of Km^R cassette	(24)
pMMB67 (EH and HE)	Broad host range plasmid	(108)
pHC79	Moderate-copy-number cosmid	(147)

1. Standard chromosomal DNA preparation

Approximately 10 ml volumes of overnight cultures of *Aeromonas* were centrifuged at 5,000 x G for 10 min. The cell pellets were resuspended in 400 µl of solution I (1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 50% (w/v) sucrose) and lysed by the addition of 100 µl of 20 mg/ml lysozyme followed by a 15 min incubation at 37°C. After lysis, 400µl of solution II (1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 20% (w/v) SDS) was added to the sample and the slurry was gently mixed. Proteins and RNA in the sample were degraded by the addition of 100 µl each of a 4 mg/ml solution of proteinase K and 10 mg/ml RNAse solutions respectively, followed by incubation at 37°C for at least 4 h. The total DNA was subsequently isolated by extraction twice with phenol and a 1:1 solution of phenol/chloroform. The DNA was precipitated by the addition of an equal volume of isopropanol to the aqueous phase followed by gentle mixing. The DNA was recovered by centrifugation at 15,000 x G for 5 min and washed with 1 ml of 70% (v/v) ethanol. The DNA was dried under vacuum and resuspended in 1 ml of sterile distilled water (sdH_20).

2. PFGE chromosomal DNA

The method of Smith *et al.* (327), was utilized in the preparation of chromosomal DNA used in PFGE, with minor modifications. *Aeromonas* cells were grown in 10 ml of TSB at 20°C to an optical density of 45 using the red 66 filter of the Klett Colorimeter (Klett Summerson Photoelectric Colorimeter, model 800-3). Cm was added and the culture was grown further for 1 h and then chilled on ice for 10 min. The cells were harvested by centrifugation at 5,000 x G for 7 min at 4°C. The cells were washed in 10 ml of Pett IV (10 mM Tris-HCl (pH 7.6), 1 M NaCl) and centrifuged as above. The cells were thoroughly resuspended in 1.6 ml of Pett IV and warmed to 30-

40°C. An equal volume of 1% (w/v) low melting point agarose (BioRad) cooled to 30-40°C was added to the cells and the sample was mixed. The mixture was dispensed into 1 ml syringes and allowed to solidify at room temperature for 2 h.

The agarose moulds containing the cells were slowly pushed out of the syringes approximately 50 µl at a time and each small cylinder (insert) was sliced off using a sterile scalpel. The inserts were placed into 6 ml of EC lysis solution (6 mM Tris-HCl (pH 7.6), 1 M NaCl, 100 mM EDTA (pH 8.0), 0.5% (w/v) Brij-58, 0.2% (w/v) Deoxycholate, 0.5% (w/v) Sarkosyl) containing 1 mg/ml lysozyme and 20 µg/ml RNAse and incubated at 37°C overnight with gentle shaking. The EC lysis solution was replaced with 6 ml of ESP solution (0.5 M EDTA (pH 9 -9.5), 1% (w/v) lauryl sarcosine, 1 mg/ml proteinase K) and was incubated at 50°C for 48 h with gentle shaking. The inserts were subsequently dialyzed twice in 10 ml of TE (10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA) containing 1 mM PMSF for 2 h to inhibit any residual proteinase K. The inserts were further dialyzed 3x in 10 ml TE for 2 h without PMSF. The TE solution was aspirated off and the DNA inserts were stored at 4°C until use.

Plasmid DNA

The alkaline lysis method described by Birnboim and Doly (35) was used for the small and large scale plasmid DNA isolation.

1. Small scale plasmid isolation

E. coli cells containing the plasmids of interest were recovered from 5 ml overnight cultures by a 1 min centrifugation at 15,000 x G. The cells were resuspended in 200 µl of solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0),

10 mM EDTA (pH 8.0)). After resuspension, 300 μl of solution II (0.2 N NaOH, 1% (w/v) SDS) was added to lyse the cells and the sample was gently mixed followed by a 5 min incubation on ice. Chromosomal DNA and other cell debris were precipitated by the addition of 300 μl of solution III (3 M sodium acetate (pH 4. 8)) and the sample was again mixed gently and incubated on ice for 10 min. The precipitated chromosomal DNA/cell debris was removed from the sample by centrifugation at 15,000 x G for 10 min. The supernatant containing the plasmid DNA was extracted twice with 400 μl of chloroform to remove contaminating proteins. The plasmid DNA was precipitated by the addition of an equal volume of isopropanol to the aqueous phase, followed by gentle mixing. The DNA was recovered by centrifugation at 15,000 x G for 15 min and washed with 1 ml of 70% (v/v) ethanol. The DNA was dried and resuspended in approximately 32 μl of sdH_20.

The plasmid DNA utilized for DNA sequencing reactions were further purified by precipitation with 8 μl of 4 M NaCl, 40 μl of 13% (w/v) Polyethylene glycol 8000 (BDH) and incubation on ice for 20 min. The DNA was recovered by centrifugation at 15,000 x G for 20 min at 4°C. The DNA was washed with 1 ml of 70% (v/v) ethanol, air dried and resuspended to a concentration of approximately 0.2 μg/μl.

2. Large scale plasmid isolation

The large scale isolation of plasmid DNA was carried out using cesium chloride (CsCl) gradient centrifugation. *E. coli* DH5α bearing the plasmid of interest was grown with shaking overnight in 500 ml of TSB supplemented with suitable antibiotics. The culture was chilled on ice for 30 min, then transferred into two chilled 250 ml tubes and the cells were harvested by centrifugation at 7,000 x G for 7 min at 4°C. The cells were resuspended in 60

ml of 20 mM Tris-HCl (pH 7.4) and centrifuged as above. The cells in each tube were resuspended in 2.5 ml of solution I and incubated on ice for 5 min. To lyse the cells 3.75 ml of solution II was added to each sample followed by a 5 min incubation on ice. A 5 ml of solution III was added to the sample to precipitate the chromosomal DNA/cell debris and incubated on ice for 10 min. The cell debris were removed by centrifugation at 18,000 x G for 30 min at 4°C. The supernatant containing the plasmid DNA was transferred into two 30 ml tubes and 6.5 ml of cold isopropanol was added to each tube to precipitate the plasmid DNA followed by a 15 min incubation at room temperature. The plasmid DNA was pelleted by centrifugation at 10,000 x G for 10 min at 15°C. The DNA was washed once with 10 ml of 70% (v/v) ethanol, air dried and then resuspended in 4.75 ml of sdH_20. After thoroughly resuspending the DNA, 4.98 g of CsCl and 0.25 ml of a 10 mg/ml ethidium bromide (Etbr) solution were added to each tube and the samples were mixed until the salt completely dissolved. The mixture was transferred to quick seal tubes for the Beckman VTi 65 rotor, and tightly sealed. The tubes were centrifuged at 55,000 x G for 19 h at 15°C. Following centrifugation, the tubes were punctured on the bottom using a needle and the plasmid DNA band was collected as the contents of the tubes flowed down. EtBr in the plasmid DNA sample was removed by extracting three times with water-saturated 1-butanol. CsCl present in the plasmid DNA sample was removed by dialysis against sdH_20 at 4°C. The plasmid DNA was stored at -20°C until use.

Oligonucleotides

Custom designed oligonucleotides were either ordered from GIBCO BRL Life Technologies or synthesized on a PCR-MATE EP DNA synthesizer model 391 (Applied Biosystems, Inc., CA).

When produced on the PCR-MATE EP DNA synthesizer, the oligonucleotides were synthesized onto beads within 40 nM columns as recommended by the manufacturer. The beads to which the oligonucleotides were linked were removed from the columns by flushing through with 1 ml of concentrated ammonium hydroxide. The oligonucleotides were released from the beads by incubation in the ammonium hydroxide solution first at room temperature for 1 h and then at 55°C overnight. The beads were removed by centrifugation and the oligonucleotides were desiccated under vacuum. The oligonucleotides were resuspended in sdH_20 and their concentrations (DNA (ng/µl) = A_{260} x 50 x dilution factor) was determined by spectrophotometry at A_{260}nm.

Gel electrophoresis of DNA

DNA fragments were separated either using the conventional horizontal gel electrophoresis "regular electrophoresis" or by Pulsed-Field Gel Electrophoresis (PFGE).

1. Regular gel electrophoresis

DNA fragments in the range of 100 bp to 20 kb were analyzed by agarose gel electrophoresis using a horizontal apparatus (GNA 100/200, Pharmacia) as described by Sambrook *et al.* (308). TAE (40 mM Tris-HCl, 40 mM acetate, 2 mM EDTA (pH 8.0)) buffer system was utilized. Typically 0.8-1.5% (w/v) agarose (BRL) gels were used for DNA separation and 0.5 µg/ml EtBr was added to the molten gel solution prior to casting in order to aid in visualization of the DNA bands under uv light. Separation of DNA fragments were conducted using a voltage range of 50-100 V.

2. Pulsed-field gel electrophoresis

Separation of large M_r DNA fragments used in the construction of the *A. salmonicida* A449 chromosomal map was carried out with both the Clamped Homogeneous Electric Field (CHEF) and Field Inversion Gel Electrophoresis (FIGE) forms of PFGE. Typically 0.7-1% (w/v) agarose gels supplemented with 1.5 µg/ml EtBr were utilized in the separation of DNA using the CHEF-DR^{R}11 pulsed field or FIGE Mapper electrophoresis systems (BioRad). The CHEF Mapper was connected to a Model 1000 Mini chiller (BioRad) to keep the buffer temperature at 14°C. The FIGE system was cooled to approximately 20-25°C by passing the rubber tubing circulating the buffer through a large bowl of ice. A 0.5x TBE buffer (45 mM Tris-HCl, 45 mM borate, 1.0 mM EDTA (pH 8.3)) was utilized for both PFGE systems.

For the CHEF system, typically two windows of DNA separation were utilized per separation operation using the long gel cast (21 x 14 cm). In the first window, large molecular weight DNA fragments in the range of 300 to 200,000 kb were separated typically using a 10-80 s pulse ramp, 5.5 V/cm, 14°C for 30 h unless where specified. The second window separated smaller DNA fragments in the range of 5-300 kb at 5-10 s pulse ramp, 5.0 V/cm, 14°C and 30 h unless where specified. For the FIGE system, typically a 0.1-0.4 s switch time ramp, 180 V forward voltage, 120 V reverse voltage and 16 h run time was utilized.

Molecular cloning

Preparation of competent cells

E. coli cells were made competent by two different methods depending on whether heat shock (competent cells) or electric voltage (electrocompetent cells) was to be utilized to transfer the plasmids into the cells.

1. Competent cells

The desired *E. coli* strain to be made competent was grown up overnight in 5 ml of TSB. A 0.5 ml portion of the culture was subcultured into 100 ml of fresh TSB and grown to an A_{600} of approximately 0.5 - 0.6. The culture was chilled on ice for 10 min, then the cells were harvested by centrifugation at 7,000 x G for 5 min at 4°C . The cells were resuspended in 20 ml of CM1 (10 mM sodium acetate (pH 5.6), 50 mM $MnCl_2$, 5 mM NaCl), left on ice for 20 min and then centrifuged as above. The pellets were resuspended in 2 ml of CM2 (10 mM sodium acetate (pH 5.6), 5% (v/v) glycerol, 70 mM $CaCl_2$, 5 mM $MnCl_2$) and 100 µl aliquots were stored at -70°C.

2. Electrocompetent cells

Electrocompetent *E. coli* cells were prepared according to the method described by Frohelich and Scott (105). As above, the *E. coli* cells to be made electrocompetent were grown overnight in 5 ml of TSB, and next day 1 ml was subcultured into 500 ml of fresh TSB and grown to an A_{600} of 0.5. The culture was chilled on ice for 30 min, then the bacterial cells were harvested by centrifugation at 5,000 x G for 10 min at 4°C. The cells were resuspended in 100 ml ice cold sdH_20 by gently swirling while on ice, centrifuged as above and the process was repeated. Next the cells were gently resuspended in 10 ml of ice cold sterile 10% (v/v) glycerol, centrifuged as above and the process was repeated. The cells were finally resuspended in 800 µl of ice cold 10% (v/v) glycerol and 60 µl aliquots were stored at -70°C.

Transformation

Prior to transformation, competent cells previously stored at -70°C were quickly thawed on ice. Plasmid DNA or ligation mixtures were added to the

competent cells and incubated on ice for 30 mins. The cells were heat shocked by quickly transferring the Eppendorf tube containing the cell/DNA mixture to a 42 - 44°C water bath for 2 min and then quickly returned to ice for 20 min. Approximately 1 ml of SOC (2% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the cells and incubated at 37°C with gentle shaking for 1 h. 50 to 100 µl aliquots of the sample were plated on media containing the appropriate antibiotics.

Electroporation

To avoid cell death as a result of arcing, DNA samples to be electroporated into electrocompetent *E. coli* had to be cleaned of all salt and resuspended in water. To clean ligation mixtures, for each 10 µl volume, 1 µl of 10 mg/ml tRNA was added as the carrier, then the DNA was precipitated with 1 µl of 3 M sodium acetate (pH 4.8) and 20 µl of 100% (v/v) ethanol. The solution was thoroughly mixed and kept at -70°C for 1 h. The sample was removed from -70°C and centrifuged at 15,000 x G for 20 min at room temperature. The supernatant was discarded and the DNA pellet was washed with 1 ml of 70% (v/v) ethanol, air dried and resuspended in 10 µl of sdH_20.

Prior to electroporation, aliquots of the electrocompetent *E. coli* cells were removed from -70°C and thawed on ice. Plasmid DNA or ligation mixtures were mixed with the cells and the cell/DNA mixture transferred into an ice cold 0.1 cm diameter cuvette (BioRad). The cuvette was placed into the holder on the Gene Pulser (BioRad) set at a resistance of 200 Ohms, a capacitance of 25 µF and the DNA was transferred into the cells by applying a pulse of 1.25 kV. The cells were resuspended in 1 ml of SOC and incubated at

37°C for 1 h. Suitable aliquots of the samples were plated on growth media with the appropriate antibiotics.

Restriction enzyme digestion

DNA samples were either completely or partially digested.

1. Complete digestion

All restriction enzymes were obtained either from Boehringer Mannheim or New England Biolabs Inc. The restriction enzyme buffers were used according to the dilutions specified by the manufacturers. The regular chromosomal or plasmid DNA to be digested was added to buffer solutions containing approximately 5-10 Units (U) of enzyme(s). The samples were digested at the optimal temperatures for the duration required for each enzyme to cleave at all its specific restriction site present in the DNA sample, as specified by the manufacturers.

With the PFGE DNA samples, for the restriction enzyme (RE) *Pac*I, DNA inserts were incubated twice in 100 µl of 1x New England Biolabs (NEB) buffer 1 for 30 min on ice. After the second incubation, a fresh 100 µl 1x NEB buffer 1 containing 10U of *Pac*I was added to the insert and incubated at 4°C overnight. Next day, digestion was performed at 37°C for at least 4 h.

With the REs *Pme*I and I-*Ceu*I enzyme (henceforth called *Ceu*I), DNA inserts were first incubated in 100 µl of 2x NEB buffer for *Ceu*I digestion or 2x NEB buffer 4 for *Pme*I digestion for 15 min on ice. The solutions were replaced with 100 µl of 1x NEB buffer for *Ceu*I containing 2U of *Ceu*I for the *Ceu*I digestions and 1x NEB buffer 4 containing 5U of *Pme*I for the *Pme*I digestions, followed by a 15 min incubation on ice. The samples were transferred to 37°C and digestion was carried out for 4 h.

2. Partial digestion

In partial digestion reactions, digestion was prematurely terminated by removing the samples from the optimal digestion temperature sooner than the length of time required to give complete digestion. With the *Ceu*I partial digests, for most cases, digestion was terminated after 40 min unless where specified. For *Pme*I partial digestion, 1.3 U of enzyme were used and the reactions were terminated after 30 mins of incubation at 37°C. The partially digested DNA inserts were separated by PFGE adjacent to complete digests obtained with the enzyme in question, to aid in the identification of partially digested DNA fragments.

After PFGE separation, the bands corresponding to partial fragments were excised from the agarose gel under low wavelength uv light, and the DNA within completely digested to the constituent *Ceu*I- or *Pme*I-derived fragments. These were subsequently separated adjacent to complete digests, again to aid in identifying the constituent fragments.

Isolation of DNA from agarose gels

DNA was isolated from agarose gels using either the QIAEX DNA purification system (QIAGEN) or by the freeze/thaw method.

1. QIAEX DNA purification system

The DNA band to be isolated was excised from the gel using a scalpel and the piece of agarose containing the DNA fragment was placed into a sterile Eppendorf tube. The DNA was transferred from the agarose unto QIAEX beads using kit-supplied buffers according to the protocol specified by the manufacturer. The beads containing the DNA were air dried for approximately 10-15 min. The DNA was eluted from the beads with the

addition of 40 µl of sdH_20 and a 5 min incubation at 50°C. The beads were pelleted by centrifugation and the supernatant containing the DNA was transferred to a clean tube.

2. Freeze/thaw method

As described above, the desired DNA fragment was excised and transferred to a sterile Eppendorf tube. The piece of agarose containing the DNA was broken to small pieces using a sterile Pasteur pipette. To extract the DNA from the agarose matrix, 200 µl of ultra pure buffer-saturated phenol (GIBCO, BRL) was added to the crushed agarose and the sample was thoroughly mixed by vortex. The slurry was kept at -70°C for 20 mins and then was centrifuged at 15,000 x G for 20 min at room temperature. The top aqueous phase was transferred into a clean Eppendorf tube. 400 µl of chloroform was added and the sample was thoroughly mixed by vortex. The sample was centrifuged at 15,000 x G for 2 min at room temperature. The top aqueous phase was again transferred to a clean tube. The DNA in the sample was precipitated by adding 0.1x the volume of 3 M sodium acetate and 2x the volume of 100% (v/v) ethanol. The sample was thoroughly mixed and kept at -70°C for at least 1 h. The DNA was recovered by centrifugation at 15,000 x G for 30 min at room temperature. The DNA was air dried and resuspended in 30 µl of sdH_20.

DNA ligation

Ligation of DNA fragments was carried out as described by Sambrook *et al.* (308). Approximately 0.2 µg each of cloning vector and DNA fragments with compatible ends were mixed in a final volume of 10 µl containing 1x ligase buffer and 1 U of T4 DNA ligase (New England Biolabs Inc.). The

ligation mixture was incubated at 16°C overnight. Following ligation, the sample was either transformed or electroporated into *E. coli*. Selection of colonies containing the correct clones were performed using selective media with appropriate antibiotics with or without X-gal and IPTG. Clones with the correct phenotypes on selective media were confirmed by RE digestion.

Klenow blunt-ending of 5′ overhangs and end labeling

The *Ceu*I sites with 5′ terminal overhangs were end-labeled by blunt-ending using Klenow and radioactive dNTPs. Each DNA insert containing the *A. salmonicida* A449 genomic DNA digested with *Ceu*I was first incubated in 100 µl of 1x Klenow buffer for 20 min on ice. The buffer was replaced with a fresh 100 µl of 1x Klenow buffer containing 10 µCi of ($\alpha^{32}P$) dATP, 10 µCi of ($\alpha^{32}P$) dTTP, 2 mM dCTP, 2 mM dGTP, and 5 U of Klenow, then the sample was incubated on ice for 20 min. The end-labeling reaction was conducted at room temperature (approximately 15-20°C) for 2 h. After end-labeling, the DNA fragments were separated by PFGE, then the gels were dried and exposed to X-OMAT AR film (Kodak Scientific Imaging Films).

Cloning of DNA from *Pme*I, *Pac*I and *Ceu*I fragments

A. salmonicida A449 genomic DNA inserts prepared for PFGE were digested with *Pac*I, *Pme*I and *Ceu*I and the resultant DNA fragments were separated in 0.7% agarose gels by PFGE. After separation, each DNA fragment was excised from the gel under low wavelength uv light. The pieces of agarose containing the DNA fragments were individually incubated in 100 µl of 1x NEB buffer 3 twice for 30 min on ice. Following the second incubation, the solution was replaced with a fresh 100 µl of NEB buffer 3 containing 20 U of *Pst*I and *Bgl*II. The sample was incubated overnight at 4°C, and then at 37°C

for 16-24 h. After digestion, the solution was aspirated off and the cloning vector pBluescript KS^- digested with both *Pst*I/*Bam*HI was added to the agarose. The DNA was extracted using the freeze/thaw method. Approximately 8 µl of the DNA extract was ligated, electroporated into *E. coli* DH5α and plated on TSA containing Ap/X-gal/IPTG. Cloned DNA fragments were used as probes in Southern blot analysis on PFGs.

Conjugation

Plasmid constructs used for allele exchange mutagenesis, or *in vivo* expression studies, were transferred from *E. coli* S17-1 into *A. salmonicida* A449 by conjugation. Plasmids pK18*mobsac*B and pSUP202 were used for allele exchange mutagenesis while pMMB67HE/EH was used for expression studies in *A. salmonicida* A449.

In the conjugal transfer experiments, equal volumes of log phase cultures of *E. coli* S17-1 bearing the construct of interest and *A. salmonicida* A449 were mixed. The mixture was centrifuged for 1 min at 7,000 x G and the supernatant was discarded. The bacterial pellet was resuspended in 50 µl of TSB and spotted to a TSA plate. The TSA plate containing the mating mixture was incubated at 20°C for approximately 4 h. The exconjugants were resuspended in 500 µl of TSB and serial dilutions of the cell suspension was plated on TSA with the suitable antibiotic selections.

Allele exchange mutagenesis

The allele exchange (marker exchange) mutation of an *A. salmonicida* A449 chromosomal gene was achieved by the homologous recombination event between the gene sequences flanking the Km cassette introduced to disrupt the Open Reading Frame (ORF) of the cloned gene and the

homologous sequences on the A449 chromosome. Double cross-over events led to the exchange of the intact chromosomal gene copy with the mutated plasmid copy.

For the marker exchange experiment, the specific *A. salmonicida* gene to be mutated was first cloned into the suicide vectors pK18*mobsac*B or pSUP202. A Km cassette was then introduced into a unique site approximately within the middle of the gene to disrupt the ORF. The suicide vector bearing the mutated gene copy was electroporated into *E. coli* S17-1 and then transferred into *A. salmonicida* A449 by conjugal mating. Serial dilutions of the exconjugants were plated on selective media.

When pSUP202 was used as the suicide vector, the exconjugants were first plated on TSA containing Tet/Km to select against *E. coli* S17-1 bearing the constructs (Ap^R/Km^R) and A449 cells (Tet^R) which did not acquire the construct during the conjugation. The first screening step selected for both single and double cross-over events in the A449 chromosome. Double cross-over *A. salmonicida* A449 mutants were subsequently identified by screening colonies in duplicate on TSA plates, one containing Tet/Km and the other Tet/Km/Ap. Double cross-over mutants grew on Tet/Km and not on Tet/Km/Ap plates, due to loss of the Ap^R suicide vector.

When pK18*mobsac*B was used as the suicide vector, the exconjugants were first plated on TSA with Tet/Km, selecting against *E. coli* S17-1 (harbouring the clones) and A449 which did not acquire the clones as above. Then a single colony was grown in TSB with gentle shaking for 24-36 h. Serial dilutions of the culture was plated on TSA containing Tet/Km and 10% (w/v) sucrose, to select for double cross-over mutants. The action of levansucrase which is encoded by the *sac*B gene of pK18*mobsac*B leads to cell lysis in the presence of sucrose (312), hence single cross-over mutants which still have the vector

integrated within the chromosome are selected against. Colonies which arose following the second screening in the presence of sucrose were confirmed for the double cross-over genotype by Southern blot analysis or PCR.

Southern analysis

Approximately 10 μg of chromosomal DNA was digested with the required enzyme(s) overnight at the appropriate temperature. The digested DNA fragments were separated on a 0.8% (w/v) agarose gel. The DNA was either transferred to Nytran$^+$ nylon membrane (Schleicher & Schuell, Inc., Keene, NH) using the capillary transfer method as described by Sambrook *et al.* (308), or the gels were dried as unblots (360).

Before transfer to nylon membrane, the agarose gel was incubated twice for 15 min in a solution of 0.25 N HCl with gentle shaking, to nick the DNA fragments. The gel was briefly rinsed with sdH_20 and the DNA was denatured by two 30 min incubations in denaturation solution (0.5 M NaOH, 1.5 M NaCl). The gel was transferred into the neutralization solution (1.5 M NaCl, 1 M Tris-HCl (pH 8.0)) and incubated with gentle shaking twice for 30 min. The DNA was transferred to nylon membrane overnight in 10x SSC (1 liter contained 87.65g NaCl, 44.1g $Na_3C_6H_50_7.2H_20$, pH 7.0) as described by Sambrook *et al.* (308). The DNA was linked to the membrane either by baking at 80°C for 1 h, or by exposure to 1,200 millijoules of uv light for 40 s, using the UV Stratalinker (Stratagene, La Jolla, CA).

With unblots (dried agarose gels), incubation in 0.25 N HCl was omitted and the gel denatured and neutralized as above. After neutralization, the gel was dried under vacuum first without heat for 30 min, and then at 80°C for 1 h. The dried gel and the nylon membrane containing the

transferred DNA were subsequently probed in Southern hybridization experiments.

Southern hybridization was performed as described by Sambrook *et al.* (308). Prehybridization and hybridization was carried out at 68°C for gene probes and 60°C for oligonucleotide probes in a solution containing 6x SSC, 10x Denhardt's solution (50x Denhardt's solution: 5 g ficoll (Sigma), 5 g polyvinyl pyrrolidone (BDH), 5g bovine serum albumin (Fraktion V, Boehringer Mannheim)), 1% (w/v) SDS, 2 mM EDTA and 0.25 mg/ml yeast tRNA. Hybridization with the labeled probes was carried out for approximately 16 h. The blots were washed twice at room temperature for 5 min in 2x SSC, 0.1% (w/v) SDS and twice at 68°C for 15 min in 0.1x SSC and 0.1% (w/v) SDS. For autoradiography, blots were exposed to X-OMAT AR film (Kodak Scientific Imaging Films).

Radiolabeling of DNA by nick translation

DNA fragments used as probes in Southern hybridization experiments were isolated from agarose gels and labeled with approximately 20-50 µCi of ($\alpha^{32}P$) dCTP (specific activity approximately 3,000 Ci/mM) (Amersham Life Science Inc. Canada) using the nick translation Kit (Amersham International U.K.) as specified in the kit protocol.

Radiolabeling of DNA by random priming

DNA fragments used as probes in Southern blot experiments were alternatively labeled with approximately 20-50 µCi of ($\alpha^{32}P$) dCTP (spec. act. approx. 3,000 Ci/mM) using the random priming kit (USB) as specified by the manufacturer.

End-labeling of oligonucleotides

Oligonucleotide probes used in Southern blot experiments were labeled by end-labeling. Approximately 200 ng of each oligonucleotide was end-labeled in a 10 µl solution containing 1x polynucleotide kinase buffer, 50 µCi of ($\gamma^{32}P$) ATP and 9.5 U of polynucleotide kinase (New England Biolabs Inc.). The sample was incubated at 37°C for 45 min. The labeling reaction was stopped by the addition of 2 µl of 0.5 M EDTA (pH 8.0), followed by incubation at 65°C for 10 min.

Polymerase chain reaction (PCR)

PCR for gene amplifications using oligonucleotide primers were performed in capillary tubes in a 1605 Air Thermo-Cycler from Idaho Technology (Idaho Fall, Idaho). Each 10 µl PCR cocktail contained 1 µl of 50 ng/µl template, 1 µl each of two different 5 µM primer solutions, 1 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP solution, 1 µl of 10x PCR buffer and 0.1 µl of Taq DNA polymerase (Pharmacia Biotech). The samples were subjected to 35 cycles of denaturation at 94°C for 2 s, annealing at 57°C for 4 s and elongation at 72°C for 70 s. The PCR products were analyzed by agarose gel electrophoresis.

Automated DNA sequencing

DNA sequencing was performed using the ABI Taq DyeDeoxy Terminator Cycle sequencing Kit (Applied Biosystems Inc.) using the M13 RUP, FUP and custom designed oligonucleotides. Sequencing reactions were performed in a Perkin Elmer Cetus model 480 Thermo Cycler (Perkin-Elmer Corp., Norwalk, Conn.) using parameters recommended by ABI. Each 20 µl sequencing reaction contained 0.5 µg of DNA template, 3.2 pM of primer and

8.0 μl of reaction premix (from the kit). After the sequencing reactions, samples were purified using the CentriSep Spin Columns (Princeton Separation) according to the method specified by the manufacturer. Sequencing reactions were finally resuspended in 4.5 μl formamide/EDTA solution (5 μl deionized formamide, 1 μl 50 mM EDTA, pH 8.0) and denatured for 3 min at 98°C, and rapidly chilled on ice. Samples were electrophoresed in a 6 % (w/v) acrylamide-8.3 M urea sequencing gel for 14 h at 2,200 V on an Applied Biosystems 373A DNA automated sequencer (Applied Biosystems Inc.).

Computer analysis

DNA sequence data were analyzed using the SeqEd program (ABI), GeneWorks release 2.5 (Intelligenetics Inc., Mountain view, CA) and DNA Strider release 1.2 (Institut de Recherche Fondamentale, CEA, France). Protein similarity searches were performed using the BLAST network service (10) at the National Center for Biotechnology Information. Clone and genome maps were drawn using the CANVAS 3.5 software (Deneba Systems, Inc.)

Separation and detection of proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining

SDS-PAGE separation of proteins was performed as described by Laemmli (192). A 12.5% (w/v) mini slab gel (8 x 5 cm separating gel) was utilized. Each 10 ml separating gel contained 2.5 ml of sdH_20, 4.1 ml of 30% acrylamide solution (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide), 2.5 ml lower gel buffer (1.5 M Tris-HCl (pH 8.8), 0.4% (w/v) SDS), 30 μl of 10% (w/v) ammonium persulphate, 10 μl of TEMED (N, N, N', N', - tetramethyl-

ethylenediamine). Each 10 ml stacking gel contained 3 ml sd$H_2$0, 1.25 ml of upper gel buffer (0.5 M Tris-HCl (pH 6.8), 0.4% (w/v) SDS), 0.75 ml 30% (w/v) acrylamide solution, 30 µl of 10% (w/v) ammonium persulphate and 10 µl of TEMED. Each liter of electrophoresis buffer contained 3.03 g of Tris, 14.41 g of glycine and 1.0 g of SDS.

Samples were first boiled in approximately 40 µl of solubilization buffer (10% (v/v) glycerol, 5% (v/v) β-Mercaptoethanol, 3% (w/v) SDS, 0.0625 M Tris-HCl (pH 6.8), 0.01% (w/v) bromophenol blue) prior to loading on the gel. The samples were stacked at 100 V and separated at 150 V. The separated proteins were visualized by staining with Coomassie brilliant blue R250 (0.5 g Coomassie R250, 90.8 ml methanol, 90.8 ml H20, 18.4 ml acetic acid). The gels were destained in 25% (v/v) ethanol and 7% (v/v) acetic acid in water, and dried at 80°C for approximately 2 h.

Amino terminal sequence determination

Protein samples for N-terminal sequencing were prepared as described by LeGendre *et al.* (203). Samples were first electrophoresed on a 12.5% SDS-PAGE gel. The separated proteins were electrophoretically transferred to Immobilon membranes (Millipore Corp., Bedford, Mass.) using a BioRad transfer apparatus. The Immobilon membrane was briefly stained in Coomassie blue, destained in 10% acetic-40% methanol-50% water, and air dried. The desired protein band was cut out of the membrane using a scalpel and the N-terminal amino acid sequence obtained by automated Edman degradation using the Applied Biosystems 470A gas phase sequencer. Phenylthiohydantoin derivatives were separated by using an on-line analyzer (ABI model 120A). The sequence data was analyzed using an ABI Biosystems model 900 Control Data Analysis module.

Immunochemical techniques

Adsorption of the *A. hydrophila* TF7 anti-flagellin anti-serum

The previously prepared TF7 anti-flagellin polyclonal rabbit anti-serum (provided by J. A. Austin) was used in the immunological detection of flagellin proteins. In order to remove contaminating antibodies produced against outer membrane proteins other than flagellin, aliquots of the serum was adsorbed against whole cells of *E. coli* S17-1 and *A. salmonicida* A449. Firstly, S17-1 cells harvested from an overnight 5 ml TSB culture were mixed with 1 ml of the anti-serum. The mixture was then incubated overnight at 4°C with intermittent shaking. The S17-1 cells were removed by centrifugation and the anti-serum was transferred to similarly prepared cells of *A. salmonicida* A449, and the adsorption was repeated overnight at 4°C. The anti-serum was subsequently used in Western immunoblots to detect the presence of *Aeromonas* flagellin.

Western immunoblotting

Protein samples were first electrophoretically separated on a 12.5% (w/v) SDS-PAGE gel. The separated proteins were transferred by electroblotting to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) at 12 V for 45 min in a semidry transblot apparatus (LKB, Baie d'Urfe, Quebec, Canada) as described by Towbin *et al.* (354). The nitrocellulose membrane was subsequently blocked using 5% (w/v) skim milk in Tris-Buffered Saline (TBS, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl) for 1 h. The blocking solution was replaced with a 1:2,000 dilution of the primary antibody (*A. hydrophila* TF7 anti-flagellin anti-serum) in 5% (w/v) skim milk TBS and the membrane was incubated with gentle shaking for 2 h. The blot was washed three times for 10 min in TBS. The secondary antibody, alkaline

phosphate-conjugated goat immunoglobulin G (IgG) (anti-rabbit IgG) (Caltag Labs, San Francisco, CA) secondary antibody was added at a 1:4,000 dilution in 5% (w/v) skim milk TBS and incubated with gentle shaking for 2 h at room temperature. After incubation, the nitrocellulose membrane was washed four times with TBS. The reactive bands were visualized using 5-bromo-4-chloro-3-indoylphosphate and 4-nitro blue tetrazolium chloride (Sigma) in 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 9.5).

***In vitro* transcription and translation**

In vitro protein expression experiments were performed using the *E. coli* S30 Coupled Transcription Translation Kit (Promega Corporation, Madison, WI, USA). Approximately 2 µg of each plasmid DNA was translated *in vitro* in the presence of 10 µCi of ^{35}S-methionine (Amersham Life Science Inc. Canada), and precipitated as specified in the kit protocol. The translation mixture with pMG3-gem was supplemented with Sp6 polymerase (Ambion, Austin Texas). The expression products were resuspended in 20 µl of SDS-PAGE solubilization buffer and boiled for 5 min. A 5 µl aliquot of each sample was electrophoresed on a 12.5% SDS-PAGE gel. Rainbow™ coloured protein M_r markers (Amersham Life Science) were used to estimate the M_rs of the translation products. The gel was dried under vacuum at 80°C for 1 h. The dried gel was exposed overnight at -70°C to X-OMAT AR film (Kodak Scientific Imaging Films).

***In vivo* expression**

The induced expression of flagellin genes were carried out as described by Furste *et al.* (108). *E. coli* DH5α or *A. salmonicida* A449 containing the expression construct was grown overnight in 5 ml of TSB containing suitable

antibiotic selections. The strains were subcultured the next day into 5 ml of fresh TSB with antibiotics and grown to an A_{600} of approximately 0.5. 1 mM of the inducing agent IPTG was added to the culture and growth was continued for 3 h. The cells were harvested by centrifugation at 15,000 x G for 1 min at room temperature. The cells were boiled in SDS-PAGE solubilization buffer for 10 min. 10 µl of each sample was analyzed on a 12.5% (w/v) SDS-PAGE gel. Following separation, the proteins were transferred to nitrocellulose membrane and analyzed by Western immunoblot analysis using the preadsorbed *A. hydrophila* TF7 anti-flagellin antibody as the primary antibody.

Electron microscopy

Negative staining

The 200-mesh formvar coated copper grids (Soquelec Limited, Montreal, Canada) were floated on bacteria resuspended in PBS or TSB for 1 min. Excess liquid was removed using Fisher brand filter paper. The grids were negatively stained by placement for 30 s on droplets of 0.5% (w/v) phosphotungstic acid pH 7.4 [or 0.5% (w/v) ammonium molybdate pH 7.0]. Excess stain was removed using filter paper and the grids were allowed to air dry. The stained grids were examined in a Hitachi 7000 electron microscope at an accelerating voltage of 75 kV.

CHAPTER 1

CHARACTERIZATION OF THE *rec*A OF *Aeromonas salmonicida* A449: FUNCTIONAL COMPLEMENTATION OF AN *Escherichia coli rec*A MUTANT.

This study was undertaken for several reasons. Firstly, because relatively very few genes of *A. salmonicida* had been cloned and sequenced, it was necessary to acquire additional genes to facilitate the construction of the *A. salmonicida* A449 chromosomal map. Hence *rec*A was selected for use as a marker on the map. We also wanted to mutate *rec*A as part of the development of a *A. salmonicida* live attenuated vaccine strain. As previously discussed, although several *A. salmonicida* vaccine strains isolated as a result of spontaneous mutations are available, none have been licensed for use because of the unknown nature of the attenuating mutation(s). The mutation of *rec*A in these strains should significantly minimize the likelihood of reversion to wild type conditions, and thus facilitate the development and licensing of attenuated vaccines for commercial use.

Finally, a number of earlier studies dealing with *A. salmonicida* have observed genetic recombination (29, 31, 162). In a number of strains, particularly those repeatedly subcultured, deletions and other rearrangements are routinely observed, often because of an associated loss of virulence (135). The identification and characterization of the recombination genes of *A. salmonicida* is likely to be an important step towards understanding the recombination events that take place in this important fish pathogen.

BACKGROUND TO RECA

The RecA protein

RecA is a multifunctional protein which has been shown to be essential in almost all types of genetic recombination (185, 294). Genetic recombinations are crucial in all organisms for the repair of damaged DNA molecules, maintenance of proper genomic integrity and in the generation of genetic diversity. Evidence has shown that the biological function, protein sequence and the gene encoding the RecA protein (*rec*A) are highly conserved among a wide variety of prokaryotes (43, 186, 243) and eukaryotes (36, 319). The *rec*A gene was first identified when a mutation in *E. coli* abolished conjugal recombination but did not disrupt the conjugal process (70). The mutation was mapped to a single locus on the *E. coli* chromosome designated *rec*A. The *rec*A of various bacteria have often been identified because of the ability of the cloned *rec*A of these organisms to complement the *rec*A phenotype of *E. coli* mutants. The complementation occurs in terms of the proteolytic, DNA repair and recombination functions of RecA, despite the fact that in some cases there may be little DNA homology in the *rec*A from the different species (175).

Several models have been put forward to explain the events which take place during homologous recombination (149, 236, 338). However, the prototypic model was the one proposed by Holliday in 1964 (Fig. 2) (149). In the Holliday model, the exchange of a DNA strand is first mediated by a single strand break in both of the homologous DNA molecules undergoing recombination. RecA assembles into a filamentous complex on the single stranded DNA generated to form the "presynaptic complex", formation of which is essential for homology search of the DNA molecules undergoing

recombination to ensue. Once the homologous region is located, a symmetrical exchange of the DNA strands occurs to produce a region of heteroduplex DNA known as a Holliday junction. The Holliday junction can be resolved in either of two possible ways to yield either a "spliced" or "patched" DNA molecule (Fig. 2). Mutational analysis have revealed that at least 25 gene products are involved in homologous recombination events in *E. coli* (185). These proteins function as DNA helicases, nucleases, ATPases, topoisomerases, DNA-binding proteins, ATP-binding proteins, DNA polymerase and DNA ligase in different steps of the recombination and DNA repair processes.

The mature RecA protein possesses several domains which are necessary to perform the recombination and repair functions. Extensive structural, genetic and biochemical analyses of the RecA of *E. coli* have revealed a binding site for nucleoside triphosphates, multiple sites for protein-protein interactions, DNA binding domains and a region which interacts with repressor proteins to promote proteolysis.

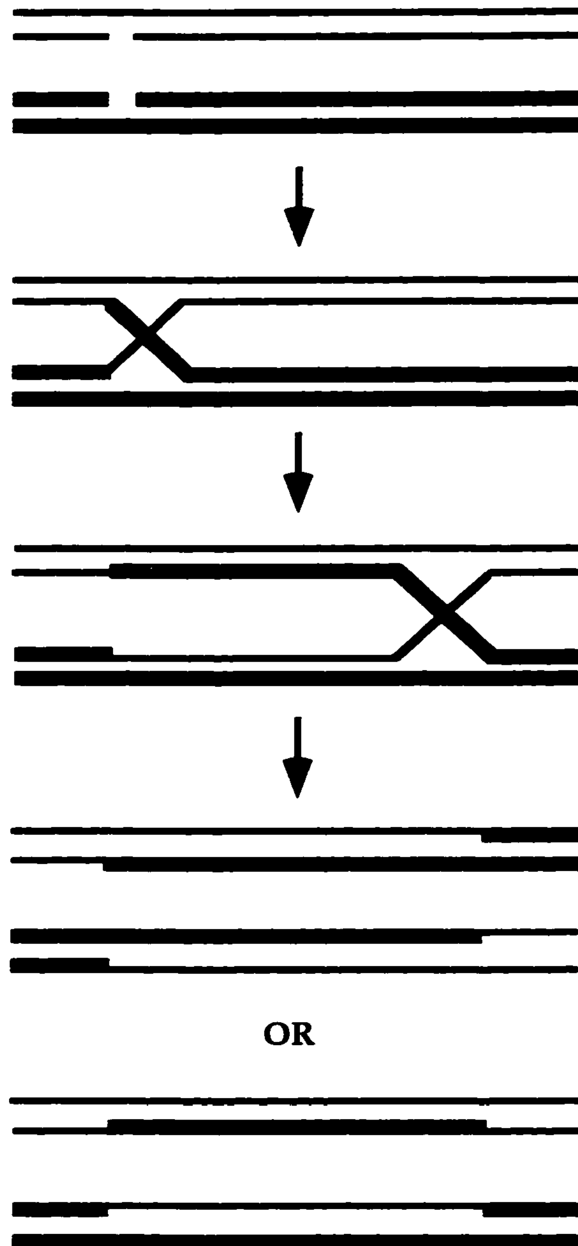

Figure 2. Holliday model for genetic recombination.
The figure depicts two double stranded DNA molecules undergoing recombination. Recombination is initiated by a single stranded break in both DNA molecules followed by a symmetrical exchange of the DNA (Adapted from Kowalczykowski *et al.* (185))

Biological functions of RecA

RecA is a multifunctional enzyme which acts as a co-protease in the cleavage of specific regulatory proteins (49, 152), catalyzes DNA dependent hydrolysis of nucleotide triphosphates (371), and DNA strand exchange steps involved in homologous recombination (75, 77). RecA plays a pivotal role in processes that maintain the genetic integrity of the cell by helping repair damaged DNA, and also allows for the diversification of its genetic material via recombination (69, 293).

Mutations in *recA* increase the sensitivity of cells to killing by uv and x-ray irradiations and by some chemical mutagens (154, 156). Such mutagens introduce lesions or mutations into the DNA molecules which can lead to cell death by blocking DNA replication and transcription (140). RecA is vital in processes that allow the cells to correct such mutations. On rare occasions, DNA polymerase can bypass such lesions with the result that mutations are introduced into the DNA molecule at the sites of the lesions (140).

RecA is involved in SOS induction and mutagenesis. The SOS network is a group of genes whose products are involved in the repair of damaged DNA molecules, such as double-strand break repair, excision repair, mismatch repair of DNA, daughter strand gap repair and mutagenesis (error prone DNA repair) (277, 368). The response is termed SOS since these functions are believed to enhance cellular survival. Damage introduced to the DNA molecule may be lethal unless the repair and recombination potential of the cell is enhanced through the induction of the SOS response (337). SOS mutagenesis is an error prone mode of DNA replication expressed in *E. coli* after exposure to DNA damaging agents (337), in which the cell appears to tolerate the presence of certain mutations. The SOS mutagenesis response depends on the action of RecA, LexA and UmuDC proteins. The

RecA and LexA proteins regulate the response while the UmuDC proteins play a significant role in the actual mutagenic event (337, 368). Evidence suggests that RecA plays an active role in the SOS mutagenic process by specifically cleaving UmuD and by forming a protein complex thought to be involved in the introduction of mutations into the DNA molecule (49, 87). SOS mutagenesis may function by relaxing the fidelity of DNA polymerase such that some mutations are tolerated.

SOS induction and mutagenesis is initiated by the ability of activated RecA to induce the cleavage of the LexA repressor which, in turn, leads to the activation of expression of at least 20 genes essential to the SOS response including *rec*A itself and the *umu*DC operon (277, 337). Critical to the SOS response is the ability of RecA to recognize damaged DNA. Evidence suggests that the intracellular inducing signal is the presence of single stranded DNA which when bound to RecA activates the protein to a form that acts as a co-protease. The activated RecA enhances the auto-cleavage and inactivation of LexA bound on the operator region of the SOS gene network, thus triggering the SOS repair and mutagenesis cascade (209, 368). The activated RecA also enhances the auto-cleavage of other temperate bacteriophage repressors and therefore is required for the induction of phages such as λ and P22 (301, 368, 208, 318, 324).

Apart from the well established roles of RecA in DNA repair and homologous recombination, genetic studies have revealed that mutations in *rec*A can have surprisingly pleiotropic effects. Disruption of the *rec*A of *Lactococcus lactis* revealed that *rec*A was involved in responses to oxygen and heat stress in this organism (89). This study showed that oxygen radicals accumulate in *L. lactis* because of inefficient elimination and *rec*A is essential to deal with the damage to DNA caused by the oxygen radicals (89). The RecA

of *L. lactis* may also regulate the heat shock response in this organism, via the regulator HflB (89). HflB downregulates the heat shock response by promoting the breakdown of the transcription factor σ^{32}. *L. lactis recA* strains survived poorly in thermal stress conditions due to the high levels of HflB and a subsequent lack of the heat shock proteins DnaK, GroEL and GrpE (89). In *E. coli*, genetic evidence also suggested that RecA played a role in proper chromosome partitioning between daughter cells (7, 323, 383). Experiments using *E. coli recA* mutants revealed that many cells in the culture population appeared to have abnormal numbers of chromosomes following completion of ongoing DNA replication (7, 383).

RecA was also shown to be essential for the expression of extracellular proteins in *Serratia marcescens*, since mutation of *recA* abolished expression of the extracellular nuclease, and chitinase expression was greatly reduced (21). Ball *et al.* suggested that the *S. marcescens* extracellular nuclease, as well as other extracellular proteins were regulated by an SOS-like system (21). The pectin lyase and carotovoricin of *Erwinia caratovora* has also been shown to be regulated by the SOS system (382).

RecA-mediated genetic recombination also plays a role in processes that increase the virulence of some microorganisms. For example, in *Neisseria gonorrhoeae* both antigenic and phase variations of the gonococcal pilus, result from DNA rearrangements within the structural genes (34, 333). The data suggests that genetic recombination is important in these events (119, 182). Also, the amplification of the cholera toxin genes, a process that enhances virulence, has also been shown to be *recA* dependent (125).

Despite its modest size of approximately 38,000, RecA may indeed be indispensable for the long term survival of numerous organisms. While the *recA* mutants of some organisms do not appear to be attenuated for virulence

(134, 286, 341), *S. typhimurium recA* strains are avirulent in mice (45). In spite of the fact that RecA may not be essential for virulence in some organisms, efforts to produce a vaccine strain of a variety of organisms usually includes mutation of *recA*. This is because elimination of RecA would be important to prevent restoration of pathogenicity through homologous recombination.

RESULTS

Isolation of the *A. salmonicida* A449 *recA*

The *A. salmonicida* A449 *recA* was identified due to the ability of the cloned gene to complement the *recA* phenotype of *E. coli* HB101. The *recA* of *A. salmonicida* A449 was isolated from an A449 gene library prepared in the cosmid vector pHC79 and transformed into HB101 (263). Inoculum from ten frozen (-70°C) stock vials, each containing approximately 10% of the cosmid library, was cultured in ten individual tubes of TSB containing Ap for 24 h. A 100 µl volume from each tube was subcultured into new tubes containing 5 ml of fresh TSB plus Ap and MMS (11 mM). Following static incubation for 48 h at 37°C, one of the tubes showed growth. A loopful of the culture was streaked out on TSA containing Ap and incubated at 37°C overnight. A single colony was picked, cultured and cosmid DNA was isolated. Analysis of the clone revealed the presence of a cloned fragment of approximately 20 kb in size (pEU1) (data not shown).

Because one of the functions of RecA is to catalyze homologous recombination, this functional property was utilized to confirm the presence of the *A. salmonicida* A449 *recA* on pEU1. The *E. coli recA* mutant (JC14604) contains a duplication of *lacZ* , with each copy containing a different missense mutation, and is therefore Lac$^-$ (231). With the introduction of cloned *recA*, homologous recombination can occur to allow the generation of a functional *lacZ* and conversion to a Lac$^+$ phenotype. The conversion can be readily detected due to the presence of Lac$^+$ papillae on colonies grown on Lactose MacConkey agar. The clone pEU1 was transformed into JC14604 and transformants were plated on MacConkey agar containing Ap. The cloned *recA* of *Francisella novicida* pJB10-4 (33) transformed into JC14604 was used as

a positive control, and JC14604 containing the cloning vector pHC79 was used as negative control. Both pEU1 and pJB10-4 resulted in the production of Lac^+ recombinants, whereas no spontaneous homologous recombination was observed with the negative control or untransformed JC14604.

To confirm the presence of sequences homologous to the *E. coli recA* on pEU1, a degenerate oligonucleotide (Rec1: GAA(G)AAA(G)CAA(G)TTC(T) GGA(C,G,T)AAA(G)GG) designed from the *E. coli* RecA protein sequence EKQFGKG was used as probe on pEU1. pEU1 was transferred to a nylon membrane and a Southern blot performed using Rec1 as the probe. The Rec1 probe hybridized strongly to pEU1 (data not shown), indicating that it contained sequences highly homologous to the *E. coli recA*.

Subcloning and nucleotide sequence of the *A. salmonicida* A449 *recA*

To subclone the A449 *recA* to a smaller DNA fragment, pEU1 was partially digested with *Sau*3AI, ligated into pUC18 digested with *Bam*HI and transformed into JC14604. Transformants were plated on MacConkey agar containing Ap and grown for 48 h at 37°C. Fifteen colonies which displayed a Lac^+ phenotype were selected and grown up for plasmid extraction. Following plasmid DNA extraction, the sizes of the recombinant plasmids were compared on an agarose gel. The smallest clone identified which was still capable of catalyzing homologous recombination in JC14604 contained a fragment of approximately 4 kb and was named pEU101 (Fig. 3).

RE analysis of pEU101 revealed a single *Eco*RI site in the 4 kb insert. A 1 kb fragment derived from *EcoR*I digestion of pEU101, obtained from cleavage at the unique site in the insert and the single *EcoR*I site in pUC18, was subcloned in pUC18 (pEU103, Fig. 3) and nucleotide sequencing was performed with RUP. The databases were searched with the nucleotide

sequence obtained using RUP. The search results revealed that the deduced amino acid sequence of the derived nucleotide sequence displayed considerable homology to the 3' region of RecA from a number of bacterial species (data not shown). The complete nucleotide sequence in both strands of the *A. salmonicida* A449 *recA* was obtained using custom designed oligonucleotides and RUP. A 1059 bp ORF encoding the *A. salmonicida* A449 *rec*A was identified (Fig. 4). The G+C content of the ORF was 57.6%, which is similar to the reported G+C content of 55.0% for the genome of *A. salmonicida* (29). The codon adaptation index (C.A.I., *E. coli*) for the ORF was 0.447. Generally, the codon usage was biased towards G or C in the third position, especially for leucine (96%) valine (89%) and proline (88%). A putative Shine-Dalgarno (SD) ribosome binding site GGAG was positioned 6 bases upstream of the ATG start site. No obvious consensus promoter sequence was identified in the nucleotide sequence immediately 5' of the *A. salmonicida* A449 *rec*A ORF, which suggests that transcription of *rec*A is probably initiated from a promoter sequence of an upstream gene. Sequences identical to the highly conserved consensus SOS-box CTG-N_{10}-CAG were identified 51 base pairs upstream of the *A. salmonicida* A449 *rec*A methionine start codon. A predicted strong termination stem-loop sequence GGCCGGGCTAACCCCCGGCC with a ΔG value of -22.4 kcal was identified 7 bases downstream of the TAA stop codon.

The evolutionary relationship of the *A. salmonicida* A449 RecA to other bacterial RecA was examined using an Unweighted Pair Group Method with Arithmetic Mean tree (UPGMA) (Fig. 5) (256). The tree suggested that the *A. salmonicida* A449 RecA was evolutionally very similar to that of *Erwinia carotovora* (381) and *Proteus vulgaris* (381). A high evolutionary relationship to the RecA of the fish pathogen *V. anguillarum* (350) was also observed.

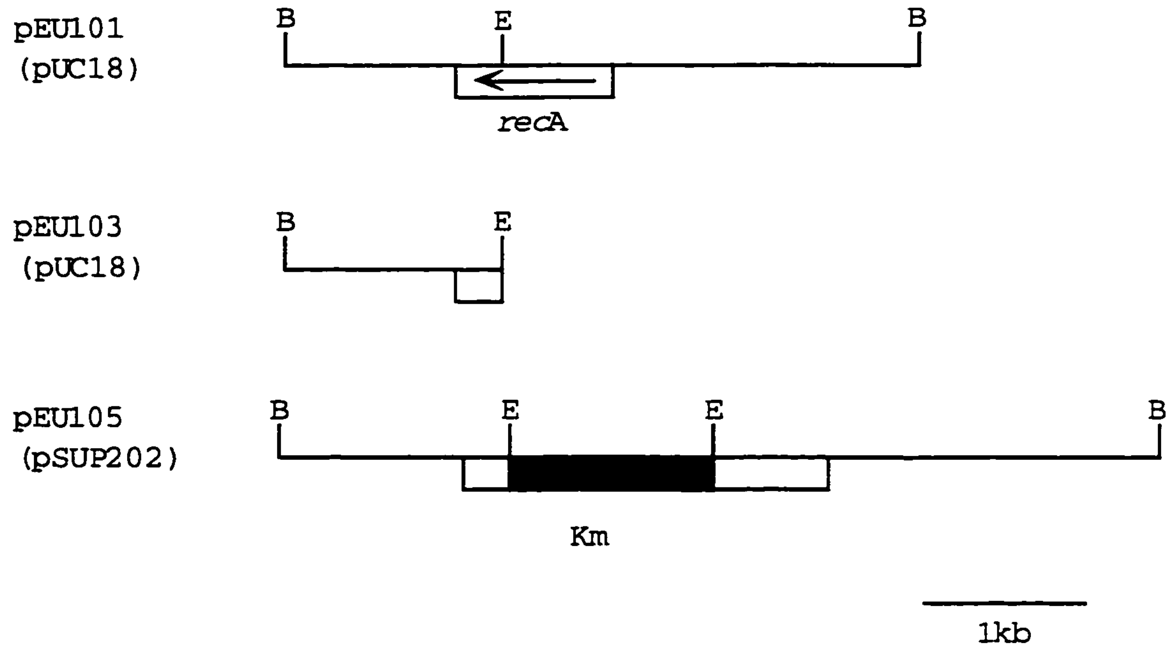

Figure 3. Physical map of the *recA* DNA clones used in this study.
B, *Bam*HI., E, *Eco*RI., Km, 1.3 kb Tn*903* Km cassette.

Figure 4. Nucleotide sequence of the *A. salmonicida* A449 *recA* and upstream sequences.
The deduced amino acid sequence is shown below the coding nucleotide sequence. The putative SD ribosome binding site (GGAG) is shown in italicized capitals and the putative consensus SOS-box (CTG-N_{10}-CAG) is indicated in bold capitals. The transcription stop codon (TAA) is shown with an asterisk (*) and the predicted strong termination loop (ΔG= -22.4 kcal) is indicated with broken arrows. The single *Eco*RI site (GAATTC) which was either blunt-ended or into which a Km cassette was cloned to disrupt the ORF is indicated. The putative ATP binding domain (GPESSGKTT) is single underlined while the amino acid sequence (EKQFGKG) homologous to the *E. coli* sequence from which the degenerate oligonucleotide probe Rec1 was designed is double underline.

tgctggcccgtttcgacggtgaccggcgtcaggtacgccagcaggcgg

tcagacaggccttgtccgactcttggccctgctcagataaattgggcttgata**CTG**tatg

aataga**CAG**tatacttagcccaacttcacttattactcacgatttccagc*GGAG*attggc

ATGGATCAGAACAAACAGAAGGCACTGGCGGCTGCGCTGGGTCAGATTGAAAAGCAGTTC 60

M D Q N K Q K A L A A A L G Q I E K Q F

GGCAAAGGCTCCATCATGCTTCTGGGCGACAGCAAGACCATGGATATCGAAGCCATCTCT 120

G K G S I M L L G D S K T M D I E A I S

ACCGGTTCCCTCTCCCTGGACGTGGCGCTGGGTATCGGCGGTCTGCCGTGCGGCCGTATC 180

T G S L S L D V A L G I G G L P C G R I

GTCGAGATCTATGGCCCGGAATCTTCCGGTAAAACCACCCTCACCCTGCAGGTGATTGCG 240

V E I Y G P E S S G K T T L T L Q V I A

GAAGCCCAGAAGAAAGGCAAGGTCTGTGCATTTATCGATGCAGAGCATGCCCTCGACCCC 300

E A Q K K G K V C A F I D A E H A L D P

ATCTATGCAGCCAAGCTGGGTGTCAACGTCGATGACCTGCTGATCTCCCAGTCGGATACC 360

I Y A A K L G V N V D D L L I S Q S D T

GGTGAGCAGGCGCTGGAAATCTGCGACATGCTGGTGCGTTCCAACGCCGTTGACGTCATC 420

G E Q A L E I C D M L V R S N A V D V I

ATCGTCGACTCGGTGGCCGCTCTGACGCCGAAAGCAGAAATCGAAGGCGAGATGGGTGAT 480

I V D S V A A L T P K A E I E G E M G D

TCCCACGTCGGCCTGCAGGCTCGTCTGATGTCCCAGGCGCTGCGCAAGCTGACCGCCAAC 540

S H V G L Q A R L M S Q A L R K L T A N

ATCAAGAACGCCAACTGCCTCTGTATCTTCATCAACCAAATCCGGATGAAGATTGGTGTC 600

I K N A N C L C I F I N Q I R M K I G V

ATGTTCGGCAGCCCGGAAACCACTACCGGTGGTAACGCGCTCAAGTTCTACGCCTCAGTA 660

M F G S P E T T T G G N A L K F Y A S V

CGTCTGGACATCCGTCGCATCGGCGCCATCAAGGAAGGTGACGAAGTGGTCGGTAACGAG 720

R L D I R R I G A I K E G D E V V G N E

EcoRI

ACCCGCGTCAAGGTGGTCAAGAACAAGGTTGCCCCTCCCTTCAAGCAGGCTGAATTCCAG 780

T R V K V V K N K V A P P F K Q A E F Q

ATTTTCTACGGTGTCGGTATTTCCAAGGAAGGTGAGCTGGTGGATCTCGGCGTCAAGCAC 840

I F Y G V G I S K E G E L V D L G V K H

AAGCTGATCGACAAGGCCGGTGCCTGGTACAGCTATAACGGCGAGAAGATAGGTCAGGGC 900

K L I D K A G A W Y S Y N G E K I G Q G

AAGGCCAACGTGATGAAGCTGTTCACCGAGAACAAGGTGATGGCGGCCGAGGTGGAAGCC 960

K A N V M K L F T E N K V M A A E V E A

AGACTGCGTGAGTTGCTGCTCTCCGGCGATGTGCCGGCCGAGAAGCCGGTCGTGGCGGAT 1020

R L R E L L L S G D V P A E K P V V A D

GCCGACGAGCTCGAAGCCGAAAGCGAACAAGAGTTCGAATAACAGTGAGGGCCGGGCTAA 1059

A D E L E A E S E Q E F E * ------>

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<------

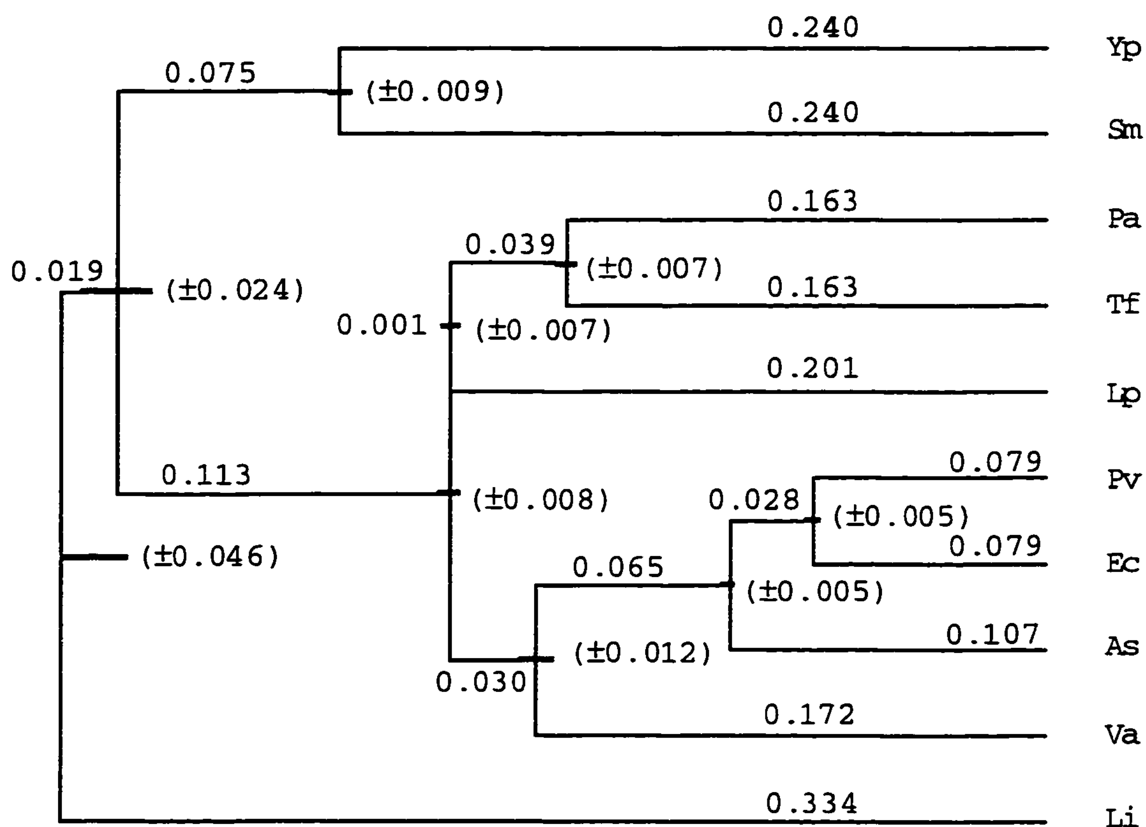

Figure 5. UPGMA tree showing the evolutionary relatedness of the *A. salmonicida* A449 RecA to some selected RecA proteins.
Numbers indicate the calculated evolutionary distances, while the numbers in parentheses indicate the standard error of the branch position. *A. salmonicida* (As), *Yersinia pestis* (Yp) (189), *Serratia marcescens* (Sm) (21), *Pseudomonas aeruginosa* (Pa) (311), *Thiobacillus ferrooxidans* (TF) (296), *Legionella pneumophila* (Lp) (380), *Proteus vulgaris* (Pv) (381), *E. carotovora* (Ec) (381), *V. anguillarum* (Va) (350) and *L. lactis* (Ll) (88).

Conservation of *recA* in *A. salmonicida* strains

Total DNA was prepared from typical and atypical strains of *A. salmonicida, A. hydrophila, A. sobria* biotype *veronii* and from *P. putrefaciens, P. aeruginosa, V. vulnificans, S. enteritidis, C. fetus* and *Enterobacter omnigens*. The DNA was digested with *Hind*III, separated on a 0.8% agarose gel and transferred to a Nytran$^+$ nylon membrane. A 1190 bp fragment containing the *recA* of A449 was amplified by PCR using the primer pair Rec6 (GAGGGTTCAGTGGGTTCGCAG) and Rec9 (CAGTATACTTAGCC CAACTTC) and labeled with (α^{32}P) dCTP. Southern blotting using the *rec*A fragment as probe was carried out under stringent conditions (6% allowed mismatch). The *recA* probe hybridized to a *Hind*III fragment of approximately 20 kb in all the typical strains checked and to different sized fragments in the lanes containing DNA obtained from atypical strains (Fig. 7). The probe hybridized to a fragment of approximately 17 kb in A400, A401 and A404, and to a 20 kb fragment in A480. The Southern blot results also indicated that there was strong similarity with the *recA* of the other aeromonads, *A. hydrophila* and *A. sobria* biotype *veronii*. The *recA* probe bound to fragments of approximately 15 kb in TF7, 17 kb in Ah300 and Ah598, and 8.5 kb in A701.

The *A. salmonicida* A449 *rec*A probe did not hybridize to total DNA obtained from *C. fetus* suggesting significant differences in the *rec*A genes of these two species. The *recA* probe bound to a fragment of approximately 9.4 kb in *V. vulnificans*, 3.2 kb in *P. aeruginosa*, 5.6 kb in *P. putrefaciens*, 17 kb in *S. enteritidis* and to two fragments of approximately 2.8 kb and 7 kb in *E. omnigens*. However, the lower hybridization signal obtained from the binding of the *A. salmonicida recA* probe to DNA from these species indicated that there was less homology at the nucleotide level of the *recA* of these species when compared to that of the aeromonads.

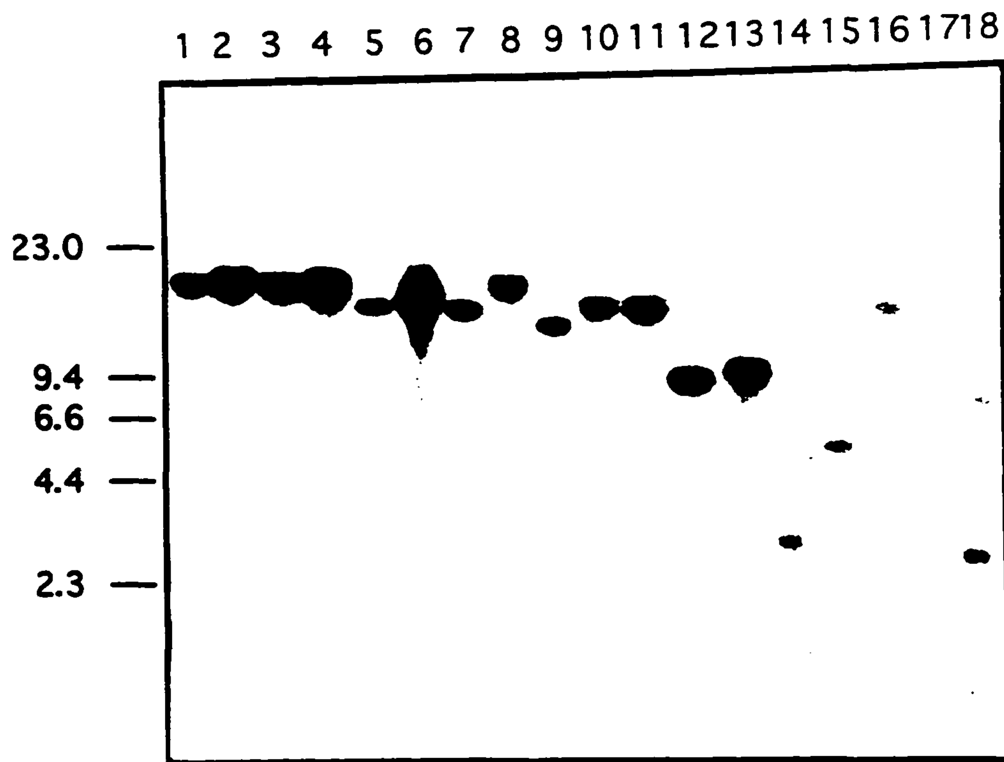

Figure 6. Southern blot analysis of *Hind*III digests of chromosomal DNA showing the conservation of *rec*A in strains of *A. salmonicida, A. hydrophila, A. veronii* biotype *sobria* and representatives from selected species.
Lanes 1. A449; 2. A251; 3. A395; 4. A438; 5. A401; 6. A400; 7. A404; 8. A480; 9. TF7; 10. Ah300; 11. Ah598; 12. A701; 13. *V. vulnificans*; 14. *P. aeruginosa*; 15. *P. putrefaciens*; 16. *S. enteritidis*; 17. *C. fetus*; and 18. *E. omnigens*. M_r markers (kD) are shown on left.

Predicted protein structure and *in vitro* expression of the *A. salmonicida* A449 *recA*

The deduced amino acid sequence of *A. salmonicida* A449 *recA* gives rise to a protein of predicted M_r of 37,900 and a predicted pI of 4.94. Analysis of the predicted amino acid composition of the *A. salmonicida* A449 RecA showed that it contained 44.19% hydrophobic, 14.17% acidic, 29.46% polar and 12.19% basic amino acid residues. The RecA protein sequence of *A. salmonicida* A449 exhibited considerable amino acid sequence homology to RecA from a diverse range of bacteria (Fig. 7). The most notable differences occurred in the C-terminal 50 amino acid residues which were quite different in all the RecA proteins compared.

The *A. salmonicida* A449 RecA was expressed from pEU101 by *in vitro* transcription and translation. From the *in vitro* expression results, the M_r of A449 RecA was estimated at approximately 42,000 (Fig. 8). This was higher than the predicted M_r of 37,900. The unprocessed Ap protein band common to all three lanes has a predicted M_r of approximately 31 kD, although the *in vitro* expressed protein also migrated slightly higher at approximately 33 kD. The 42,000 protein was confirmed to be the *A. salmonicida* A449 RecA since pEU102 which has a mutated copy of *rec*A (filled-in *Eco*RI site in *rec*A) does not produce any protein corresponding to this size. The blunt-ending of the *Eco*RI site would result to the production of a truncated protein of M_r 27,574. A faint protein band corresponding to approximately 29 kD was present in the lane with pEU102 but not in either lanes with the negative control or pEU101. The 29 kD protein could be the truncated RecA protein produced as a result of the disruption of the *rec*A ORF. The elimination of the *Eco*R1 site inactivated the complementing A449 RecA activity in *E. coli* because cells bearing the mutated *rec*A allele were unable to grow in media containing MMS.

Figure 7. Alignment of the deduced amino acid sequence of the *A. salmonicida* A449 RecA with the RecA from selected organisms.
A. salmonicida A449 RecA (As), *V. anguillarum* (Va) (350), *S. marcescens* (Sm) (21), *P. vulgarus* (Pv) (381), *E. carotovora* (Ec) (381)), *P. aeruginosa* (Pa) (311). Colons (:) indicate amino acid identity with the *A. salmonicida* A449 RecA.

```
As     MDQNKQKALAAALGQIEKQFGKGSIMLLGDSKTMDIEAISTGSLSLDVALGIGGLPCGRI    60
Va     ::E:::::::::::::::::::::::R:::NR:::V:T:::::::::I:::A::::M:::    60
Pa     ::E::KR::::::::::R:::::AV:RM::HERQA:P:::::::G::I::::::::K:::    60
Sm   MAI:E:::::::::::::::::::::::R::EDRS::V:T:::::::::I:::A::::M:::    62
Pv   MAI:E:::::::::::::::::::::::R::EDRS::V:T:::::::::I:::A::::M:::    62
Ec   MAI:E:::::::::::::::::::::::R::EDRS:NV:T:::::::::::::A::::R:::    62

As     VEIYGPESSGKTTLTLQVIAEAQKKGKVCAFIDAEHALDPIYAAKLGVNVDDLLISQSDT   120
Va     ::V:::::::::::::EL::A::RV::T:::::::::::::::K:::::I:E::V::P::   120
Pa     ::::::::::::::::S:::::::Q:AT:::V::::::::D::G::::::::::V::P::   120
Sm     ::::::::::::::::::::A::RE::T:::::::::::::::K::::DI:N::C::P::   122
Pv     ::::::::::::::::::::A::RE::T:::::::::::::::K::::DI:N::C::P::   122
Ec     ::::::::Y:::::::::::A::RE::I:::::::::::::::Q::::DI:N::C::P::   122

As     GEQALEICDMLVRSNAVDVIIVDSVAALTPKAEIEGEMGDSHVGLQARLMSQALRKLTAN   180
Va     :::::::::::A::G:I:::VI::::::::::::::::::::M:::::ML:::M::::G:   180
Pa     :::::::T::::::::::::::::::::V:::::::::::A:::::::::::::::I:G:   180
Sm     :::::::::A:T::G::::::::::::::::::::::I::::M::A::M::::M:::AG:   182
Pv     :::::::::A:T::G::::::::::::::::::::::I::::M::A::M::::M:::AG:   182
Ec     :::::::::A:S::G:::::V::::::::::::::::I:::::::A::M::::M:::AG:   182

As     IKNANCLCIFINQIRMKIGVMFGSPETTTGGNALKFYASVRLDIRRIGAIKEGDEVVGNE   240
Va     L:QS::M::::::::::::::::N::::::::::::::::::::::T:S::::::A::::   240
Pa     :::::::V:::::::::::::::N::::::::::::::::::::::T::V::::::::S:   240
Sm     L::::T:L:::::::::::::::N::::::::::::::::::::::::::::::::::S:   242
Pv     L:Q::T:L:::::::::::::::N::::::::::::::::::::::T::::D:E::::S:   242
Ec     L::S:T:L:::::::::::::::N::::::::::::::::::::::::SV:N::::::S:   242

As     TRVKVVKNKVAPPFKQAEFQIFYGVGISKEGELVDLGVKHKLIDKAGAWYSYNGEKIGQG   300
Va     ::I::::::I:A:::::DT::L::Q:FNR:::::::::::::VE::::::::::D:::::   300
Pa     ::::::::::S:::R::::::L::K::YRT::II::::QLG:VE:S::::::Q:S:::::   300
Sm     :::::::::I:A:::::::::M::E::NSR:::::::::::M:E::::::::::::::::   302
Pv     :::::::::::A:::::::::L::E::NIH:::::::::::::E::::::::::D:::::   302
Ec     :::::::::I:A:::::::::M::E::NTF:::I::::::::VE::::::::::::::::   302

As     KANVMKLFTENKVMAAEVEARLRELLLSGDVPAEKPVVADADELEAESEQEFE          353
Va     :::AC:FLR::PAA:MALDTK:::M::NPAELIVEEPILSEMPQ:E:L               348
Pa     :::AA:YLED:PEIGSVL:KTI:DQ::AKSG:VKADAEEV::AEAD                 346
Sm     :::ACNFLK::PAI:::LDKK::D:::HSGGELVAASGD:FEDD:::TSEQ:           354
Pv     :::ACNFLK::SLVKETKNFNGC                                        325
Ec     :::ATTYLK:HPE:YN:LNTK:::M::NHAGEFTSARDTC                       342
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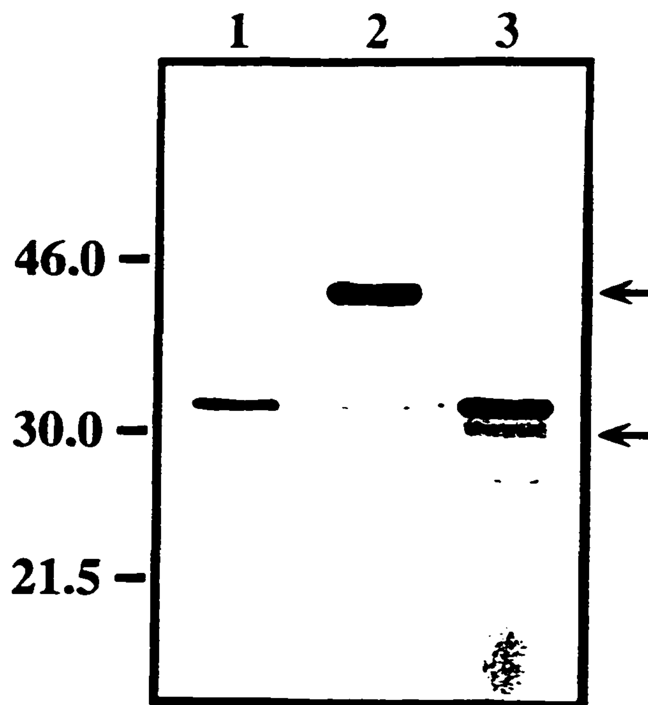


Figure 8. *In vitro* transcription and translation of *rec*A of *A. salmonicida* A449.
Lanes 1, pUC19; 2, pEU101; 3, pEU102. The estimated 42 kD RecA protein band in lane 2 is indicated with a arrow. The faint 29 kD protein band in lane 3, which could be the truncated RecA is also indicated with an arrow. Mr markers (kD) are indicated on left.

Allele exchange mutagenesis

Attempts to generate a *recA* strain of *A. salmonicida* A449 by either allele exchange or single insertion mutagenesis were unsuccessful. In the allele exchange mutagenesis experiments, the first strategy employed involved the disruption of the *rec*A ORF with the introduction of a Km cassette at the *Eco*RI site, 771 bp into the *recA* ORF (Fig. 3). The interrupted *recA* allele was cloned into pSUP202 and pK18*mobsac*B, which were transferred into A449 by conjugation. Allele replacements were not achieved with either vector, despite numerous attempts. The sucrose sensitivity imparted by pK18*mobsacB* can be used in *A. salmonicida* (E. Umelo, unpublished data) to select for double cross-over mutants, but sucrose-resistant isolates in these experiments were *recA*$^+$. Similarly, pSUP202 has been successfully used a number of times to generate *A. salmonicida* mutants (66, 265, 267). Only *A. salmonicida* A449 cells with single-crossover phenotypes were observed when pSUP202 was used as the suicide vector, these strains contained a mutated and a wild type copy of *rec*A (data not shown).

In *V. cholerae*, attempts to mutate the *Vibrio rec*A by allele replacement when the ORF was disrupted by either of three different antibiotic cassettes encoding Km-, Mercury-, or Cm-resistances also failed. However an allele replacement *V. cholerae rec*A strain was finally obtained when the *rec*A ORF was disrupted by blunt-ending an internal *Xba*I site. A similar strategy was attempted in *A. salmonicida* A449. The single *EcoR*I site in the gene (Fig. 4) was blunt-ended to disrupt the ORF. In the *E. coli* HB101 background, the *rec*A containing the blunt-ended *EcoR*I site was unable to complement the cells for the ability to survive in growth media containing MMS. The mutated allele was cloned into pSUP202 and transferred to *A.*

salmonicida A449 by conjugation. Without the convenience of an antibiotic selection, the *A. salmonicida* A449 exconjugants had to be screened for loss of RecA activity by inability to grow on media containing MMS. At least 3000 exconjugants were screened on duplicate TSA plates, with and without MMS. *A. salmonicida* A449 colonies which have undergone a double cross-over event leading to loss of RecA activity were not expected to grow on the media containing MMS. However, all the colonies screened grew on TSA with and without MMS indicating the presence of an intact *rec*A. Southern blots were performed on a selected number of the colonies, and the results obtained confirmed the presence of an intact *rec*A (data not shown).

In a final attempt to obtain a *rec*A strain of *A. salmonicida* A449, an internal 490 bp fragment of the A449 *rec*A was PCR-amplified using the primer pair Rec6 and Rec7 (GCCTCAGTACGTCTGGACATC) and cloned into both pK18*mobsac*B and pSUP202. The vectors containing the 490 bp *rec*A fragment were transferred into *A. salmonicida* A449 by conjugation. Since these vectors were suicidal and could not replicate autonomously in *A. salmonicida*, they had to integrate at the chromosomal locus containing the homologue of the cloned fragment. Such an integration would result to a disruption of the *rec*A ORF. However, although *A. salmonicida* A449 exconjugants were obtained with antibiotic phenotypes that suggested the vectors were present in the cells, Southern blot analysis confirmed that these plasmids had not integrated into the *rec*A locus (data not shown). The blot results did not show random integration all over the chromosome but suggested integration in at least two different loci. Because the Southern blot results presented in Figure 6 suggested that *A. salmonicida* strains had a single copy of *rec*A, and because the suicide vectors cannot replicate autonomously in *A. salmonicida*, the interpretation of the Southern blot

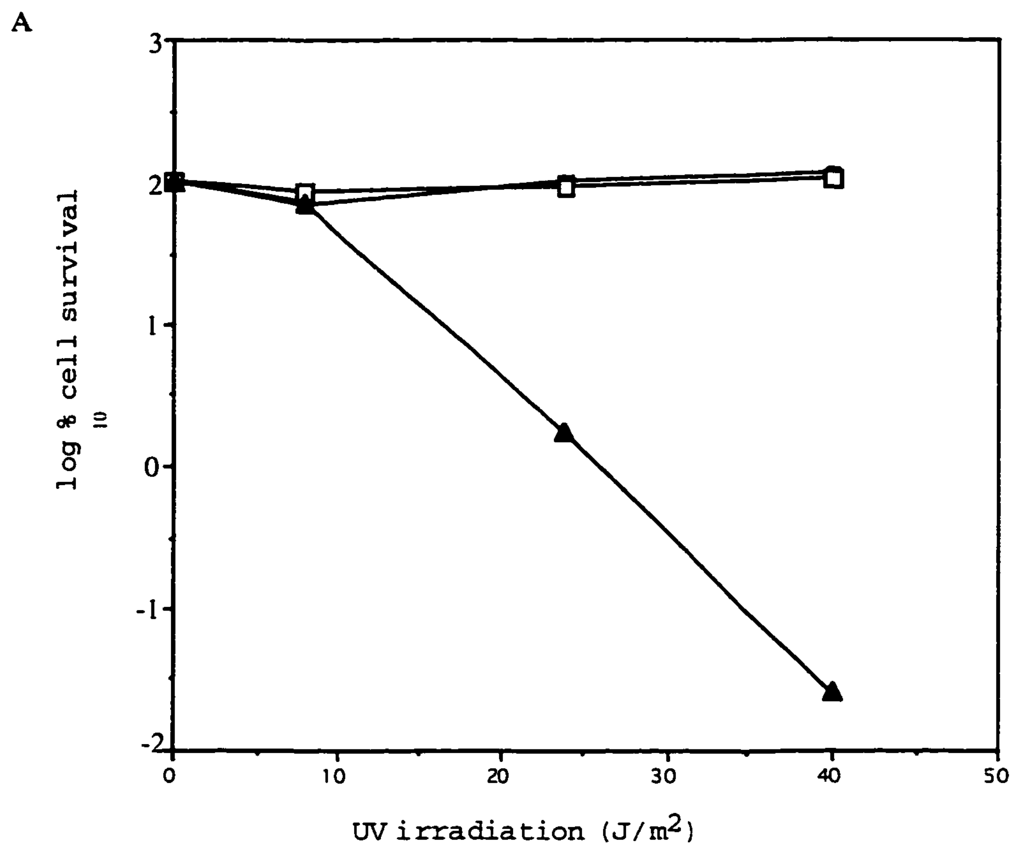
results was that the vectors containing the internal *rec*A fragment must have integrated into the A449 chromosome at a locus homologous to some vector sequences.

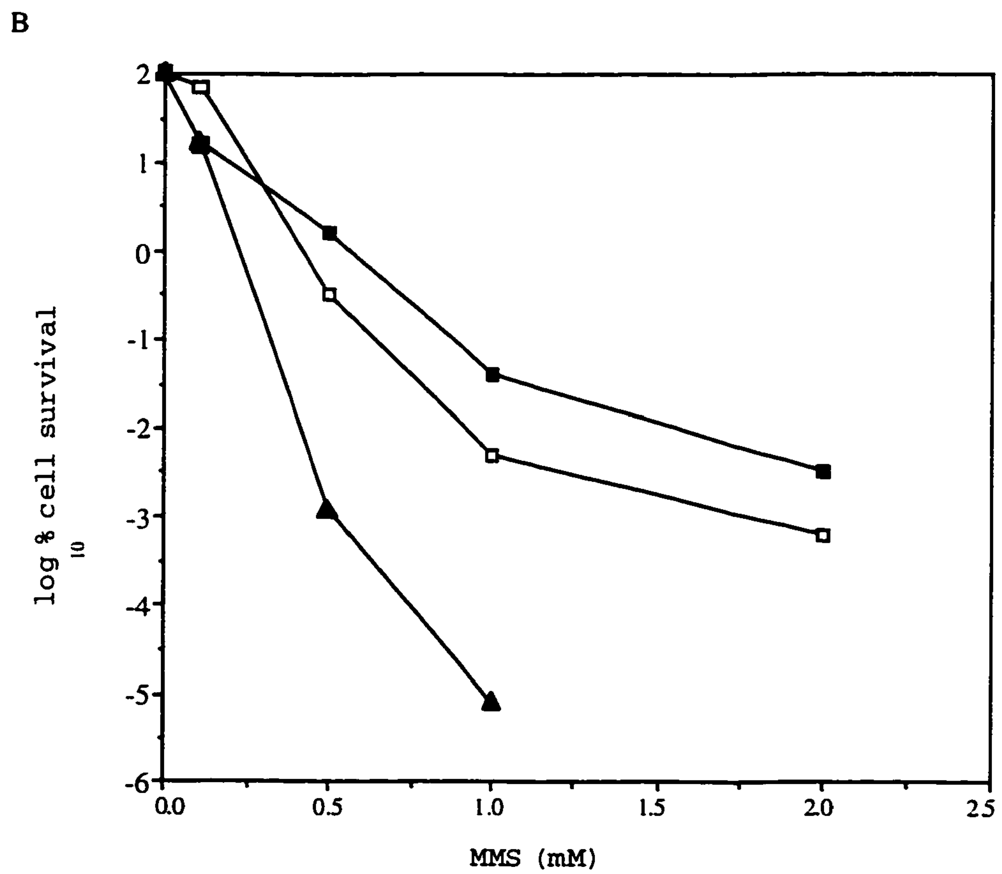
Complementation of an *E. coli rec*A mutant with *A. salmonicida* A449 *rec*A

The effect of *A. salmonicida* A449 *recA* on the survival of an *E. coli recA* strain, JM109, was investigated (By Dr. B. Noonan). The parent strain JM107 was used as a positive control. JM109 was transformed with pEU104 (*A. salmonicida recA* in pSUP202) and pEU105 (Fig. 3). The three strains, JM107, JM109(pEU104) and JM109(pEU105) were exposed to varying doses of uv irradiation for different amounts of time (Fig. 9A). The results show that the rate of survival of JM107 was unaffected by the levels of uv irradiation tested (Fig. 9A). Similarly, the survival of JM109(pEU104) was indistinguishable from JM107. However, increasing the levels of uv irradiation greatly reduced the survival of JM109 (pEU105), indicating that the insertion of the Km cassette inactivated the complementing RecA activity (Fig. 9A).

JM107, JM109(pEU104) and JM109(pEU105) were also tested for growth on media containing varying concentrations of MMS (Figure 9B) The results also shows that the presence of pEU104 resulted in increased survival of JM109 on MMS (Fig. 9B). pEU105 was unable to complement JM109 and these cells were completely unable to grow at levels of MMS higher than 1 mM (Fig. 9B).

Figure 9. Complementation of uv (A) and MMS (B) sensitivity of *E. coli* JM109 with cloned *A. salmonicida* A449 *rec*A.
■-■ = JM107, □-□ = JM109 (pEU104), ◆-◆ = JM109 (pEU105).


A
log10 % cell survival
3
2
1
0
-1
-2
0
10
20
30
40
50
UV irradiation (J/m2)


B
log10 % cell survival
2
1
0
-1
-2
-3
-4
-5
-6
0.0
0.5
1.0
1.5
2.0
2.5
MMS (mM)

Induced recombination with *A. salmonicida* A449 *recA* in *E. coli* JC14604

To investigate whether the *A. salmonicida* A449 *recA* can be induced in *E. coli* in conditions that activate the SOS response, the ability of *E. coli* JC14604 to undergo induced recombination with the *A. salmonicida* A449 *recA* was investigated (By Dr. B. Noonan). Cells were grown on MacConkey agar for 48 h and the amount of recombination to Lac^{+} relative to the number of colonies examined was determined (Table 6). Exposure to the DNA damaging effects of uv irradiation resulted in an increase in the number of Lac^{+} papillae formed by JC14604 containing pEU101. The uv irradiation did not give rise to any recombination in JC14604 containing pEU102 which has a mutated *recA* allele. This suggested that the *A. salmonicida* A449 *recA* was inducible in *E. coli* in response to DNA damage.

The regulatory region 5' of the *E. coli recA* is known to contain a LexA repressor binding site (SOS-box) (Fig. 10, (309 1087)). However it has been shown that some organisms such as *R. meliloti* (317) and *A. tumefaciens* (241) do not have a consensus SOS-Box 5′ of the *recA* gene. Therefore nucleotide sequence analysis of the DNA 5′ of the *A. salmonicida* A449 *recA* was performed and compared to those of *E. coli* and *S. marcescens* (21). Alignment of the sequences demonstrated the presence of a putative LexA-like repressor binding site 5′ of the *A. salmonicida* A449 *recA* (Fig. 10). The identification of the consensus SOS-box 5′ of the *A. salmonicida* A449 *recA* supported the finding described above that the *A. salmonicida* A449 *recA* was inducible in *E. coli* in response to DNA damage.

Table 4. UV-induced recombination in *E. coli* JC14604 with *A. salmonicida* A449 *recA*

Plasmid	UV irradiation (J/m^2)	Recombination frequency[a]
pEU102	0	0.00 (0)
pEU102	16	0.00 (0)
pEU101	0	0.58 (0.10)
pEU101	16	1.15 (0.12)

[a] Colonies with Lac^+ papillae were scored after 48 h. Mean of 3 experiments with 45-50 colonies per measurement. Standard deviation is indicated in parentheses.

```
Ec  cttgataCTGtatgagcataCAGtataatt--44--AGGAGtaaaa-ATGgctatc
Sm  cttgataCTGtatgaccataCAGtataatt--65--AGGAGcaaaa-ATGgctatt
As  cttgataCTGtatgaatagaCAGtatactt--33--CGGAGattggcATGgatcag

              SOS-box                      SD          M ——→
```

Figure 10. Comparison of the upstream regulatory regions from the *recA* of *A. salmonicida* A449 with those of *E. coli* and *S. marcescens*.
The *recA* of *A. salmonicida* A449 (As), *E. coli* (Ec) (309) and *S. marcescens* (Sm) (21). The highly conserved bases from the consensus SOS-box are indicated in bold capitals, the putative SD ribosome binding region (GGAG) and the Methionine start codon (ATG) are indicated in capitals. Hyphens (-) were utilized to facilitate alignment and the number of the intervening nucleotides between the consensus SOS-box and SD sequence are indicated.

DISCUSSION

The *recA* of *A. salmonicida* A449 was cloned based on its ability to allow the survival of an *E. coli recA* mutant in the presence of the chemical mutagen, MMS. Southern blot analysis using the *A. salmonicida* A449 *recA* as a probe demonstrated that it is highly conserved among both typical and atypical strains of *A. salmonicida* as well as among other aeromonads including *A. hydrophila* and *A. sobria* biotype *veronii*. The *A. salmonicida* A449 *recA* also hybridized under stringent conditions to total DNA from a number of other bacterial species. The amino acid sequence similarities between *A. salmonicida* A449 RecA and other bacterial RecA were highlighted by sequence alignment and by a comparison of the amino acid sequences using an UPGMA plot (256). Of the RecA proteins compared, the *A. salmonicida* A449 RecA was found to be most closely related to the RecA of *E. carotovora* and *P. vulgaris* (381). A high degree of relatedness to *V. anguillarum* RecA was also observed (350).

A. salmonicida A449 RecA has a predicted M_r of 37,900. Determination of the apparent M_r of the *A. salmonicida* A449 RecA protein using *in vitro* transcription/translation and SDS-PAGE analysis, identified RecA as having a M_r of 42,000. This difference between predicted and measured M_r has also been observed with the *V. cholerae* RecA protein which has a predicted M_r of 38,248 and an apparent M_r of 44,000 on SDS-PAGE gels (124). The deduced amino acid sequence of *A. salmonicida* RecA was compared to other bacterial RecA proteins. This demonstrated a high degree of sequence identity between all the RecA proteins, except at the C-termini where significant variation occurs. It has been shown that as much as the last 50 amino acid residues (representing 15%) of the C-terminal of *E. coli* RecA is dispensable as

truncated proteins still retain certain biochemical activities such as assimilation of homologous single stranded DNA into duplex DNA and ATPase activity, both *in vitro* and/or *in vivo* (32, 153, 194). Other studies showed that RecA proteins lacking 62, 77, 93, 149 amino acids (153) or 21% of the C-terminal amino acids (379) were affected in several functions including those essential for the SOS response. It therefore appears that the C-terminal of RecA may have a role in regulating the induction of the SOS response (153, 194). In this study, the results also showed that mutations leading to production of a truncated *A. salmonicida* A449 RecA protein missing the last 94 amino acids residues (26%) of the C-terminus are completely inactive in *E. coli* in terms of the SOS response, homologous recombination and DNA repair functions.

In *E. coli,* the RecA protein has been characterized in detail and amino acid residues which are involved in the biochemical activities of the protein have been identified. Among these are the 66-GPESSGKT-73 residues which constitute part of an nucleotide-binding domain. The deduced *A. salmonicida* A449 RecA protein also possesses this consensus nucleotide-binding sequence. Similarly, regions of the *E. coli* RecA protein known to be involved in DNA-binding (residues 156-165 and 194-210) and target protein binding (G-229 and R-243) are also conserved in the *A. salmonicida* RecA. Amino acid residues throughout the *E. coli* RecA protein have been implicated in protein-protein interactions (185) and once again a significant level of amino acid sequence identity with *A. salmonicida* A449 RecA was observed.

Numerous attempts to interrupt the *recA* of *A. salmonicida* by allele exchange mutagenesis using a Km resistance cassette cloned into the ORF were unsuccessful. While a number of *recA* mutants have been generated in a variety of bacteria, similar problems with allele exchange mutagenesis of

recA have been reported for *Synechococcus* sp. strain PCC 7002 (254), *M. xanthus* (269) and *V. cholerae* (124). *M. xanthus* has two *recA* genes, *recA*1 and *recA*2 which have a 67.0% identity with each other and a 60.5% and 60.9% identities with the *E. coli recA* respectively. Expression of *recA*1 was not detected in *M. xanthus* in both uninduced and induced conditions with nalidixic acid, while *recA*2 was detected in uninduced conditions although its concentration increased approximately threefold on induction with nalidixic acid treatment (269). However both *recA*1 and *recA*2 were expressed in an *E. coli recA* strain and were both functional in *E. coli,* although strains harbouring a plasmid with *recA*2 were more resistant to UV irradiation than those harbouring plasmids with *recA*1. *M. xanthus recA*1 mutants were easily obtained by insertion of a Km cassette into the *recA*1 locus in the chromosome by allele replacement. In contrast *M. xanthus recA*2 mutants could not be isolated by a similar method, despite numerous attempts (269). In the case of *V. cholerae,* the problem was overcome by blunt-ending an *Xba*I site in the *recA* gene to generate a frameshift or nonsense mutation and transferring the mutated allele to the chromosome of a *V. cholerae thyA* mutant. Selection for *recA* mutants involved the use of lysogenic phage and thymine starvation (124). A similar approach with *A. salmonicida* would have to await the development of a suitable screening procedure. The evidence suggests that a large interruption of the coding sequence, such as is caused by the insertion of a Km resistance cassette, has polar effects on essential genes 3' of *recA* in some species. In *H. pylori, rec*A is cotranscribed with a downstream gene which encodes enolase and mutation of *rec*A had a polar effect on expression of the enolase encoding gene (344). The *rec*A strain of *H. pylori* survived poorly at low pH, probably because of polar effects preventing expression of some gene(s) whose products are essential for

survival at low pH. It was concluded that the *rec*A region of *H. pylori* was involved in resistance to low pH conditions (344). Indeed in *A. salmonicida* A449 *rec*A may constitute part of an operon since no consensus promoter sequence was identified in the DNA sequences immediately 5′ of the methionine start codon. Mutation of the *A. salmonicida* A449 *rec*A may be lethal because of other essential gene(s) whose expression are prevented by the disruption of the *rec*A ORF.

A. salmonicida A449 *recA* complements the *E. coli rec*A strain JM109 and allows growth after irradiation with uv light. Indeed, the growth of JM109 containing the cloned *A. salmonicida* A449 *recA* was indistinguishable from the parent strain, JM107, under the conditions used in this study. Growth of JM109 on media containing MMS was also complemented by *A. salmonicida* A449 *recA*. Exposure of the *E. coli recA* mutant JC14604, containing the cloned *A. salmonicida* A449 *recA*, to uv irradiation increased the frequency of recombination in this strain. By measuring the amount of recombination to a Lac^{+} phenotype, it was demonstrated that exposure to uv damage resulted in an almost two-fold increase in the number of Lac^{+} papillae formed. This is strong evidence that the *A. salmonicida* A449 *recA* is being induced in *E. coli*, presumably via cleavage of the LexA repressor. In *E. coli*, RecA interacts with single stranded DNA, which results in the activation of RecA to a form that is now able to act as a co-protease in the cleavage of the LexA repressor protein (152, 209). This results in the derepression of approximately 20 unlinked genes in *E. coli*, including *recA* itself (reviewed in (185). The observation that *A. salmonicida* A449 *recA* responds to induction due to DNA damage in *E. coli* and also the presence of a consensus SOS-box sequence 5' of the gene indicates that the mechanism of *recA* induction in *A. salmonicida* closely parallels that of *E. coli*. This is further evidence of the

high degree of evolutionary relatedness between recombination systems from diverse bacteria.

Studies with a number of bacterial species have determined that divergence occurs in the regulatory region 5' of *recA*. An SOS-box has been shown to be present in front of the *recA* genes of *E. coli* (309), *P. aeruginosa* (311), *Proteus mirabilis* (1) and now *A. salmonicida* A449. There is no SOS-box, however, in front of the *recA* genes of *R. meliloti* (317), *A. tumefaciens* (241) or *Thiobacillus ferrooxidans* (296). An earlier study involving the promoter regions of the *recA* genes of *A. tumefaciens, R. meliloti, R. phaseoli*, and *R. sphaeroides* fused to *lacZ* demonstrated that the mechanisms of *recA* induction can vary between species (299). The *recA* genes from these species, which do not have an SOS-box 5' of the gene, are not inducible in *E. coli*. Likewise, the *recA* of *E. coli* is not inducible in any of the other four species. In fact, the regulatory mechanisms of *R. sphaeroides* only operated on *Rhodobacter recA* (299). Recent analysis of the regulatory region of the *recA* of *R. etli* has revealed that the sequence TTG-N_{11}-CAA may be the consensus SOS-box of the three Rhizobiaceae, *R. meliloti, R. etli* and *A. tumefaciens* (340). This suggests that there may be several classes of *recA* regulation in bacteria. The *A. salmonicida recA* would appear to be grouped with *recA* genes possessing the consensus sequence CTG-N_{10}-CAG at the SOS-box operator region. Interestingly, the *A. salmonicida recA* also shares the highest degree of nucleotide sequence identity with the *recA* from species containing SOS-box operator regions, such as *E. coli, P. aeruginosa*, and *P. mirabilis*.

The elucidation and study of the virulence determinants of *A. salmonicida* will require the generation of defined mutations and the subsequent characterization of their effects on pathogenesis. In order to be able to state unequivocally that a particular mutation results in a specific

effect, it will be necessary to ensure that recombination events have not occurred leading to unknown secondary mutations. For these reasons, it is important to elucidate the mechanisms of recombination in *A. salmonicida* with a view to generating mutants with a reduced ability to undergo recombination. Similarly, with the advent of mutant *A. salmonicida* strains for use as live attenuated vaccines (264, 346, 347), it will be important to limit the possibility of genetic recombination in vaccine strains. This is especially a concern as *A. salmonicida* plasmids are transmissible by conjugation (31) and therefore the possibility exists of DNA transmission between strains.

CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF A *fla* OPERON CONTAINING FOUR FLAGELLAR STRUCTURAL GENES IN *Aeromonas salmonicida* A449

This study was initiated following the serendipitous discovery of a *fla*A gene during one of our searches for additional genes to facilitate construction of the *A. salmonicida* A449 chromosomal map. The *fla*A gene encoded a protein whose deduced amino acid sequence had strong homology to other bacterial flagellins. Given the fact that *A. salmonicida* is classified as non-flagellated and non-motile, we were intrigued by the presence of a flagellin gene in the organism's genetic makeup. Because some strains of *A. salmonicida* have been reported to express flagella at extremely low frequency (234), it was decided to further characterize the *fla* gene. The *fla* gene was also used as a marker on the *A. salmonicida* A449 genome map.

BACKGROUND TO FLAGELLA

Bacterial flagella

Flagellum is an organelle utilized primarily for motility in prokaryotes. For the majority of flagellated bacteria such as *Escherichia, Salmonella* and *Pseudomonas*, the flagella is localized extracellularly and is used for swimming or swarming motility. Other bacteria such as spirochetes, have internal flagella (periplasmic flagella) localized in the periplasmic space which are more suited for gliding through viscous environments. The *Borrelia* spp. which possess the periplasmic-type flagella, exhibit a planar waveform-type of motility also suited for efficient movement through viscous environments (126). Finally, the complex-type flagella of *Rhizobium* are more rigid and are adapted for movement through environments of even higher viscosity such as the gelatinous layer surrounding the root hairs of leguminous plants (188).

The flagella consists of three main structural regions, a complex membrane-associated structure of rings and rods known as the basal body, an externally located curved hook and a helical filament (Fig. 11) (217). Rotation of the filament driven by the membrane-associated flagellar motor apparatus results in the propulsion of the bacteria (217). The source of energy for rotation of the filament is obtained either from the proton motive force or the sodium transmembrane potential, where rotation is coupled to the transport of these ions across the membrane (247).

The flagellar filament represents the major component of this complex and biologically important organelle. The filament is a polymer composed of thousands of copies of the self assembling protein, flagellin (255). The N- and C-terminal regions of the flagellin are highly conserved across numerous

bacterial species. This conservation is very likely a result of function-related selective pressure during evolution since the N- and C- terminii of the flagellin have been shown to be involved in export and self-assembly of the monomers (151, 268). Electron density mapping studies of intact flagella has revealed that the conserved terminii are confined within the intact flagellar filament, while the surface exposed areas are formed from the folding outward of the central region (99, 255, 355, 366). Therefore the central region of the flagellin is more variable, more strain specific and is responsible for antigenic determinant of each bacterial strain (168, 191, 259, 316, 328).

In some bacterial species, the production of flagella can be influenced by external stimuli. For example, in *Legionella pneumophila* and *Yersiniae*, flagella expression is temperature regulated (58, 170). In addition, *V. parahaemolyticus* produces two distinct types of flagella, polar or peritritous, depending on the viscosity of its environment, each kind adapted to a different type of motility (14, 247). The lateral flagella of *V. parahaemolyticus* are induced in viscous conditions which inhibit the efficient rotation of the polar flagella. Polar flagella either singly, or in bundles, are more suited for swimming in liquid environments while the peritritous lateral flagella are adapted for swarming on solid and viscous surfaces (14, 224, 225). In *Proteus mirabilis*, a bacterium also capable of expressing swimmer or swarmer flagella, the two flagella are composed of identical flagellin monomers (247). However in *V. parahaemolyticus*, the swimmer and swarmer flagella do not share any structural components in common (225), and are even powered by two separate systems. The rotation of the polar flagella is powered by the sodium transmembrane potential while the lateral flagella are powered by the proton motive force (14).

The morphogenesis of the flagella organelle is a very complex process involving the products of over 40 genes (207, 217). These genes are grouped into three or four classes which are expressed in a sequential order. Expression of some classes of genes are dependent on prior expression of others, in an order that is coupled to the assembly of the flagella (217). Regulatory proteins and those required for assembly of the basal body structure are among the first produced, next the proteins required to assemble the hook are synthesized and finally filament proteins are expressed (217). As a result, the assembly of the flagella organelle is achieved from the base to the tip by the addition of new components through a flagellin specific export channel located within the growing organelle. This complex regulation ensures that the highly synthesized flagellin monomers are not made unless the other preceding structures required for proper assembly have been synthesized.

The best described filaments are those of the enteric bacteria *Escherichia* and *Salmonella*. These filaments are formed by the polymerization of a single species of flagellin, which is encoded by a single copy gene (255). Another group of bacteria produce flagella filaments composed of two or more species of flagellin, and contain multiple flagellin genes. These "complex" filaments have been described for species of *Campylobacter* (132), *Helicobacter* (183), *Caulobacter* (372), *V. anguillarum* (232), *Rhizobium* (285), and *Halobacterium halobium* (117). Some organisms have flagella filaments that are covered by a sheath which can be membranous, as the case with *Vibrio* (322) and *Helicobacter* (116), or proteinaceous as in the Spirochetes (247).

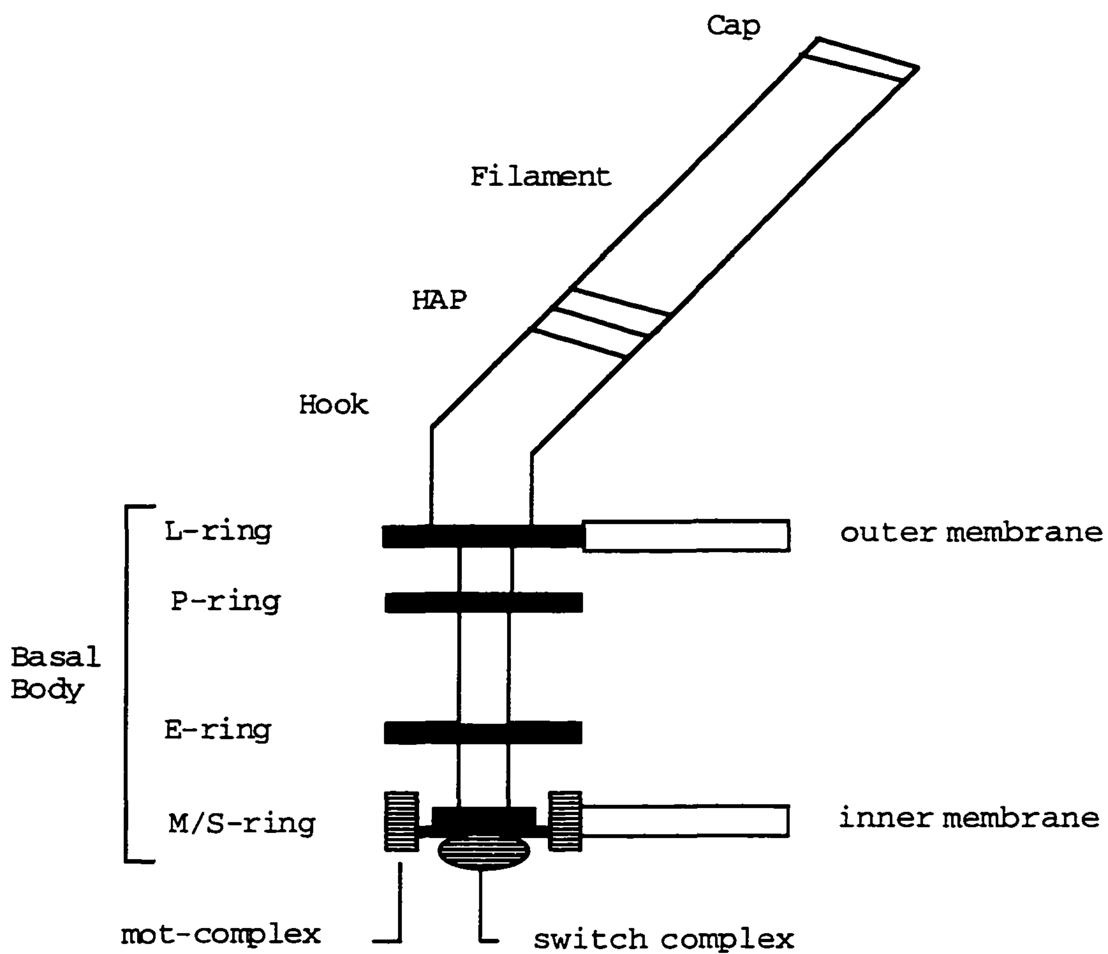


Figure 11. General structure and substructure of the flagellum of a Gram-negative bacterium.
The cellular localization of the three structural components of the flagellum, the basal body, hook and filament are indicated.

Role of flagella in pathogenesis

The process of bacterial colonization can be considered in three distinct steps. Firstly, the bacteria needs to reach the target site within the host, then it needs to colonize the site and finally establish and proliferate either at the site or after invasion into the site. Logically, motility should be an advantage for a bacterium to reach its preferred site in the host, particularly within animal hosts where some surfaces are covered by a film of dense mucus which the pathogens have to swim through to avoid getting trapped (133, 196). Chemotaxis-directed motility is likely to be very important in pathogenesis, since it would enable bacteria to move towards favourable environments and away from unsuitable sites. The contribution of motility to disease can best be established by comparing the disease caused by motile wild type strains and non-motile mutants.

Using a burned mouse model of infection, the flagella of *P. aeruginosa* was shown to be essential for invasion, since non-motile mutants could proliferate in the wound, but could not cause the characteristic systemic infection associated with this organism (85). Other studies using different flagella mutants of *V. anguillarum* showed that the FlaA, either as part of the motility organelle or in association with some other virulence mechanism, was essential for invasion of the fish host (245). The flagella of *V. anguillarum* also played a role in the dissemination of the pathogen within fish after the initial crossing of the integument (245).

Some studies have reported that the flagellar organelle also contributes to pathogenesis by facilitating adhesion of bacterial pathogens to host cell surfaces (15, 167, 235, 378). For example, the flagella of *C. jejuni* was reported to play a significant role in adherence to epithelial cell lines (235), and in the internalization of the organism into the cultured cells (128). On the other

hand, analysis of several *L. pneumophila* flagella mutants revealed that although the flagellar structure was not essential for virulence, the ability of *L. pneumophila* to infect amoebae and human phagocytic cells appeared to be linked to flagellar expression (292), suggesting a role for motility. In other studies using *V. cholerae* flagella mutants, it was suggested that either motility (131), or an adhesin which was proposed to be associated with the flagella (15, 167) was needed for the colonization of intestinal tissues. However, a later study using different *V. cholerae* flagella mutants suggested that motility rather than the flagellar structure itself was essential for the colonization of rabbits, although the sheath of the flagella may play a minor role in virulence (298). In studies such as these in which Fla mutants are used to determine the role of flagella as an adhesin, the effect of lack of motility needs to be established. Thus the role of flagella in adhesion may best be investigated by comparing flagellated non motile bacteria against aflagellate non motile mutants.

Flagella are often highly immunogenic and elicit an immune response in the host. This property may be more of an advantage to the host than to the bacteria since it enhances the ability of the host's immune system to clear the invading microorganism. For example, infection by *P. aeruginosa* can be inhibited using antiflagella antibody (193). However bacteria have developed mechanisms to evade the immune response. Some species such as *C. jejuni*, can undergo flagella phase variation such that they can exist as either flagellated or aflagellated (342). In such situations, immune response elicited against the flagella selects for the aflagellate transformants. Others such as *C. coli* VC167 can undergo antigenic variation in which they can produce flagella of different antigenic types (342). Immune response elicited against each antigenic type selects for transformants exhibiting the new antigenic

type. In these situations, the immunogenic property of flagella may be considered an advantage for the bacterium since both antigenic and phase variation of flagella can prolong the survival of the invading pathogens within the host and probably produce significant stress to the host.

Flagellation of psychrophilic aeromonads

As discussed previously the psychrophilic aeromonads have always been easy to differentiate from the mesophilic aeromonads, not only due to their markedly different optimal growth temperatures, but also because of their apparent lack of motility. Despite this, there have been a few reports suggesting that some strains of *A. salmonicida* and *A. media* may exhibit motility (16, 44, 199, 234). Four isolates from old cultures of *A. media* were reported to possess either a single polar flagella or groups of two or three polar flagella by day 28 following repeated subculturing on TSA (16). McIntoch and Austin reported that incubation of *A. salmonicida* at supra-optimal temperatures (30-37°C) and in broth medium with high viscosity resulted in the expression of motility by polar flagella in about 1% of the cells (234). These findings have remained unconfirmed.

RESULTS

Cloning and sequencing of *A. salmonicida* A449 *fla*A, *fla*B and *fla*G

A fragment of the *fla*A gene of *A. salmonicida* A449 was initially cloned serendipitously in pBluescript on an approximately 2.8 kb *Pst*I fragment (pBSKfla) (Fig. 12). The insert in pBSKfla was sequenced from both ends with FUP and RUP and the data bases were searched with the sequences obtained. The search results showed that the amino acid sequence translated from the RUP-derived nucleotide sequence was highly homologous to the flagellin proteins of other bacteria (data not shown). The homology search also suggested that the cloned gene was truncated, missing a few bases from the 3' end. The 2.8 kb *Pst*I fragment from pBSKfla was therefore used as probe on an *A. salmonicida* A449 chromosomal digest in order to clone an approximately 8 kb *Bam*HI fragment in pUC18 (pMG1) containing the full length *fla*A (Fig. 12). Further sequence analysis of pMG1 revealed another ORF (*fla*B) 582 bp downstream of *fla*A, which encodes a second flagellin protein. A 2.3 kb *Hin*dIII/*Bam*HI fragment was subcloned from pMG1 into pUC18 to construct the clone pMG2 which contains *fla*A (Fig. 12). A 2.5 kb *Hin*dIII/*Cla*I fragment of pMG1 which contained *fla*B was subcloned into pUC19 (pMG3) (Fig. 12). The complete nucleotide sequences of *fla*A and *fla*B were then obtained using RUP, FUP and custom designed oligonucleotides and pMG2 and pMG3 as the sequencing templates.

The *fla*A gene with an ORF of 915 bp, encodes a protein with 305 amino acids and a predicted M_r of 32,351, while *fla*B with an ORF of 909 bp, encodes a protein with 303 amino acids and a predicted M_r of 32,056 (Fig 13). The two A449 flagellin genes share 79% nucleotide sequence identity. The G+C contents of 52% and 53% for *fla*A *fla*B, respectively, are only slightly

lower than the reported G+C content of 55% for the *A. salmonicida* genome (30). Putative SD sequences are positioned 7 bps upstream of the ATG start site of *fla*A and 9 bp upstream of *fla*B, and there are also several potential σ^{28} and σ^{54} promoter sequences upstream of both genes. The predicted σ^{28} promoter sequence TAAA-N_{15}-GCCGTTAA is identical to that of the *V. anguillarum* *fla*B gene (232).

In the case of *flaA*, the nucleotide sequence AGGCCGGTTTTCCGGC CT 180 nucleotides downstream of the TAA stop codon could provide for a strong termination stem-loop structure with a ΔG value of -18.6 kcal. No terminator encoding sequence is apparent 3' of the *fla*B stop codon, although a weak predicted stem-loop encoding sequence, TCCCTGCTGGGTTAACAGG CA, encompasses the *fla*B stop codon. Beginning 35 bp downstream of *fla*B (Fig. 13) is a 441 bp ORF which encodes a 146 residue protein of predicted M_r 15,965 with homology to FlaG of *P. aeruginosa* (367), *V. anguillarum* (245) and *V. parahaemolyticus* (226). *fla*G has a G+C content of 51.5%, consistent with that of the *fla*A and *fla*B genes. The *fla*G gene also terminates at a TAA stop codon. The exact role played by the FlaG protein in flagella morphogenesis is unknown (226, 232).

Another partially characterized ORF (*fla*H, Fig 12) whose deduced amino acid sequence has homology to the Hook-Associated Protein II (HAP-II) of other bacteria occurs immediately downstream of *fla*G. There are no obvious consensus promoter sequences in the nucleotide sequences immediately 5′ of either *fla*G or *fla*H. In addition, there are no obvious termination loop sequences located downstream of either *fla*B or *fla*G, suggesting that transcription initiated at the *fla*B promoter region continues through *fla*G and into *fla*H. The predicted strong termination loop structure located downstream of *fla*A suggests that transcription initiated at the *fla*A

operator region terminates after *fla*A expression and does not read through into the rest of the downstream genes.

In *V. parahaemolyticus*, the order of the flagellin gene arrangement are identical to those of *A. salmonicida* A449. Thus the two flagellin genes are tandemly located next to the *fla*G, which in turn is next to *fla*H encoding the HAP-II.

Figure 12. Linear maps of the constructs containing the cloned *A. salmonicida* A449 *fla* genes. Transcription direction as determined by nucleotide sequencing is indicated by the arrows in *flaA*, *flaB*, *fla*G and *fla*H. P, *Pst*I; B, *Bam*HI; Bs, *Bsp*EI; C, *Cla*I; H, *Hin*dIII, Hp, *Hpa*I, A, *Afl*II. FL7, FL8 and FL10 are oligonucleotides described in the text.

Figure 13. The coding and upstream nucleotide sequences of the *fla*A, *fla*B and *fla*G genes of *A. salmonicida* A449.
The deduced amino acid sequences are shown below the coding regions. The putative SD ribosome binding sites of the three genes are highlighted in bold while the TAA stop codons are indicated by the star sign (*). The predicted consensus σ^{28}-type promoter sequences TAAA-N_{15}-GCCGTTAAAT and TAA-N_{15}-GCCGATAAAT upstream of both *fla*A and *fla*B are single underlined while the possible σ^{54}-type consensus promoter sequence (GG-N_{10}-GC) are double underlined. The underlined sequence AGGCCGGTTTTCCGGCCT flanked by broken arrows pointing in opposite directions 180 nucleotides downstream of *fla*A encodes a predicted termination loop structure. FL10, FL8 and FL7 are oligonucleotide primers described in the text. The unique RE sites for *Afl*II, *Bsp*EI, *Hin*dIII and *Hpa*I are indicated.

```
          GGTTACCGCCGCCAGTACCATGACGCCGAAGGCATCCATCCTGAGCCGCCCTGCC   55
                                    FL8----------->
ACCAGGACGCCCGAAAACGCGAATACCGCAGTACCAAACATGTCGCTGATGTAAACAATC   115

TGCTCAACCAGTGTCTGCATGGAGAACAACTCACGTCCAATTGTTGATGGGGTGCGATTT   175

TAGGGGGTGGACAGGCTTCTGACCAATGATGGCCTGCAAAAATGAGAGCCAGCCCCAGTT   235

TTTGCAAACGGGATGTGGGTGCGCGGATGAGCGCTGACACCTTGCAGGTCAGGGCACTTG   295

CTTTGCCATGGCCTTTGGCCTGTGCGGCATCCTTCTGATAAGGGGAACTTTTTGGATTTT   355
   AflII                                          F17---------->
TTCTCTTAAGTAAAACGGGAATGTGCCGATAAATAGGGTAAGCGGCGTTAATGCGATGAA   415

GCGTTAAAAATAATGCTAAAGACATCGAAAAAGAGGCCGTTAAATAAGACGCATCGGACA   475
                                                 FL10---------->
GGAAAGACAATAAAGTCCGACCAAATTAGATAGTAAATTTAATAGCTTAGGAGAATGGTT   535
flaA
ATGGCACTTTATATCAATACGAACGTTTCATCGCTTAACGCCCAGCGTAACCTGATGAAC   595
M  A  L  Y  I  N  T  N  V  S  S  L  N  A  Q  R  N  L  M  N

ACCAACAAATCTCTGGATACTTCTTACACCCGTCTGGCATCTGGTCTGCGCATCAACAGC   655
T  N  K  S  L  D  T  S  Y  T  R  L  A  S  G  L  R  I  N  S

GCAAAAGACGATGCGGCAGGTCTGCAAATATCCAACCGTTTGACCTCCCAGATCAATGGA   715
A  K  D  D  A  A  G  L  Q  I  S  N  R  L  T  S  Q  I  N  G

CTGGATCAAGGTAACCGTAACGCCAACGATGGCATTTCCGTTGCCCAGACCGCAGAAGGT   775
L  D  Q  G  N  R  N  A  N  D  G  I  S  V  A  Q  T  A  E  G

GCCATGGATGAAGTGACCTCCATGCTGCAACGGATGCGGACCCTTGCTCAGCAATCTGCC   835
A  M  D  E  V  T  S  M  L  Q  R  M  R  T  L  A  Q  Q  S  A

AACGGTTCCAACAACACCGATGACCGTACCGCTTTGCAGCAAGAGTACGACCAATTGATG   895
N  G  S  N  N  T  D  D  R  T  A  L  Q  Q  E  Y  D  Q  L  M

ACCGAGATAGATCGTGTCGCCGGTGATACTACATTTGGCGGCCAGAATTTGCTTAATGGT   955
T  E  I  D  R  V  A  G  D  T  T  F  G  G  Q  N  L  L  N  G
                                                     BspEI
GGGTATGAAGGGACTTTCCAAGTGGGTGCTGATGCGGGTCAGACCATTACTTTCCGGATG   1015
G  Y  E  G  T  F  Q  V  G  A  D  A  G  Q  T  I  T  F  R  M

ACCACTGCCTTTACCATATCTGGTATGGCATCTGCTACTAAGGGGAATGCAGTAGTTGCA   1075
T  T  A  F  T  I  S  G  M  A  S  A  T  K  G  N  A  V  V  A

GCCACGACCGCAGGAAGCAAAGAACCGTATAAAGTCACGCCTTCGACTACGGGGAATGTT   1135
A  T  T  A  G  S  K  E  P  Y  K  V  T  P  S  T  T  G  N  V

GCTGTTGCGGACATGAACAGTATCACCGCTGCCAGCTCAGCCCAGTCTGCCATGGCTAAC   1195
A  V  A  D  M  N  S  I  T  A  A  S  S  A  Q  S  A  M  A  N

CTGGACTACATGATCAAGGTGGTGGACAGCAAGCGTGCGGAATTGGGTGCGGTACAGAAC   1255
L  D  Y  M  I  K  V  V  D  S  K  R  A  E  L  G  A  V  Q  N

CGGTTTGACTCGACCATCCGCAACCAAGCGAACATCTCCGAGAACGTCAATGCAGCTCGC   1315
R  F  D  S  T  I  R  N  Q  A  N  I  S  E  N  V  N  A  A  R
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TCACGTATTCGTGACGCAGACTTCGCGACCGAAACGGCCAACCTGACCAAGCAAAACATT   1375
S  R  I  R  D  A  D  F  A  T  E  T  A  N  L  T  K  Q  N  I

TTGCAACAGACTGCATCCACCATACTGTCACAGGCAAACCAGCGTCCTCAGTCTGCCCTC   1435
L  Q  Q  T  A  S  T  I  L  S  Q  A  N  Q  R  P  Q  S  A  L

TCGTTGCTGCAGGGCTAACAAAAGAAAACAGGGCGGTTGTTCAGGCCGCCCTGGTCAAGA   1495
S  L  L  Q  G  *

GATGGAACAAAGGGGTGTTCGGTTTGCACTCTGTTCTATCGGGAGAGAATCAATTCTCTA   1555

TATTAGTAATGCCAAGTGTTGTACAGCTGTTCAGTTCTGTCATCCTTGGTCATATTCTCC   1615

TGAGCGTATGCTCATTTTAGGCCGGTTTTCCGGCCTCTTTTTATTGTGCCGTCAAATTGA   1675
    --------->            <---------
CGCCACTCCCCCATATTCAAGCACAAAGCGTGCCAAAAGCAACCAACGCCGCTATCCGGT   1735

TCCTCGACCAACTCTAGACCCCCTGGCTATGCTCCTGTCCTCTTTCCCTTTGCCGCCTGC   1795
                                    HindIII
CGGATAAAATTGCCACCTTCGGCAATTATTTTCCTAAAGCTTGTGTCCCTGCAGCCGTTA   1855

AATAAAGTGAAGCAATCGAGGGGTGTTCGGAATCCAACGCTTGCACTACTGCGGGTGGCC   1915

TGTTCGTTCAAACAGACACCCGCCAGAAGACACAGGATTGTTCAACCTGTTCTTCTAACG   1975

GTGGGACGTCGCTTTGTCGGCGCAACCCGCAGTCGCATGTTCTTCGGGAGAATATTTTT    2034
flaB
ATGGCCATGTACATCAACACGAATACTTCATCACTGAACGCCCAGCGTAACCTGATGAAT   2094
M  A  M  Y  I  N  T  N  T  S  S  L  N  A  Q  R  N  L  M  N

ACCAACAAGTCCTTGGATACCTCCTATACCAGACTGGCCTCCGGTTTGCGTATCAACACC   2154
T  N  K  S  L  D  T  S  Y  T  R  L  A  S  G  L  R  I  N  T

GCCAAGGACGATGCAGCAGGTCTGCAGATCTCCAACCGTTTGACCTCACAAGTCAACGGT   2214
A  K  D  D  A  A  G  L  Q  I  S  N  R  L  T  S  Q  V  N  G

CTGGACCAAGGTAACCGCAACGCCAACGATGGCATCTCTGTTGCTCAGACCGCTGAAGGC   2274
L  D  Q  G  N  R  N  A  N  D  G  I  S  V  A  Q  T  A  E  G

GCCATGGATGAAGTCACTTCCATGCTGCAGCGTATGCGTACTCTGGCTCAGCAATCAGCC   2334
A  M  D  E  V  T  S  M  L  Q  R  M  R  T  L  A  Q  Q  S  A

AACGGTTCCAACAACACTGATGACCGTACTGCGTTGCAGCAAGAGTATGATCAGTTGACC   2394
N  G  S  N  N  T  D  D  R  T  A  L  Q  Q  E  Y  D  Q  L  T

ACAGAGATCGATCGTATCTCCAGCGATACTACCTTTGGTGGCCAAAAACTGCTGGATGGT   2454
T  E  I  D  R  I  S  S  D  T  T  F  G  G  Q  K  L  L  D  G

AAGTACAAAGGTTCCTTCCAGGTCGGTGCCGATGCTGGTCAGACCATAACCTTCAAGATG   2514
K  Y  K  G  S  F  Q  V  G  A  D  A  G  Q  T  I  T  F  K  M

ACGAGCGCTTTCACTATCAGTGGTATTGCCGCCGCCTCCAAGGGTAGTGCAACCGTTGCA   2574
T  S  A  F  T  I  S  G  I  A  A  A  S  K  G  S  A  T  V  A

GCCACTACGGCAGGTAGCAAAGAGCCCTATAAAGTAACATCTGGGACAACACCGGTGACC   2634
A  T  T  A  G  S  K  E  P  Y  K  V  T  S  G  T  T  P  V  T
```

```
GTCGCCAACATGAATAGCATCAAGGCGGCGAGTTCTGCTCAATCGGCCATGGCCAACCTT   2694
V  A  N  M  N  S  I  K  A  A  S  S  A  Q  S  A  M  A  N  L

GACTTCATGATCAAGGCGGTTGACAGCAAACGGGCAGAGCTGGGTGCGGTGCAAAACCGG   2754
D  F  M  I  K  A  V  D  S  K  R  A  E  L  G  A  V  Q  N  R

TTTGACTCGACCATTCGCAACCAGTCCAACGTCTCTGAAAACCTGAGTGCAGCACGCTCT   2814
F  D  S  T  I  R  N  Q  S  N  V  S  E  N  L  S  A  A  R  S

CGTATTCGCGACGCGGACTTTGCGACTGAAACAGCCAACCTGACCAAGCAGAACATTCTG   2874
R  I  R  D  A  D  F  A  T  E  T  A  N  L  T  K  Q  N  I  L

CAACAGGCGGCCTCCAGCATCTTGGCGCAAGCCAACCAAAGACCGCAGTCTGCCCTGTCC   2934
Q  Q  A  A  S  S  I  L  A  Q  A  N  Q  R  P  Q  S  A  L  S

    303 HpaI                                    flaG
CTGCTGGGTTAACAGGCAGTGATGAGATAGAAAGAGGAGCGACGATATGGCCAATGACATT   2995
L  L  G  *                                     M  A  N  D  I

GGAATACCCTCATCGGTCACTCCTTCTTCTCTTCAGTCAAGTAGCAAGAGCGGCCAATCG   3055
G  I  P  S  S  V  T  P  S  S  L  Q  S  S  S  K  S  G  Q  S

GCCGCTCAAGTGCTATCTGGCACGACGGAAGAGCAGCGGGTGATGGCACAGGATGTGAAG   3115
A  A  Q  V  L  S  G  T  T  E  E  Q  R  V  M  A  Q  D  V  K

AAACAGCAAGAGGAGAAGGCACAACCAAAAGCCAGCAAACAGGATATTTCTGAGGTTGAT   3175
K  Q  Q  E  E  K  A  Q  P  K  A  S  K  Q  D  I  S  E  V  D

ATTGAAAAGGAGGTCCAAAACTTGCAAGAGTTCAGTAAGTTGCAAGGATGGACTGTCAAC   3235
I  E  K  E  V  Q  N  L  Q  E  F  S  K  L  Q  G  W  T  V  N

TTCAGTGTCGAAAAGGATCTCGAGCAGGTTGTCATCAAGGTGATGGATGCCCACACCAAG   3295
F  S  V  E  K  D  L  E  Q  V  V  I  K  V  M  D  A  H  T  K

TCGATGATCCGCCAGATCCCGAGCGAAGAGTTACTGGCCATCAGCAAACGGATCAAGGAT   3355
S  M  I  R  Q  I  P  S  E  E  L  L  A  I  S  K  R  I  K  D

CTGCGCGAAGGTGATGCGACCGGCGGAGGTTCCCGTGTCGGGCTGCTGCTCGATAACGAG   3415
L  R  E  G  D  A  T  G  G  G  S  R  V  G  L  L  L  D  N  E

ATCTAATACGCAAGCATAA                                            3434
I  *
```

Comparison of FlaA with FlaB and other bacterial flagellins

The two *A. salmonicida* flagellins share an overall 88% amino acid sequence identity. Of the 23 residue substitutions in FlaB, eight are conservative replacements. Seventeen of the substitutions between residues 140 and 223 lie in the central domain (Fig. 14). The most notable compositional difference predicted between the two proteins is in their lysine content, FlaA having eight and FlaB having thirteen. The different content of this basic amino acid presumably contributes to the different predicted pIs of the two proteins, FlaA having a predicted pI of 5.03 while the predicted pI of FlaB is 9.68. Consistent with other flagellins, cysteine, histidine and tryptophan are absent from the deduced *A. salmonicida* flagellin sequences.

Both *A. salmonicida* flagellins display extensive amino acid sequence homology to other bacterial flagellins at the N- and C-termini (Fig. 14). The flagellins most similar in molecule length and sequence homology to those of *A. salmonicida* are the flagellins of *P. aeruginosa* and *V. anguillarum*. At the N-terminus, the first 150 amino acid residues of *A. salmonicida* FlaA show a 60% identity with *P. aeruginosa* flagellin (353) and a 55% sequence identity with *V. anguillarum flaA* flagellin (245). At the C-terminus, the last 98 amino acid residues of *A. salmonicida* FlaA exhibit a 53% sequence identity with the last 97 amino acids of *P. aeruginosa* flagellin and a 55% sequence identity with those of *V. anguillarum*. Predictably, the principal sequence divergences between these three flagellins occurs in the central regions of the various molecules. Comparison of the sequence between the first 150 and last 98 amino acids residues of *A. salmonicida* FlaA with those between the first 150 and last 97 amino acid residues of the *P. aeruginosa* and *V. anguillarum* flagellins reveals only 10% sequence identity with the *P. aeruginosa* protein and 9% identity with the *V. anguillarum* protein.

Figure 14. Alignment of the deduced amino acid sequences of the *A. salmonicida* A449 flagellins with the flagellins of selected bacteria.
A. salmonicida A449 FlaA (AsA) and FlaB (AsB) flagellins, *P. aeruginosa* PAk (Pa) (353), *V. anguillarum* (Va) (245), *S. fremantle* (Sfr) (205), *E. coli* strain U5-41 (Ec) (313), and *Shigella flexneri* Sf (351). Hyphens indicate gaps introduced to facilitate sequence alignment and residues identical to those of A449 FlaA are indicated as colons (:). Residue number is indicated on the right.

```
AsA  MALYINTNVSSLNAQRNLMNTNKSLDTSYTRLASGLRINSAKDDAAGLQI   50
AsB  ::M:::::T::::::::::::::::::::::::::::::T::::::::::   50
Pa   :::TV:::IA:::T::::N:SSA::N::LQ::ST:S::::::::::::::   50
Va   :TITV:::::AMT:::Y:NKATGE:N::ME::S::NK:::::::::::::   50
Sfr  ::QV::::SL::LT:N::NKSQS::SSAIE::S::::::::::::::QA:   50
Ec   ::QV::::SL::IT:N:INKNQSA:SS:IE::S::::::::::::::QA:   50
Sf   ::QV::::SL::IT:N:INKNQSA:SS:IE::S::::::::::::::QA:   50

AsA  SNRLTSQINGLDQGNRNANDGISVAQTAEGAMDEVTSMLQRMRTLAQQSA  100
AsB  :::::::V::::::::::::::::::::::::::::::::::::::::::  100
Pa   A::::::V:::NVATK:::::::L:::::::LQQS:NI:::::D:SL:::  100
Va   :::::A:SR:::VAM::::::::I::::::::N:S::I:::::D::L:::  100
Sfr  A::F::N:K::T:AS::::::::I:::T:::LN:INNN:::V:E:SV:AT  100
Ec   A::F::N:K::T:AA::::::::::::T:::LS:INNN:::I:E:TV:AS  100
Sf   A::F::N:K::T:AA::::::::::::T:::LS:INNN:::I:E:TV:AS  100

AsA  NGSNNTDDRTALQQEYDQLMTEIDRVAGDTTFGGQNLL------------  138
AsB  :::::::::::::::::::T:::::ISS:::::::K::------------  138
Pa   ::::SDSE::::NG:AK::QK:L::ISNT:::::RK::------------  138
Va   ::T:SASE:Q::DE:STA:QD:LN:I:ET:S:::RK::------------  138
Sfr  ::T:SDS:LKSI:D:IQ:RLE:::::SNQ:Q:N:VKV:SQDNQMKIQVGA  150
Ec   T:T:SDS:LDSI:D:IKSRLD:::::S:Q:Q:N:V:V:AKDGSMKIQVGA  150
Sf   T:T:SDS:LDSI:D:IKSRLD:::::S:Q:Q:N:V:V:AKDGSMKIQVGA  150

AsA  -NG-------------GYE-GTFQV-GADAGQTIT---------------  157
AsB  -D:-------------K:K-:S:::-:::::::::---------------  157
Pa   -D:-------------SFGVAS:::-:SA:NEI:SVGIDEMSAE--SLNG  171
Va   -::-------------SFGEAS::I-::SS:EA:IMGLTSIRAD--D---  168
Sfr  ND:ETITIDLQKIDVKSLGLDG:N:N:P-----KEATVGDLKSSFKNVTG  195
Ec   ND:ETITIDLKKIDSDTLGLNG:N:N:KGTITNKAATVSDLTSAGAKLNT  200
Sf   ND:QTITIDLKKIDSDTLGLNG:N:N:GG:VANTAASKADLVAANATVVG  200

AsA  -------------FR------------MTTA-----FTIS-G----MASA  172
AsB  -------------:K------------::S:-----::::-:----I:A:  172
Pa   T---Y--------:KAD------GGGAV:A:TASGTVD:AI:----ITGG  200
Va   -------------::MG------GQSFLAEQGKDKDW----:----VPAG  191
Sfr  YDT-YA--AGADKY:VD-----INSGAVV:-------DAAAPDKVYVNA:  230
Ec   TTGLYGLKTENTLLTTDAAFDKLGNGDKV:VGGVDIYNAKS:DFTTTK:T  250
Sf   NK--YTVSAGYDAAKASDLLAGVSDGDTVQ:TINNG:GTAASATNYKYDS  248

AsA  T--------KGNAV----------VAAT----------------------  182
AsB  S--------::S:T----------::::----------------------  182
Pa   SAVNVKVDM:::ETA---EQAAAKI::AVNDANVGI--------------  233
Va   -ARDLKFEFT-TQAG---ELVTLD:L:KDGDDIEEL--------------  222
Sfr  NG---QLTTDDAENNTAVDLFKTT------KSTAGTAEAKAIAGAIKGGK  271
Ec   AGTGVDAAAQATDSAKKRDALAATLH:DVGKSVNGSYTTKDGTVSFVTDS  300
Sf   ASKSYSFDTTTASAADVQKYLTPG:-GDTAKGTI-TIDGSAQDVQISSDG  296
```

```
AsA   ----TAGSKEPY--K-----------VTPST-------TGNVAVADMNSI   208
AsB   ----::::::::--:-----------::SG:-------:P-:T::N::::   207
Pa    -GAFSD:DTIS:VS:AGK---DGSGAI:SAVSGVVIAD::STG:GTAAGV   279
Va    -ATYIN:QTDKLKASVDQ---DGKLQIFAAEPNLQGDLAISGGL:TELGL   268
Sfr   EGDTFD-----:-----------KGVSFTIDTKA----GNDGNGTVSTT:   301
Ec    AGNI:I:GSQA:VDDAGNLTTNNAGSARKADMKALLKAASEGSDGASLTF   350
Sf    K-ITASNGDKL:IDTTGRLTKNGSGASLTEA--SLSTLAA:NTK:TTID:   343

AsA   TAASSA----QSA-----------------------------------MANL   221
AsB   K:::::----:::-----------------------------------::::   220
Pa    APSAT:FAKTNDT-----------------------------------V:KI   296
Va    NGGPG--VKT--T-----------------------------------VQDI   281
Sfr   NGEKVTLT---------VADITAGA----ADVNAATLQSSKNVYTSVV:G   338
Ec    NGTEYTIAKATP:TTSPVAPLIPGGITYQATVSKDVVLSETKAAAATSSV   400
Sf    GGT:ISFT-GN:TTPDTITYSVTGAKVDQAAFDKAV-------STSGN:V   385

AsA   DY------------------------------------------------   223
AsB   :F------------------------------------------------   222
Pa    :ISTAK--------------------------------------------   302
Va    :ITSVG--------------------------------------------   287
Sfr   QFT---FDDKTKNESAKLSDLEANNAVKGESKITVNG--AEYTANATGDK   383
Ec    TFNSGVLSKTIGFTAGESSDAAKSYVDDKGGITNVADYTVSYSVNKDNGS   450
Sf    :FTTAGYSVN-GTTGAVTKGVDSVYVDNNEALTT-SD-TVDFYLQ-DDGS   431

AsA   ------------------------------------------M-------   224
AsB   ------------------------------------------:-------   223
Pa    -----------------------------------------ALSRRAGDR   311
Va    -----------------------------------------GSQNAVGVL   296
Sfr   ITLAG-------KTMF---IDKTA-SGVSTLINEDAAAAKKSTANPLAAI   422
Ec    VTVAGYASATDTNKDYAPAIGTAVNVNSAGKITTETTSAGSATTNPLAAL   500
Sf    VTNGS---------------GKAVYKDADGKLTTDAETKAATTADPLKAL   466

AsA   ---IKVVDSKRAELGAVQNRFDSTIRNQANISENVNAARSRIRDADFATE   271
AsB   ---::A:::::::::::::::::::::S:V:::LS:::::::::::::::   270
Pa    TTA::QI:ASVPTSV:::::::N::N:LK::G:::S:::G::E:T:::A:   361
Va    DAALRY:::Q::::::K:::LSHS:N:L:::Q:::E:SN:::K:T:::K:   346
Sfr   DSALSK::AV:SS:::I::::::A:T:LG:TVT:L:S:::::E:::Y:::   472
Ec    DDA:SSI:KF:SSV::I:::L::AVT:LN:TTT:LSE:Q:::Q:::Y:::   550
Sf    DEA:SSI:KF:SS:::::::L::AVT:LN:TTT:LSE:Q:::Q:::Y:::   516

AsA   TANLTKQNILQQTASTILSQANQRPQSALSLLQG                   305
AsB   :::::::::::A::S::A:::::::::::::G-                    303
Pa    :::::NQV:::AGTA::A::::L:::V::::R-                    394
Va    :TQM::AQ::::AGTS::A::K:L:N::M:::::-                  379
Sfr   VS:MS:AQ::::AGTSV:A::::V::NV::::R-                   505
Ec    VS:MS:AQ:I::AG:SV:AK:::V::QV::::::                   584
Sf    VS:MS:AQ:I::AGNSV:AK:::V::QV::::::                   550
```

FlaG protein comparison

Unlike the flagellin proteins, the FlaG proteins of the species *Aeromonas, Vibrio* and *Pseudomonas* have less amino acid sequence identities. The *A. salmonicida* A449 FlaG is only 28% identical to that of *P. aeruginosa,* 16% identical to *V. anguillarum* FlaG and 21% identical to *V. parahaemolyticus* FlaG (Fig. 15). Comparison of the *V. parahaemolyticus* and *P. aeruginosa* FlaG proteins revealed only 32% identities. However the size of *A. salmonicida* FlaG (146 amino acids) is closer to that of *V. parahaemolyticus* (144 amino acids), while *V. anguillarum* and *P. aeruginosa* FlaG consists of only 134 and 91 amino acids, respectively.

```
As    MANDIGIPSSVTPSSLQSSSKSGQSAAQVLSGTTEEQRVMAQDVKKQQEE   50
Pa    :-----------------------------------::-------:PLP:    8
Vp    :EIS-SYA:NIQ:YGTPNGTNVANKNGNGIGTPSTASSTGDVSPQ:AKGT   49
Va    :EIP-SYT:NIQ:YGS::GI:FASEND---GA:RASSKQNEVNRTE:LRN   46

As    KAQPKASKQDISEVDIEKEVQNLQEFSKLQGWTVNFSVEKDLEQVVIKVM  100
Pa    VTASREASESRDDLGL--A:SDI:S:VQSVKRNL:::IDDSSG:::V::I   56
Vp    EHDFSVQAAIEMAESRQELNREER:KMVE:MNEFVS:IN:GVA-FRVDEE   98
Va    RQNQSVEAAIELAQQR:QINKSERAKMVE:MNEFIS:IN:::A-FRVDEE   95

As    DAHTKSMIRQIPSEELLAISKRIKDLREGDATGGGSRVGLLLDNEI      146
Pa    :GDSGEVV::::::V:KLAA:LD:VH-----------SV:FETRA        91
Vp    SGRDVVT:YETNTGDVIRQFPDEEL:VVLRRLAEHTANSG::VEKV      144
Va    SGRDVVT:YEASTGDIIRQIPNEEM:EVLRRLARQKDHS-------      134
```

Figure 15. Alignment of the deduced amino acid sequences of the *A. salmonicida* A449 FlaG with the FlaG proteins of *Pseudomonas* and *Vibrio* spp. *A. salmonicida* A449 *(As)*, *P. aeruginosa* (Pa) (254), *V. anguillarum* (Va) (245) and *V. parahaemolyticus* (Vp) (226). Hyphens indicate gaps introduced to facilitate sequence alignment and residues identical to those of A449 FlaA are indicated as colons (:). Residue number is indicated on the right.

The evolutionary relationship of the *A. salmonicida* A449 flagellins with selected bacterial flagellins

The phylogenetic relationship of the *A. salmonicida* flagellins to selected bacterial flagellins was also examined using an UPGMA tree comparison (256) (Fig. 16). Not surprisingly, given the similarity in molecule length and sequence homology, the *A. salmonicida* flagellins appear to be evolutionally closely related to the *P. aeruginosa* and *V. anguillarum* flagellins. The flagellins of each of these three genera appear to have evolved on the same branch as the M_r 28,500 flagellin of *Caulobacter crescentus*, but on a different branch to the longer flagellins of the Enterobacteriaceae and *Campylobacter* FlaA (132).

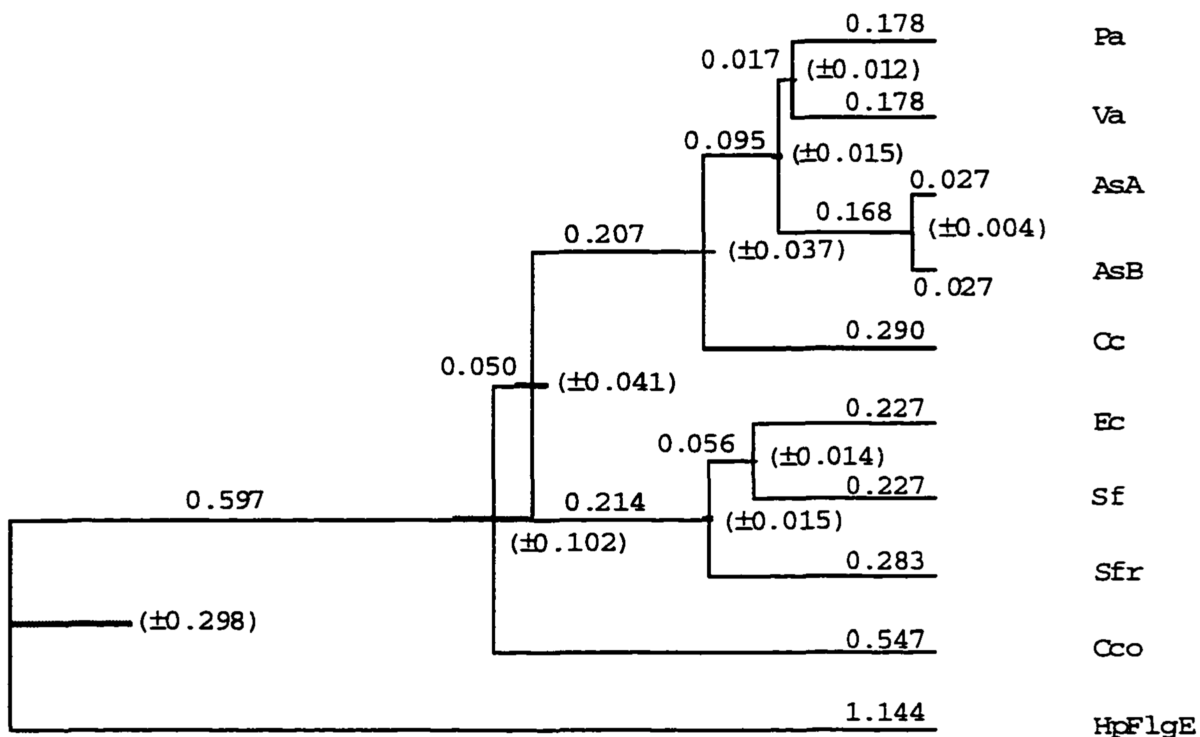


Figure 16. UPGMA tree showing the evolutionary relationship of the *A. salmonicida* A449 flagellin proteins to some selected bacterial flagellins. Numbers indicate the calculated evolutionary distances, while the numbers in parentheses indicate the standard error of the branch position. The bacterial flagellin proteins compared are A449 FlaA (AsA) and FlaB (AsB), *E. coli* strain U5-41 (Ec), *S. flexneri* (Sf), *S. fremantle* (Sfr), *P. aeruginosa* (Pa), *V. anguillarum* (Va), *C. crescentus* (Cc) (120), *C. coli* (Cco) (132). The hook protein FlgE of *H. pylori* (Hp FlgE) is included as an outlier.

Conservation of *fla*A and *fla*B in *A. salmonicida*

To determine whether the presence of two flagellin genes was a strain specific phenomenon, or was a more common property of *A. salmonicida*, total DNA was prepared from a range of typical and atypical strains of *A. salmonicida*. In addition, to evaluate the genetic relatedness of the *A. salmonicida fla* genes with those of the motile aeromonads, total DNA was also prepared from several strains of *A. hydrophila* and *A. veronii* biotype *sobria* (Table 2). The DNA was double digested with *Hin*dIII and *Bam*HI, separated on a 0.8% agarose gel and transferred to nylon membranes in duplicate, one to be probed with *fla*A, and the other with *fla*B. The *fla*A ORF was PCR amplified from pMG2 using the primer pair FUP and FL10 (GCTTAGGAGAATGGTTATGGC) and cloned into pGemT (pFL10*fla*A) (Fig. 12). The cloned DNA fragment from pFL10*fla*A which contains the *fla*A coding region and no upstream sequence was used as probe on the *fla*A blot. For the *fla*B blot, the probe was a PCR product containing only *fla*B sequence and no downstream sequence, and was obtained from pMG3 template using the primer pair RUP and FLB6 (GTTCTGCTTGGTCAGGTTGG). Both DNA probes were labeled with (α^{32}P)dCTP and Southern hybridization was performed under conditions of high stringency, with a 4.51% and 5.97% allowed mismatches for *fla*A and *fla*B probes respectively.

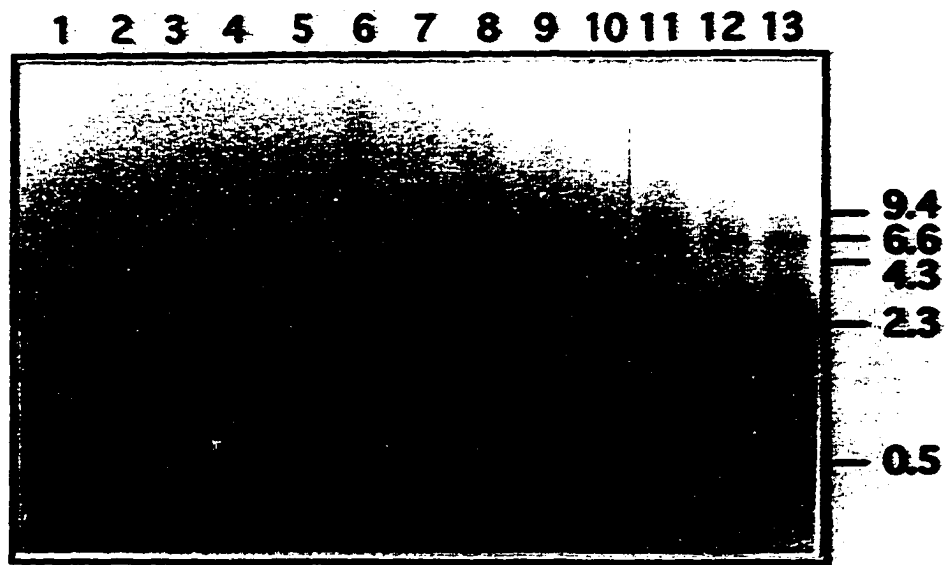
Figure 17 shows that *fla*A (Fig. 17a) and *fla*B (Fig. 17b) were conserved in all the typical and atypical strains of *A. salmonicida* examined. The *fla*A probe hybridized strongly to a DNA fragment of approximately 2.3 kb and weakly to a 6.0 kb and a 9.4 kb in all the typical strains. In the atypical strains A401, A402 and A404, the *fla*A probe bound strongly to a DNA fragment of approximately 3.6 kb and weakly to 6.6 kb band and in A460, to three fragments of approximately 5.5 kb (strong), 3.6 kb and 800 bp (weak). The

*fla*A probe also bound very weakly to DNA fragments from *A. hydrophila* strains Ah300 (5 kb and 800 bp) and TF7 (~800 bp), Ah598 (5.0 kb and 800 bp) and with a stronger hybridization signal to *A. veronii* biotype *sobria* strain A701 (~7 kb).

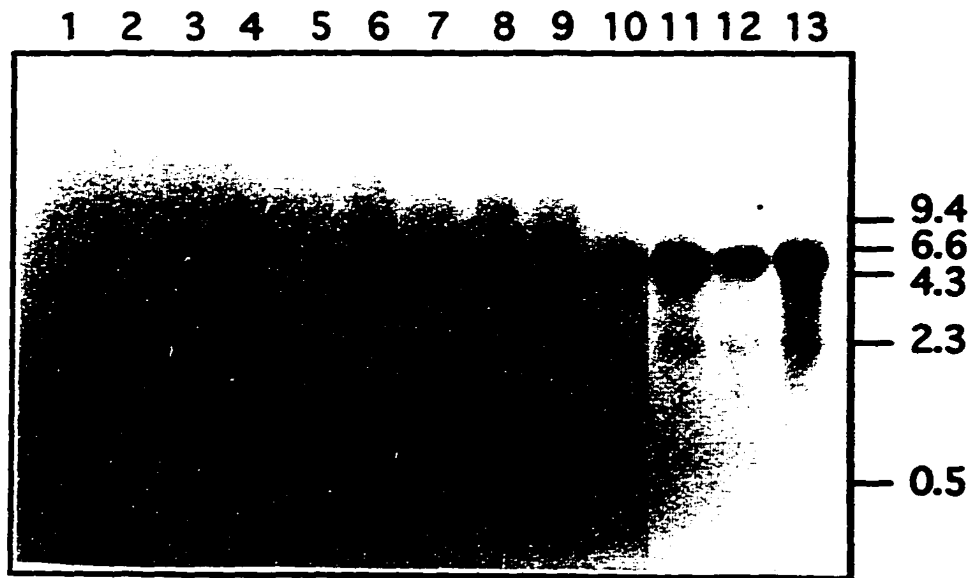
For the *A. salmonicida* strains, the hybridization pattern of *fla*B is essentially opposite to the pattern observed for the *fla*A blot. This means that the *fla*B probe hybridized strongly to the 6.0 kb DNA fragment and weakly to the 2.3 kb fragment in the typical strains. In DNA from some of the atypical strains A401, A402 and A404, the probe bound strongly to the 6.6 kb band and weakly to the 3.6 kb band, while in A460 *fla*B hybridized to two fragments of approximately 5.5 kb (weak) and 3.6 kb (strong). The *fla*B probe also hybridized weakly to DNA from *A. hydrophila* TF7 (~800bp) and Ah300 (~5.0 kb and 800 bp), Ah598 (5.0 kb and 800 bp) and to DNA from *A. veronii* biotype *sobria* A701 (~9.4 kb, 7 kb, 2.6 kb and 1.8 kb).

Figure 17. Autoradiograms of Southern blots of total DNA isolated from *Aeromonas* strains showing the conservation of *fla*A and *fla*B.
The DNA was digested with *Bam*HI and *Hin*dIII and the blots were probed with (α^{32}P)dCTP labeled 1.2 kb fragment containing A449 *fla*A (A) and a 1.1 kb PCR fragment containing A449 *fla*B (B). Blots were washed under high stringency conditions at 68°C in 1X SSC and 0.1% SDS. *A. hydrophila* strains Ah598 (lane 1); TF7 (lane 2); Ah300 (lane 3); *A. sobria* As701 (lane 4); and *A. salmonicida* strains A460 (lane 5); A404 (lane 6); A402 (lane 7); A401 (lane 8); A202 (lane 9); A440 (lane 10); A395 (lane 11); A251 (lane 12); A449 (lane 13). Numbers on right indicate size in kb.

17A



17B



***In vitro* expression of *fla*A and *fla*B**

To express *A. salmonicida* A449 FlaA, the oligonucleotide FL7 (GGCGTTAATGCGATGAAGCG) was designed to DNA located 137 bp upstream of *fla*A (Fig. 13) and the FL7/FUP primer pair was used in PCR to amplify an approximately 1.4 kb fragment containing *fla*A from pMG2. This fragment was ligated to pGEM-T to construct the clone pFL7*fla*A (Fig. 12). In order to express *A. salmonicida* FlaB, the *fla*B-containing insert from pMG3 was cloned into pGem to construct pMG3-gem. The two clones pFL7*fla*A and pMG3-gem had the *fla*A and *fla*B gene inserts in an orientation such that the 5' end of the genes were directly downstream of the SP6 promoter of pGem.

For *in vitro* transcription and translation, pFL7*fla*A and pMG3-gem were prepared by CsCl gradient centrifugation and expressed using the S30 Coupled Transcription Translation System with radioactive methionine. The autoradiograph of the translation (Fig. 18) shows the production of a protein of approximate M_r 39,000 from pFL7*fla*A (lane 2) and a protein of approximate M_r 38,000 from pMG3-gem (lane 3). Both flagellins migrated aberrantly in SDS-PAGE, displaying apparent M_rs higher than those predicted from their nucleotide sequences. A protein migrating at approximately M_r 16,000 in the lane with pMG3-gem (lane 3) could be the FlaG protein. No protein corresponding to these sizes were produced by the negative control (lane 1).

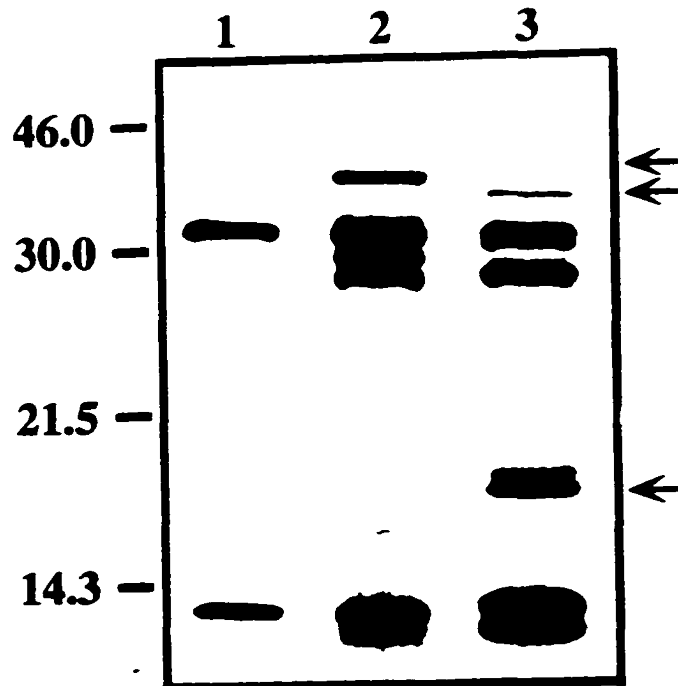


Figure 18. Autoradiogram of SDS-PAGE analysis of proteins obtained from *in vitro* transcription and translation of the *fla*A, *fla*B and *fla*G genes of *A. salmonicida* A449.
Lanes 1, pGem; 2, pFL7*fla*A; and 3, pMG3-gem. Arrows indicate FlaA (lane 2), FlaB and FlaG (lane 3) respectively. M_r markers (kD) are indicated on the left.

***In vivo* expression of *fla*A and *fla*B and antigenic cross-reactivity with *A. hydrophila* anti-flagellin antibody**

Our studies suggested that FlaA was poorly expressed from *in vivo* expression constructs, in contrast to FlaB. Comparison of sequences 5′ of *fla*A and *fla*B showed they were different except for the putative consensus σ^{28} promoter sequence (data not shown). To evaluate the possibility that the upstream sequence might be influencing the expression of *fla*A, we examined the effect of deleting the DNA upstream of *fla*A on FlaA production using three clones containing varying amounts of the *fla*A regulatory region. The clone pFL8*fla*A was constructed similarly to pFL10*fla*A and pFL7*fla*A, except the primer used in the initial PCR amplification was designed to DNA located 451 bp upstream of *fla*A. The inserts in pFL7*fla*A, pFL8*fla*A and pFL10*fla*A (Fig. 13) excised using *Hin*dIII/*Sal*I double digestions were cloned into the expression vector pMMB67EH to construct the clones pFL7*fla*AEH, pFL8*fla*AEH and pFL10*fla*AEH respectively. Similarly, a *Hin*dIII/*Bam*HI fragment of pMG3 with the *fla*B gene was cloned into pMMB67HE (p*fla*BHE). All four expression constructs contained the cloned genes in the orientations such that the 5' end of both genes were immediately downstream of Ptac. The constructs were transformed into *E. coli* strains DH5α and S17-1. The expression constructs in S17-1 were then transferred into *A. salmonicida* A449 by conjugation. Expression of the *fla* genes from the Ptac promoter was then induced with IPTG in both DH5α and A449, and flagellin detected in Western immunoblots of whole cell lysates using polyclonal anti-*A. hydrophila* TF7 flagella anti-serum. Whole cell lysates of *A. hydrophila* TF7 and pMMB67EH(DH5α) were used as positive and negative controls respectively.

Figure 19 shows flagellin expression by the various constructs in the *E. coli* DH5α background, in which flagellin expression from the Ptac promoter

was comparatively higher than in *A. salmonicida* A449. The anti-*A. hydrophila* flagellin antibody bound to the approximately 39 kD flagellin(s) in the positive control lysate of *A. hydrophila* TF7 (lane 1), and the 39 kD *A. salmonicida* FlaA (lane 2 - 4, 6) and 38 kD FlaB proteins (lanes 9, 10). Interestingly, deletion of the upstream region of *fla*A (pFL10*fla*AEH) (lane 2) did indeed appear to result in an increase in FlaA protein production upon induction with IPTG, in comparison to pFL7*fla*AEH(DH5α) (lane 3) or pFL8*fla*AEH(DH5α) (lane 4). FlaA was barely detectable in the uninduced lanes of pFL7*fla*EH(DH5α)(lane 7) and pFL8*fla*AEH(DH5α)(lane 8) while FlaA could be strongly detected in uninduced lysates of pFL10*fla*AEH(DH5α) (lane 6). No flagellin protein was detected in control pMMB67EH (lane 5). In contrast, *A. salmonicida* FlaB protein was readily detectable in the lysates of uninduced p*fla*BHE(DH5α) (lane 9) and its production was further increased upon induction by IPTG (lane 10). Indeed, based on immunoblot signal strength, significantly more FlaB protein appeared to be produced from an equal amount of cells of p*fla*BHE(DH5α) (lane 10) compared to FlaA from pFL7*fla*AEH(DH5α) (lane 3) or pFL8flaAEH(DH5α) (lane 4). No flagellin was detected in control lysates of *A. salmonicida* A449 (lane 11). Results similar to those above were also obtained when the various constructs were expressed in *A. salmonicida* A449, except that FlaA was barely detectable in the uninduced lanes of cell bearing pFL10*fla*AEH (data not shown). However although the flagellin proteins could be detected in Western immunoblots when the cloned *fla* genes were expressed from Ptac in *A. salmonicida* A449, no flagella were detected either by examination of cells in the electron microscope, or by growth on motility agar.

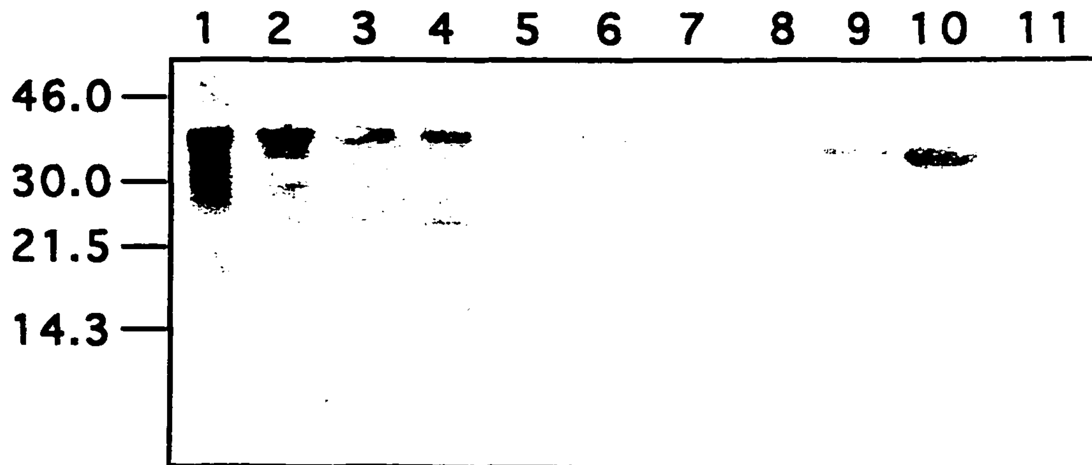


Figure 19. Western immunoblot showing the antigenic cross reactivity of *A. hydrophila* TF7 anti-flagella polyclonal antibody with *A. salmonicida* A449 flagellin proteins.
Whole cell lysates of *A. salmonicida* A449, *A. hydrophila* TF7 and *E. coli* DH5α bearing the specified expression constructs are shown. Lanes: 1, TF7; 2, pFL10*fla*AEH (+); 3, pFL7*fla*AEH (+); 4, pFL8*fla*AEH (+); 5, pMMB67EH (+); 6, pFL10*fla*AEH (-); 7, pFL7*fla*AEH (-); 8, pFL8*fla*AEH (-); 9, p*fla*BHE (-); 10, p*fla*BHE (+); 11, A449. (-) indicates without IPTG and (+) indicates IPTG added. M_r markers (kD) are indicated on the left.

FlaB N-terminal sequence analysis

Because ample amounts of the putative FlaB protein could be produced by inducing p*fla*BHE with IPTG, N-terminal sequence analysis was performed on the apparent M_r 38,000 antigenically cross-reactive protein to confirm its identity. After IPTG induction of strain DH5α(p*fla*BHE) for 3 h, a sample of the thick very obvious band corresponding to the putative FlaB protein (data not shown) present in a SDS-PAGE separated whole cell lysate was subjected to Edman degradation. The 28 residue sequence obtained (AMYINTNTSSLNAQRNLMNTNKSLDTSY) corresponded to the predicted FlaB sequence from residue #2 to #29 (Fig. 14). The N-terminal methionine residue encoded by *flaB* has presumably been removed by post translational processing in the *E. coli* host background.

The derived N-terminal amino acid sequences of the *A. salmonicida* FlaB and the deduced N-terminal amino acid sequences of *A. salmonicida* FlaA were compared with those obtained from N-terminal amino acid sequencing of flagellin proteins from several *A. hydrophila* strains (J. A. Austin, unpublished data) (Table 7). The results in table 5 show the high degree of conservation in the N-terminal amino acid sequences of the strains from both genera. The N-terminal amino acid sequences from *A. hydrophila* A6 and A421 were the most similar to the FlaB flagellin of *A. salmonicida* A449 with just a single conservative amino acid substitution from N-22 to T. Others had only a minimum of 2 conservative changes in some strains to a maximum of 5 conservative changes in others. The result in table 5 shows that unlike the nucleotide sequences of the flagellin genes from both genera which are not very similar as determined by high stringent Southern blot analysis, the amino acid sequences are quite similar.

Organism	N-terminal amino acid residues
	5 10 15 20 25 30
A. salmonicida A449 *fla*A[a]	MALYINTNVSSLNAQRNLMNTNKSLDTSYT
A. salmonicida A449 *fla*B[b]	:M::::T:::::::::::::::::::::
A. hydrophila TF7 polar[b]	:MF:::T::::::::::::T::::::::
A. hydrophila TF7 peritricious[b]	:MF:::T::::::::::::T::
A. hydrophila A434[b]	:MF:::T::::::::::::T::::R::
A. hydrophila A421[b]	:M::::T:::::::::::::::::I:
A. hydrophila A6[b]	:M::::T::::::::::::T:::::

a, Sequence deduced from DNA sequence
b, Sequence derived from N-terminal protein sequence

Table 5. Comparison of the N-terminal amino acid sequences of the flagellin proteins of *A. salmonicida* A449 and several *A. hydrophila* strains. (*A. hydrophila* N-terminal amino acid sequences were obtained by J. A. Austin.)

Electron microscopic detection of flagella on *A. salmonicida* A449

Cells of *A. salmonicida* A449 grown aerobically for various periods of time on TSA, TSB and motility medium at 14°C, 20°C, 30°C and 37°C, in BHI broth supplemented with 18% ficoll or incubated in sterilized sea water for 4 hours, were negatively stained and examined for the presence of flagella by electron microscope. Flagellated cells were not observed when cells were grown at 30°C or 37°C. However, cells producing unsheathed flagella (approximately 11-14 nm diameter) (Fig. 20) were observed at a frequency significantly <1% in some of the other conditions. Cells producing one, two or four polar flagella from the same pole, or distributed at both poles were observed.

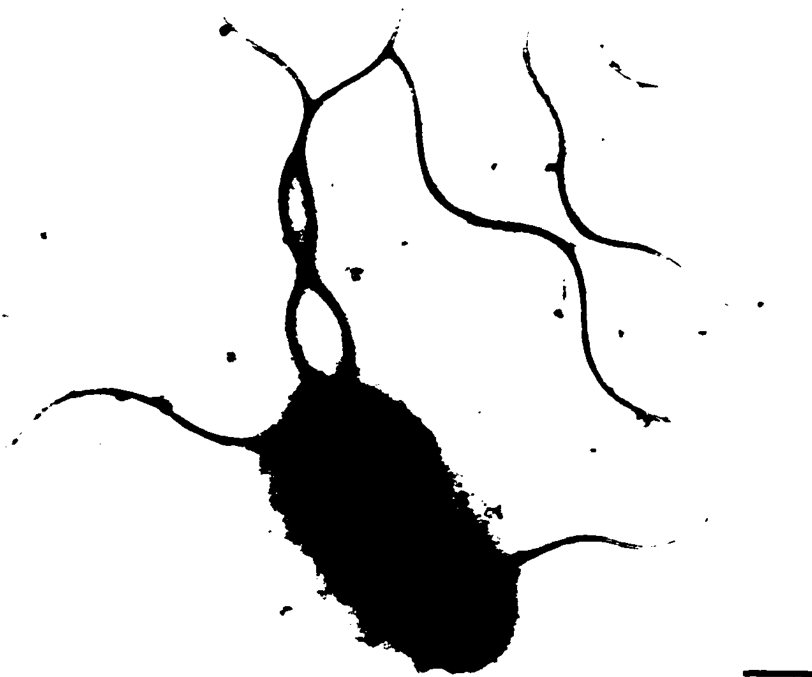
Figure 20. *A. salmonicida* A449 cells with either one, two, or four polar flagella.
The preparation was negatively stained with 0.5% phosphotungstic acid (pH 7.4) [or with 0.5% (W/V) ammonium molybdate pH 7.0]. The bar indicates 0.25 μM (A) and 0.5 μM (B-D).

A

B

C

D



DISCUSSION

This study has shown that *A. salmonicida*, an organism defined taxonomically as being non-motile, possesses two tandemly linked flagellin genes. Both *fla* genes appear to be conserved in the species, and in strain A449 the tandemly linked flagellin genes were also tandemly linked with the genes encoding a FlaG and FlaH protein homologues. The *flaA* and *flaB* genes were cloned and expressed *in vitro*, and *in vivo* in *E. coli* and *A. salmonicida*, and the resulting proteins were antigenically cross-reactive and of similar apparent subunit M_r to the flagellin produced by the highly motile relative *A. hydrophila*.

The predicted M_r of the unprocessed *A. salmonicida* FlaA flagellin was 32,351 while that of FlaB was 32,056. However, the flagellin proteins of *A. salmonicida* migrated higher than the M_r predicted from their nucleotide sequences when the proteins are expressed both *in vitro* and *in vivo*. Some bacterial flagellins have been shown to be posttranslationally modified which alters their mobility on SDS-PAGE gels (8, 122). The aberrant migration of the *A. salmonicida* A449 flagellins is unlikely to be due to post translational modifications since it is observed in both the *in vivo* and *in vitro* expressed Fla proteins. Other proteins have been known to migrate at an M_r contrary to those predicted from their nucleotide sequences (27, 290, 295). The *fla*B gene of *Proteus mirabilis* on expression in *E. coli* minicells also produced a protein which was larger than predicted from the nucleotide sequence (27). The reasons for the aberrant migration of the *A. salmonicida* flagellin proteins in SDS-PAGE gels are unknown.

The flagellin proteins of *A. salmonicida* A449 displayed amino acid sequence typical of other flagellins, with conserved N- and C-termini, linked

by a relatively short central variable domain. The central portion of the FlaA and FlaB flagellins of *A. salmonicida* A449 showed extensive variations when compared to those of other bacterial flagellins. One significant difference was that the FlaA and FlaB of A449 appeared "deleted" within this region. However it could well be that the larger flagellin proteins have evolved as a result of "insertions" into the central region of the protein. In comparison with its closest evolutionary relatives *Vibrio* and *Pseudomonas* spp., the central portion of *A. salmonicida* A449 FlaA has only 57 amino acids and FlaB has 56 while *P. aeruginosa* and *V. anguillarum* have approximately 147 and 132 amino acid residues respectively. Some bacteria have even smaller flagellin proteins, such as *C. crescentus* with flagellins of 25 kD, 27 kD and 29 kD (246) and *Roseburia cecicola* flagellin is 31.3 kD (221). The central portion of the flagellin does not appear to play any role in the export or assembly of the flagellin monomers. Kuwajima showed that as much as 187 nucleotides could be deleted from the central region of the *E. coli fla* gene and the approximately 33 kD truncated flagellin protein produced was still functional and could be exported and assembled into flagella (190). Therefore although the flagellin proteins of A449 are smaller than those of some bacteria, the essential N- and C-termini regions appear intact.

The *fla*B gene of *A. salmonicida* A449 when cloned under the control of the Ptac promoter is highly expressed when induced in *E. coli* DH5α, and the FlaB protein is strongly detectable even in the uninduced state. In contrast, in *flaA* clones containing intact upstream sequence, the FlaA protein is poorly produced on induction and is barely detectable in the uninduced state. However deletion of the DNA upstream of *fla*A down to the 16 nucleotides immediately 5′ of the start codon increased induced FlaA protein expression approximately three-fold, and also resulted in increased

FlaA expression in uninduced conditions. It is unclear why the presence of certain DNA sequence upstream of *fla*A appears to decrease the efficiency of the Ptac promoter, or why *fla*A is poorly expressed in *E. coli* compared to *fla*B, however the findings raise the possibility that *flaA* may be the subject of negative regulation. Certainly, with the exception of a potential σ^{28} promoter sequence, the DNA in the 5' region of *fla*A and *fla*B is quite dissimilar, and may contain different regulatory sequences.

The tandem arrangement of the *A. salmonicida* A449 *fla*A and *fla*B genes argues for production of a complex flagellum. Both *C. coli* (133) and *R. meliloti* (285) have also been shown to possess two highly related flagellin genes which are stably maintained tandemly on the chromosome. In *C. coli* VC167 T1 and T2 cells, the *fla*A and *fla*B genes have more than 93% nucleotide sequence identity (133), compared to the 79% nucleotide sequence identity shared by the *A. salmonicida fla* genes. In the case of *R. meliloti* both flagellins appear to be present in approximately the same amounts in the flagella filament, and mutants in either *fla* gene can form functional flagellar filaments (285). In *Campylobacter*, FlaA is present in significantly higher copy number in the filament than FlaB, and while mutants in *flaB* can assemble an apparently full length flagella filament, mutants in *flaA* can only produce a truncated filament (133). In the case of *A. salmonicida*, based on the relative expression levels of the *flaA* and *flaB* genes in *E. coli*, it is possible that FlaB could comprise the major filament protein of the flagellum.

The presence of two homologous flagellin genes has been shown to provide the host bacterium with additional advantages. For example, in *C. coli* in which motility is essential for the initial establishment of infection by allowing the bacterium to colonize the viscous intestinal mucus lining, it has been shown that bacterium possessing flagella polymerized from both FlaA

and FlaB flagellins have greater motility than cells with flagella of equal length made from just FlaA (133). Indeed *Campylobacter flaB* expression is subject to environmental regulation, such that environmental factors which increase the amount of FlaB in the flagellum result in increased cell motility (9). The existence of two highly homologous flagellin genes may also ensure that a functional back up copy is present if mutations were to occur in one copy and also could allow recombinational events to take place to generate new copies or to repair defective ones. Such recombinational events involving *fla* genes have been shown in *Campylobacter* (9).

Electron microscopic examination showed that *A. salmonicida* was capable of producing unsheathed polar flagella at a low frequency, consistent with the presence of a full complement of flagella genes in the genomic make-up of this important fish pathogenic bacterium. With such a phenotype, *A. salmonicida* appears to be similar to the human enteric pathogen *Shigella*. Recently, species of *Shigella*, a genus which has also long been regarded as non-motile and non-flagellated, were shown to possess intact flagellin genes. These genes were initially thought to be cryptic (351), however Girón later showed that prototypic strains of all four *Shigella* spp., including fresh isolates, could be flagellated and motile (121), albeit at low frequency. Depending on the strain, the ratio of motile to non-motile cells was estimated to be approximately 1:300 to 1: 1,000. The loss of motility in some *Shigella* strains has been attributed to mutations by endogenous insertion sequence (IS) elements in the *flh*D or *flh*C genes which encode proteins involved in the positive regulation of the flagella regulon (4). These workers have proposed that repeated culturing of clinical isolates in the laboratory together with long term storage may select for non motile strains. In *Bordetella pertussis* which is also regarded as a non-motile and non-

flagellated species, Southern blot analysis has suggested that flagellin genes are present (3, 204). While the reason for the non-motile phenotype of this species is unknown, a recent study has suggested that this could be due to differences within gene(s) in the flagellar regulon of this species compared to motile *Bordetella* species rather than on the regulatory BvgAS loci (78).

Like *Shigella*, *A. salmonicida* also possesses endogenous IS-elements (135). Transposition of these IS-elements can result in mutations affecting expression of the A-layer. However, growth conditions which select for these IS-element induced mutations in A-layer expression do not appear to result in the selection of flagella producing variants. We have observed flagella on old *A. salmonicida* A449 isolates which have undergone repeated laboratory culturing and long term storage. In addition, Austin had also observed that motility was evident in some *A. media* isolates only following repeated laboratory subculturing (16). It therefore suggests that the production of flagella in *A. salmonicida* must be under intricate negative regulation in the laboratory conditions. Indeed the presence of putative σ^{54} promoters directly 5′ of the flagellin genes of *A. salmonicida* suggests the genes might be under environmental regulation. We have not found conditions such as growth on M9 minimal agar, TSA or TSB at 14°C, 20°C, 30°C, 37°C, anaerobic conditions at 20°C, BHI broth supplemented with 18% ficoll or TSA supplemented with 2.5% and 5.0% polyvinyl pyrrolidone to be such appropriate conditions for optimal flagella expression. That environmental conditions can indeed modulate expression of *A. salmonicida* flagella is suggested by our unconfirmed results that incubation in sterilized sea water resulted in a significant increase in the number of flagella bearing cells.

The production of flagella is a very costly commitment by the bacterium in terms of resources and energy (142, 217). It would, therefore,

seem to be advantageous to a bacterium to be able to produce flagella only when required. While most motile bacterial species appear not to regulate flagellum expression under normal laboratory growth conditions, a few species are capable of some form of regulation. For example, in *E. coli*, production of flagella is repressed by growth on media with glucose and several other catabolites (217). In *C. crescentus*, biogenesis of the complex filamented organelle occurs during only one phase of the species complex life cycle (123). In the case of *B. bronchiseptica*, *flaA* synthesis is negatively controlled at the transcriptional level through a complex regulatory hierarchy in response to environmental conditions (2, 3). While *A. salmonicida* rarely produces flagella under conventional laboratory culture conditions, the stable genetic maintenance of all the machinery required for the production of flagella in *A. salmonicida* argues for a role for this organelle under yet to be identified environmental conditions.

The fact that *A. salmonicida* rarely produces flagella in the laboratory suggests that flagella and/or motility are not essential under such conditions and therefore the cells remain predominantly nonflagellated. There are probably some as yet unidentified conditions that are encountered by this major fish pathogen in nature or during infection of its host which would induce predominant expression of flagella and subsequent motility on *A. salmonicida*.

CHAPTER 3

THE PHYSICAL AND GENETIC MAP OF THE *Aeromonas salmonicida* A449 CHROMOSOME

It was decided to construct the physical and genetic map of the *A. salmonicida* A449 chromosome for several reasons. Firstly, to obtain a working blueprint of the chromosome in terms of the position of specific landmarks such as RE sites. The approximate location of the genes of *A. salmonicida*, particularly those encoding proteins which have been implicated in virulence, would be determined. Knowledge of the genome organization of *A. salmonicida* could facilitate the rational development of an attenuated live vaccine strain because the virulence factor-encoding genes which are far apart on the chromosome would be targeted for mutation. This would reduce the chances of complementation of the multiple mutations by a single incoming plasmid or piece of DNA.

Comparison of the genome maps of numerous organisms have revealed that bacterial genomes are very complex and more flexible than was originally believed (181). The determination of the *A. salmonicida* A449 chromosome map would allow for an estimation of its relationship with those of other organisms. Finally, since the origin of *A. salmonicida* strains have been suggested to be clonal, it was decided to compare the *Ceu*I digestion fingerprint of the genomes of typical and atypical strains of *A. salmonicida*, as well as representative strains of other members of the genus *Aeromonas* to determine indeed the extent of clonality in the species.

BACKGROUND TO GENOME MAPPING

Bacterial genomes

The genomes of bacteria consist of both chromosomes and extrachromosomal DNA which are called plasmids. Chromosomes and plasmids are composed of deoxyribonucleosides linked together by phosphodiester bonds to form a continuous entity. The genome essentially comprises discreet genes and operons, linked together by non-coding regions which may contain regulatory sequences. The genes encode proteins required for the structural and functional integrity of the organism. The minimum size of a bacterial genome required to sustain life has been estimated at 550 kb (163). However, the genomes which have been analyzed to date range in size from as low as 600 kb in *Mycoplasma genitalium* (71), and up to 9,454 kb in *M. xanthus* (62).

Chromosomes are generally regarded as the genomic component containing the *rrn* genes which encode the ribosomal RNA and other essential genes such as *gap* which encodes the glyceraldehyde 3-phosphate dehydrogenase, both of which are required for normal growth (20, 181, 336). Although chromosomal genes encode the majority of the proteins produced by a bacterium, resident plasmids can be quite large, ranging up to 400-1,500 kb in size (23, 146, 157).

The structure of bacterial genomes appear to be quite varied, contrary to original dogma. Bacteria were classically considered to contain a single circular chromosome (181, 187). While the majority of prokaryotic chromosomes which have been analyzed are circular (51, 59, 213, 240, 326, 334), linear chromosomes have been identified in *Borrelia burgdorferi* (56), *Streptomyces lividans* 66 (206) and *Streptococcus coelicolor* A3(2)

(195). *A. tumefaciens* possesses another possible chromosome structure which is comprised of one linear (2,100 kb) and one circular (3,000 kb) chromosome (5).

A surprising number of bacterial species have quite complex genomes composed of several different replicons. For example, *R. sphaeroides* 2.4.1 carries two circular chromosomes and five resident plasmids, seven different replicons making a total genome size of 4,400 kb (336). *Brucella melitensis* 16M also possesses two independent chromosomes (239). The complexity of genomes can vary extensively even within closely related species. Two chromosomes of 3050 and 900 kb were identified in *R. sphaeroides* (336), while the genome of *R. capsulatus* comprised of one chromosome of 3700 kb and sometimes a plasmid of 130 kb (103). In addition, *P. aeruginosa* PAO possesses a single chromosome of 5900 kb (304), while *P. cepacia* has three different chromosomes of 3400, 2500 and 900 kb (63).

The structure and number of resident plasmids have also been found to vary extensively. While most are circular, the presence of linear plasmids have been identified in several bacteria. A giant linear plasmid of approximately 550 kb has been described in several *Streptomyces* strains (176, 177), and *B. burgdorferi* Sh-2-82 contains five linear plasmids in addition to two circular plasmids (56).

Bacterial genomes vary extensively both in base compositions and the order in which these bases occur. As a result, REs which cleave at specific sites produce varying numbers and sizes of fragments when used in the digestion of different bacterial genomes. To some extent, the relative frequency of a particular RE cleavage site within a specific bacterial genome can be predicted with prior knowledge of the G+C content of the genome. Genomes with a low G+C content are expected to be cleaved infrequently by REs which

recognizes G+C-rich cleavage sequences. It has been reported that the tetranucleotide CTAG occur rarely in genomes with G+C contents greater than 45% (229). Hence REs such as *Spe*I (ACTAGT), *Xba*I (TCTAGA), *Avr*II (CCTAGG) and *Nhe*I (GCTAGC) would be expected to cleave rarely within such genomes. In those bacteria with G+C contents of approximately 50% such as *N. gonorrhoeae* (148), it is more difficult to predict rare cutting REs based on G+C values alone. The genomic DNA fragments derived from RE digestion can be separated using electrophoretic techniques. However, resolution using regular gel electrophoresis is limited and DNA fragments larger than approximately 50 kb co-migrate together independent of size.

Approaches utilized for genome mapping

Until quite recently, the determination of genome maps of both prokaryotic and eukaryotic organisms had been limited by the lack of technologies available for the resolution of large DNA fragments. Four approaches which have been applied in deducing genome maps include purely genetic methods, PFGE, gene encyclopedia construction and genome sequencing projects.

The approach that was first applied in determining bacterial genome maps consisted of purely genetic methods, and this was used to construct genetic linkage maps of *E. coli* (19), *Bacillus subtilis* (282) and *S. typhimurium* (310). Genetic linkage maps were constructed using plasmids which were able to mobilize large sections of a donor bacterial chromosome to recipient cells. This approach provided relatively detailed maps however it had several disadvantages. Apart from being very labour intensive, genome mapping by purely genetic approaches was limited to organisms which were genetically easy to manipulate and which had previous extensive genetic

characterization. The fact that there are few genetic linkage maps available may be a fair indication of the difficulties associated with this approach as well as its unsuitability for a wide variety of organisms.

PFGE was introduced in 1983 by a landmark study conducted by Schwartz (315). PFGE has made it possible to investigate both prokaryotic and eukaryotic organisms at the genome level since DNA fragments greater than 10,000 kb can be efficiently resolved (314, 315, 325). The use of PFGE as a means of constructing bacterial genome maps is described in detail below. However as a comparison with the purely genetic approach described above, over 120 different bacterial genome maps have been deduced using PFGE since its discovery fourteen years ago. Genome mapping using the PFGE technique can be more easily applied to diverse organisms and a prior genetic characterization is not a necessity.

The determination of bacterial genome maps via construction of gene encyclopedias have also been recently introduced (180). Construction of gene encyclopedias involves the ordering of cloned DNA fragments into contigs, which are uninterrupted sequences, until the whole genome is represented. The first step in this approach involves the construction of a gene library. The second step involves the ordering of clones within the gene library by detection of overlaps. Overlapping clones have been identified either by DNA hybridizations or by comparison of fragments derived after digestion of the clones with different REs. Kohara *et al.* determined the physical map of the *E. coli* genome by ordering DNA clones in the *E. coli* gene library (180). Construction of gene encyclopedias lead to the derivation of detailed genome maps. This approach is particularly useful for constructing genome maps of bacteria such as *Mycobacterium* spp. that possess cell walls which are refractile to the treatments utilized in the isolation of intact bacterial chromosome

necessary for genome mapping with PFGE. Eiglemeier *et al.* deduced the genomic organization of *Mycobacterium leprae* by ordering clones in the gene library using both fingerprinting and hybridization techniques (91). They obtained 4 different contigs of overlapping clones which together accounted for 2800 kb of DNA. Although the four contigs could not be linked, they reported that the gaps between the contigs were small and that the contigs gave a good coverage of the *M. leprae* chromosome (91). An ordered cosmid library has also been used to deduce the high resolution genome map of the *H. pylori* strain NCTC11638 (47).

The latest contribution to genome mapping has been the genome sequencing projects. This is the ultimate solution to determining genome maps. Some workers have suggested that construction of gene encyclopedias appear to be the first step before large scale genome sequencing projects are initiated (102). The first complete bacterial genome sequence was reported for *H. influenza* Rd (101). Since then, the genomes of several bacteria including *M. genitalium* have been completely sequenced (104). Although knowledge of the total genome sequence is very useful, such information becomes fully powerful only following the functional characterization of the genome.

PFGE

PFGE is a technique capable of resolving mb-sized DNA molecules (315). In PFGE, large DNA molecules are forced to periodically change the direction of migration within the pores of an agarose gel by the alternation of the electric field between spatially distinct electrodes. The speed at which the DNA fragments reorient is dependent on the M_r of the molecules whereas in the ordinary gel electrophoresis, mobility of large DNA fragments are

independent of M_r. Thus smaller DNA fragments reorient quickly and are able to move past the larger fragments which spend comparatively longer times in changing directions. The time required to change direction can be varied to facilitate the optimal separation of specific DNA fragment size windows.

Two main types of PFGE, CHEF (64) and FIGE (52) have been described, although several derivatives of these have been reported (22, 80, 335). In contrast to the one-anode/one-cathode set up of the conventional gel electrophoresis, the CHEF system has a hexagonal arrangement of electrodes which allows for current to alternate from side to side, with net movement of DNA fragments from top to bottom (Fig. 21). The resultant zigzag pattern of DNA migration allows for the smaller fragments to move past the larger fragments and separation depends on the time taken by each fragment to reorient. The CHEF system utilizes two important properties which allows for the optimal resolution of mb-sized DNA molecules:

1. A uniform electric field which is achieved within the agarose gel by the use of an array of twenty-four electrodes held to intermediate potentials to eliminate lane distortion. This results in lanes which are straight at the end of the electrophoretic separation.
2. A 120° reorientation angles generated by the hexagonal geometry of the twenty-four electrodes, which is the reported optimal angle for the separation of DNA molecules within the size range of 100 kb to 6 mb.

The FIGE system efficiently separates smaller DNA fragments of 100 bp up to 200 kb. Like the regular gel electrophoresis system, FIGE uses a one anode/one cathode electrode setup. However, in the FIGE system, the polarity of the electrodes are periodically reversed during the electrophoretic separation, and the times taken for the inversion of the polarity (switch

times) are short. An asymmetric switch time is utilized to achieve a net downward movement of DNA fragments through the agarose gel. Typically, the duration of the forward switch time is approximately 3x the reverse switch time.

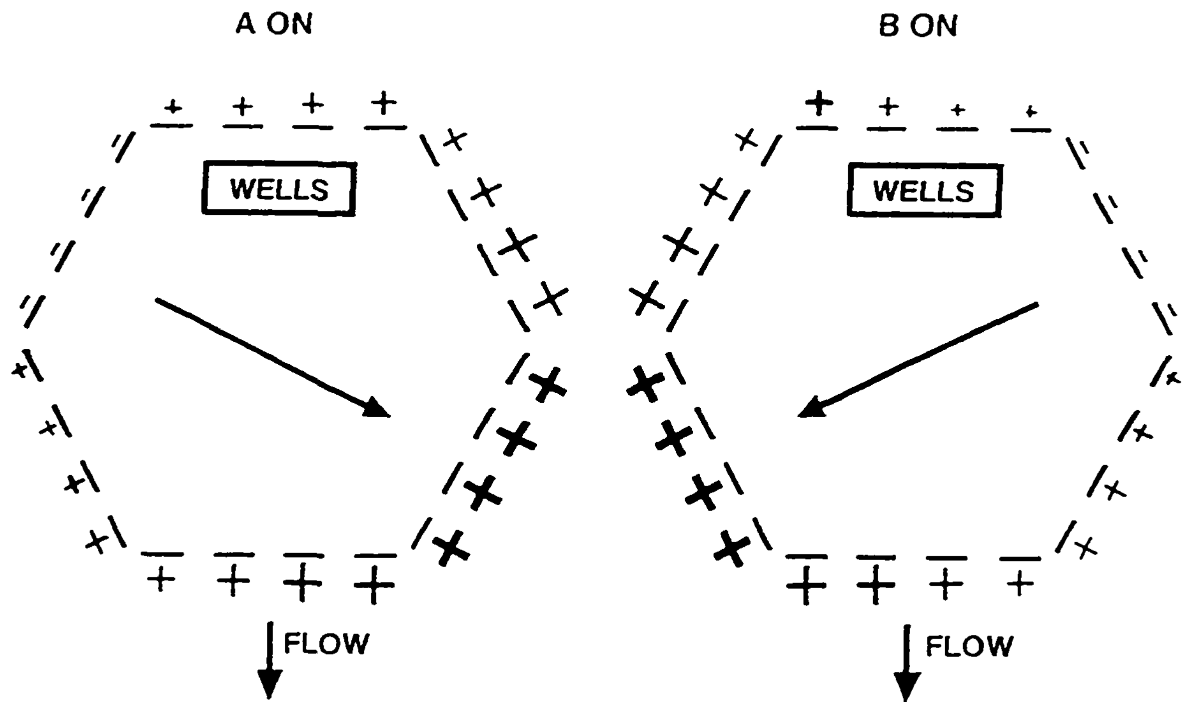


Figure 21. The CHEF PFGE system.
The figure depicts the hexagonal arrangement of the electrodes in the CHEF PFGE system, the side (A on) to side (B on) alternation of electric current which leads to a zigzag pattern of DNA migration and the ultimate direction of the DNA migration from the top (wells) to bottom. (Adapted from CHEF-DR^{R}II PFGE systems instruction and applications guide (BioRad)).

Bacterial genome mapping using PFGE

The first bacterial genome to be mapped using the PFGE technique was that of *E. coli* K-12 (326). Since then, the genome maps of over 120 different bacteria including *H. influenzae* Rd (197, 198), *S. flexneri* (271), *B. subtilis* (281), *Clostridium perfringens* (50) and *Campylobacter* spp. (343, 376) have been determined. In genome mapping, the two terms, physical and genetic mapping are often employed. Construction of a physical map involves the identification of the approximate distances in bp between landmarks (such as RE sites) in a bacterial genome, and the linkage of fragments obtained from RE digestion of the genome either in the linear or circular form representing the chromosome of interest. Physical maps are converted to genetic maps with the localization of specific genes on the physical maps.

A critical requirement for the construction of genome maps using PFGE is the isolation of total genomic DNA which is devoid of any unintentional double stranded breaks. Hence total DNA is isolated from the bacteria using procedures that are aimed at protecting the genome from cleavage during isolation. Due to the large axial ratio of DNA molecules, preparation of total bacterial DNA in liquid results in numerous single and double stranded breaks. To avoid such unwanted breaks, intact bacterial cells in solution are mixed with an equal amount of 1% (w/v) low melting point agarose and the mixture are allowed to solidify. The agarose blocks containing the embedded bacterial cells (inserts) are subsequently incubated in solutions which lyse and degrade all cellular materials except the DNA molecules. Since the DNA molecules are cushioned by the agarose matrix from all mechanical or shearing forces subjected to the sample during isolation, the

molecules presumably remain intact in the original state in which they existed within the bacteria.

The DNA molecules within the inserts are subsequently digested with rare cutting RE which produce few large M_r fragments. The digested inserts are placed in the wells of an agarose gel and the DNA fragments within are separated by PFGE using suitable parameters which resolve the M_rs of interest. Since the DNA fragments are resolved according to size, once separated by PFGE, the resultant fragments may be mixed up from the order in which they originally occurred on the chromosome. The construction of the genome map involves the determination of the correct order in which the fragments obtained following PFGE are linked to form the genome of the bacteria.

Genome map linkage strategies

Several strategies have been successfully used in the linkage of bacterial genomic DNA fragments obtained following digestion with rare cutting REs and PFGE separation. All existing linkage procedures have their own set of weak points, best results are often obtained by combining more than one of these strategies. In the simplest of cases where a limited number of fragments are involved, comparison of fragments obtained following complete digestion of partial fragments can be used to deduce the genome map. This approach was used to link six fragments obtained from digesting the *Thermus thermophilus* genome with *Hpa*I (40). Other strategies have been based on DNA hybridization experiments. For example, fragments generated by one RE have been used as probes on fragments generated by another RE to construct the genome maps of *H. influenzae* (198) and *M. xanthus* (62). The genome maps of several *Campylobacter* spp. were also constructed mainly by using

different RE fragments as probes on others in Southern analysis, although partial digestion and/or natural transformation studies facilitated the map constructions (343, 376). A different approach involving two dimensional PFGE was used to construct the genome maps of *M. mobile* (26), *P. aeruginosa* (303) and *Stigmatella aurientica* DW4/3.1 (258).

In the majority of cases however, a combination of more than one strategy have been employed in the construction of numerous bacterial genome maps. The genome maps of *C. crescentus* (96), *B. burgdorferi* (56), and several *Salmonella* spp. (210, 212, 213) and *E. coli* K-12 (326), have been constructed using different combinations of single and double RE digestion, probing RE fragments using cloned genes, oligonucleotides or PCR fragments in Southern hybridization, and localization of transposons inserted at different positions around the chromosome of different mutants. The genomic maps of *P. aeruginosa* PAO, *Listeria monocytogenes*, *Streptococcus pyogenes* and *R. sphaeroides* were constructed by partial digestion, probing with cloned genes and junction fragments in Southern blot analysis (240, 297, 334, 336).

The genome of *A. salmonicida* A449

The most comprehensive work in the characterization of *A. salmonicida* genome was reported by Belland and Trust (30). They estimated the genome size of different strains of *A. salmonicida*, both typical and atypical, calculated the sequence divergence following reassociation and the %mol G+C contents. Belland and Trust determined the average G+C content of *A. salmonicida* to be 55% and the genome size estimated by DNA-DNA reassociation was approximately 3600 kb (2.4×10^9 D) (30). The typical *A. salmonicida* strains were found to be quite a homogenous group, the level of

relationship was estimated to be 97±6.4% at 70°C and a sequence divergence of 0.9±0.8°C. Atypical strains were more divergent, with a mean homology value estimated to be 76±9.4% at 70°C, a sequence divergence value of 2.6±1.2°C. From their work, it appeared there was a relationship between the types of diseases caused by atypical strains and their geographical origins. Atypical strains isolated from goldfish ulcer in one location had a 99±3.0% homology but were only 79±2.9% related to atypical strains isolated from carp erythrodermatitis from another location.

The presence of cryptic plasmids in both typical and atypical strains of *A. salmonicida* have been reported by several workers, (25, 31, 137, 162, 331, 352), and it has been suggested that some of these plasmids may provide essential functions. Hackett *et al.* reported a similarity in the low M_r plasmids profiles (137). Belland and Trust carried out a more detailed study of the *A. salmonicida* plasmids from fourteen typical and eleven atypical strains (31). They found that the atypical strains had two to four plasmids depending on isolate source or biotype, and these plasmids ranged in size from 3.5 to 150 kb. The typical strains had four to seven plasmids sizes ranging from 3.5 to 145 kb. All typical strains tested possessed three low M_r plasmids of 5.0, 5.2 and 5.4 kb, while ten out of the fourteen had a 6 kb plasmid in common. The 6 kb plasmid did not appear to be essential since strains lacking it were both viable and fully virulent. They cloned the three low M_r plasmids from strain A449, pAsa 1 (5.0 kb), pAsa 2 (5.2 kb) and pAsa 3 (5.4 kb), as well as most of the DNA fragments obtained from the 145 kb plasmid, and transcribed plasmid encoded proteins both *in vitro* and in *E. coli*. Seventeen plasmid encoded proteins with sizes ranging from 12-90 kD were identified. The proteins included two putative exported proteins and a Cm acyltransferase (31).

R-plasmids have also been found in some strains of *A. salmonicida,* which could be mobilized into other *A. salmonicida* strains as well as into *E. coli* (11, 12, 13, 25, 31). The R-plasmids encoded resistance to Tet while one strain had a second R-plasmid encoding resistance to both Cm and streptomycin (31).

Until this study, nothing was known about the organization of any *Aeromonas* genome in terms of the physical and genetic maps.

RESULTS

Identification of enzymes suitable to map the *A. salmonicida* A449 genome

The *A. salmonicida* A449 genomic DNA was digested with at least 60 different REs in order to identify rare cutters that would produce few fragments on complete digestion of the genome. All of the tetra- and hexa-nucleotide cutters, as well as majority of the octanucleotide cutters examined produced too many fragments, and were therefore unsuitable for genome mapping. However, the three enzymes, *Ceu*I, *Pac*I and *Pme*I, produced relatively few fragments (Fig. 22), and were selected to map the *A. salmonicida* A449 genome. *Ceu*I (isolated from *Clamydomonas eugamatous*) cleaves at a 26 bp site (TAACTATAACGGTCCTAA/GGTAGCGA) within the gene encoding the 23S rRNA and produced nine fragments on complete digestion of the *A. salmonicida* A449 genome. *Pme*I (isolated from *P. medicinia*) cleaves the sequence GTTT/AAATC and produced eleven fragments from the A449 genome. *Pac*I (isolated from *P. alcaligenes*) cleaves the sequence TTAAT/TAA and produced approximately twenty-two fragments from the A449 genomic DNA.

A. salmonicida A449 chromosome size estimation

Due to uncertainty about the actual number of fragments obtained following *Pac*I digestion of the *A. salmonicida* A449 chromosome, only the *Pme*I- and *Ceu*I-derived DNA fragments were used for size estimation. The *Pac*I-derived DNA fragments were only used to show or confirm the linkage between adjacent *Ceu*I- and *Pme*I-derived fragments as discussed below.

To estimate the size of the *A. salmonicida* A449 chromosome, DNA digested with *Ceu*I and *Pme*I were separated by PFGE adjacent to λ concatemer

PFG markers (BioRad) and yeast chromosome PFG marker (BioRad). The size of each of the DNA fragments generated by the two enzymes were estimated from at least twenty gels and the average taken (Table 6). The sizes of the individual fragments was summed to obtain a total estimated genome size of 4620.25 ± 28.5 kb with *Ceu*I, and 4697.41 ± 31.0 kb with *Pme*I. The size of the *A. salmonicida* A449 chromosome is therefore estimated at 4658.83 ± 29.75 kb.

Comparison of the genome size with previous estimates

Belland and Trust had earlier estimated the genome size of typical strains of *A. salmonicida* to be approximately 3600.00 kb (2.4 x 10^9 D) using DNA reassociation methods (30). This value is lower than the 4658 ± 29.75 kb which we have deduced in this study using PFGE. Similarly, in the *E. coli* K-12 genome size estimation, the size determined by DNA reassociation techniques was approximately 4000 kb (2.7 x 10^9D) (178), but was determined to be 4600 kb by PFGE (326). PFGE is likely to be a more accurate means of estimating genome size because it involves direct comparison of genomic DNA fragments with M_r standards.

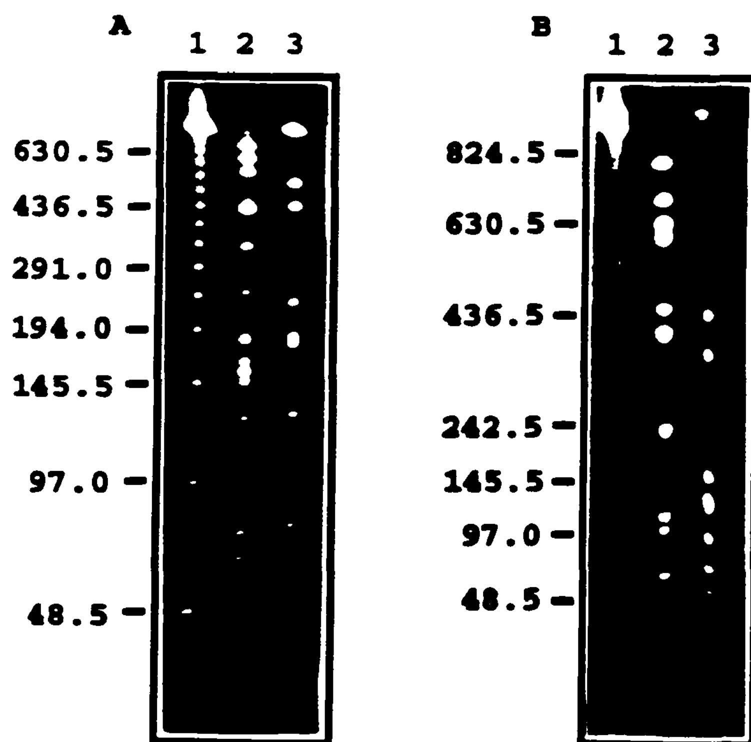


Figure 22. The genomic DNA of *A. salmonicida* A449 digested with *Pac*I, *Ceu*I and *Pme*I.

In Panel A, lanes 1, λ concatemer PFG marker; 2, *Pac*I digest; 3: *Ceu*I digest. In panel B, lanes 1, λ concatemer PFG marker; 2, *Pme*I digest; 3, *Ceu*I digest. M_rs in kb are indicated on the left. Panel A was separated first using a pulse ramp of 5-10 s for 30 h at 5.0 V/cm and then 10-80 s pulse ramp for 30 h at 5.5 V/cm. Panel B was separated using a pulse time of 10-80 s at 5.5 V/cm for 53 h.

fragment	*Ceu*I	*Pme*I
1	2800.00 ± 10.0	876.00 ± 3.5
2	525.25 ± 3.0	766.00 ± 4.0
3	436.50 ± 2.0	710.81 ± 2.5
4	238.06 ± 1.5	670.00 ± 2.0
5	186.50 ± 2.0	515.50 ± 1.5
6	178.50 ± 3.0	460.75 ± 5.0
7	130.94 ± 2.0	315.60 ± 3.0
8	76.50 ± 2.0	160.75 ± 2.5
9	48.00 ± 3.0	144.00 ± 2.0
10		68.00 ± 3.0
11		10.00 ± 2.0
Total	4620.25 ± 28.5	4697.41 ± 31.0

Table 6. Estimated sizes of the *Ceu*I- and *Pme*I-derived fragments of *A. salmonicida* A449 chromosome.
The sizes were estimated from at least twenty different gels by comparison with λ concatemer and Yeast M_r markers. The size of Ceu#1 was estimated by summation of the sizes of the constituent six *Pme*I-derived sub-fragments.

Localization of genes to the *A. salmonicida* A449 chromosomal DNA fragments generated by *Ceu*I and *Pme*I

Southern blot analysis was used to localize specific genes to the *A. salmonicida* A449 chromosomal DNA fragments generated by *Pme*I and *Ceu*I. The DNA fragments used as probes were either cloned *A. salmonicida* genes, PCR fragments amplified using primers designed to published *A. salmonicida* gene sequences or heterologous genes from *E. coli* (Table 7) Each DNA fragment utilized as probe was first labeled with (α^{32}P)dCTP and used in Southern hybridization under high stringency. Figure 23 shows an example of a Southern blot performed using the *vap*A/*abc*A genes from pSC150 (67) as probe on *Pme*I and *Ceu*I A449 DNA digests. To aid in the identification of the fragments to which the respective probes hybridized, λ DNA was also labeled and included in the hybridization cocktail to highlight the M_r markers. Using this approach, several genes were localized to the *Pme*I- and *Ceu*I-derived DNA fragments of the *A. salmonicida* A449 genome (Table 8).

As discussed previously, searches were performed for additional genes to facilitate construction of the chromosome map. As a result, the *fla*A, *fla*B, *fla*G, *fla*H, *rec*A, *Ssyn*A (encoding a siderophore synthase), pBSKpd (encoding a phosphogluconate dehydratase) and pBSKrep (encoding a gene with homology to a repressor) were identified and localized to *A. salmonicida* A449 DNA fragments (Table 8). In addition, the use of the *E. coli* genes (*uvr*D, *lyt*B and *ile*S) encoding UvrD Helicase II, LytB and isoleucyl tRNA synthase respectively, lead to the identification of the homologous copies of these genes on the *A. salmonicida* A449 chromosome (Table 8). While the identity of the *A. salmonicida* A449 genes to which the *ile*S, *lyt*B *and uvr*D genes hybridized were not confirmed, the fact that the hybridization signal was

specific under the stringent conditions used in the Southern analysis suggested that the homologous genes were localized.

Table 7. Source of the genes localized on the *A. salmonicida* A449 chromosomal map.
The DNA fragments used as probes were either amplified by PCR, or inserts excised from plasmid clones. When amplified by PCR, the identity of the amplified product was confirmed by DNA sequencing prior to being used as probe in Southern analysis. For the sequence of the primers used in DNA amplification, see "LIST OF OLIGONUCLEOTIDES" on page "xviii".

Gene	Probe	Reference
*fla*A,B,G,H	8 kb *Bam*HI fragment from pMG1	(363)
*aso*A,b	6 kb *Bam*HI fragment of pB42	(267)
*aps*E	4 kb *Hin*dIII fragment of pHI	(265)
*vap*A/*abc*A	3.2 kb *Hin*dIII/*Sal*I fragment of pSC150	(67)
*pil*B,C,D	2.8 kb *Bam*HI fragment of pPilD1	(214)
*rec*A	4 kb *Sal*I/*Eco*RI fragment from pEU101	(362)
*lyt*B	900 bp *Pst*I/*Eco*RI fragment from pXY10	(136)
*ile*S	601 bp *Cla*I/*Stu*I fragment of pMT521	(158)
*ISAS*1	800 bp *Hinc*II/*Pst*I fragment from pCG106	(135)
*ISAS*2	900 bp *Eco*RI/*Pst*I fragment from pSC153.	(135)
*uvr*D	900 bp *Pst*I/EcoRI fragment of pKS14	Dr. E. E. Ishiguro (unpublished data)
pBSKpd	2.3 kb *Pst*I fragment from pBSKS54 #7	This study
pBSKrep	2.4 kb *Pst*I fragment from pBSKS54 #5	This study
*asp*A/*Ssyn*A	2.2 kb *Pst*I fragment of *asp*ApUC18	This study
*vap*A	421 bp AP1/AP2 PCR fragment	(65)
*pil*A	600 bp PilAF/PilAR PCR product	(215)
*asp*A	351 bp EU1/EU2 PCR product	(373)
*lam*B	1185 bp MIPG1/MIPG2 PCR product	(81)

*aro*A	1155 bp EU3/EU4 PCR product	(365)
GCAT	500 bp GCAT1/GCAT2 PCR product	(257)
*exe*D	950 bp exeDF/exeDR PCR product	(171)
5SrRNA	30 bp oligonucleotide	(216)
16SrRNA	439 bp EU5/EU6 PCR fragment.	(223)
23SrRNA	2 kb *Hin*dIII fragment of H3pNEB193 #15	This study

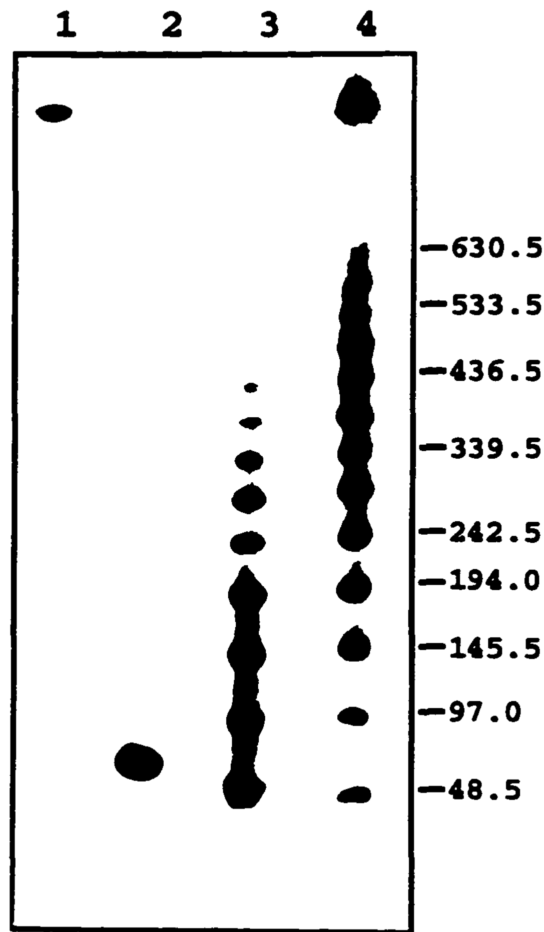


Figure 23. Southern blot analysis performed using as probe, an *A. salmonicida* A449 DNA fragment containing the *vap*A/*abc*A genes.

Lanes 3-4, low and high M_r λ PFG markers respectively; 2, A449 *Pme*I digest; and 1, A449 *Ceu*I digest. The genes localized to the 68 kb Pme#10 fragment and 2800 kb Ceu#1 fragment.

*Ceu*I	*Pme*I	Gene	Reference
1	2	*fla*A,B,G,H	(363)
1	2	*asp*A	(373)
1	1	*aso*A,B	(267)
1	1	*lam*B	(81)
1	10	*aps*E	(265)
1	10	*vap*A	(65)
1	10	*abc*A	(67)
1	5	*aro*A	(365)
2	4	*GCAT*	(257)
2	4	*pil*A,B,C,D	(214)
2	4	*recA*	(362)
2	4	*lytB*	(136)
2	4	*ileS*	(158)
3	7	*exe*D	(171)
6	8	*ISAS*1	(135)
7	6	*uvr*D	Dr. Ishiguro.
7	6	pBSKpd	This study
7	6	pBSKrep	This study

Fragments with multiple copy genes

*Ceu*I	*Pme*I	Gene	Reference
1	2,5	*Ssyn*A	This study
1,6,8	2,6,8	*ISAS*2	(135)
2,3,4,5,6,7,8,9	1,3,4,6	*rrf*	(216)
2,3,4,5,6,7,8,9	1,3,4,6	*rrs*	(223)
1,2,3,4,5,6,7,8,9	1,3,4,6	*rrl*	This study

Table 8. Localization of genes of *A. salmonicida* by Southern blot analysis to the fragments generated by *Ceu*I and *Pme*I.
The genes used as probes were cloned *A. salmonicida* DNA, PCR amplified DNA fragments and heterologous genes of *E. coli*. The probes were labeled using (α^{32}P)dCTP and hybridization performed under stringent conditions.

Genome comparison of *Aeromonas* strains

The RE *Ceu*I was used to examine the gross genomic similarities and differences between the typical and atypical strains of *A. salmonicida* isolated from different fish diseases and from diverse geographical origins. The *Ceu*I digestion pattern of representative strains of *A. hydrophila* and *A. sobria* biotype *veronii* were also compared with those of the *A. salmonicida* strains.

Genomic DNA isolated from nine typical strains, sixteen atypical strains, four *A. hydrophila* strains and two *A. sobria* strains (Table 2) were digested with *Ceu*I and the resultant DNA fragments separated by PFGE. Figure 24 shows the overall *Ceu*I digestion fingerprint of the thirty-one *Aeromonas* strains examined. The nine typical strains yielded nine fragments of apparently near identical fingerprint following *Ceu*I digestion (Fig. 24A). In contrast, the atypical strains showed significant variation both in the number and sizes of fragments produced following *Ceu*I digestion (Fig. 24B (lanes 1-11) and 24C (lanes 8-12). While nine of the atypical strains yielded nine fragments, A602 yielded three, A475 yielded seven, A460, A461, A462 and A600 yielded eight while A480 yielded ten fragments. Since *Ceu*I cleaves only within the 23S rRNA-encoding gene, the results suggested that there was a great variation in the copy number of this gene present in the atypical strains. In the lanes with the atypical strains, while some fragments appeared to be common in several strains, there were several fragments which did not migrate at similar sizes. This suggested that some regions of the chromosome of the atypical strains were identical or very similar, and regions that were not common between strain resulted in the observed polymorphism.

Like the nine typical *A. salmonicida* strains, the two *A. sobria* strains (As701 and As702) also yielded nine fragments each that appeared to have an identical fingerprint (Fig. 24C, lanes 2-3). In addition, there was an observed

similarity in size between the five smallest fragments of the *A. sobria* strains in comparison with the typical *A. salmonicida* strains (Fig. 24A, lanes 1-9). The observed differences between the *Ceu*I digests of *A. sobria* As701, As702 and the nine typical strains of *A. salmonicida* analyzed in this study appeared to reside in the *Ceu*I-derived fragments #2, #3 and #4.

Like the atypical strains of *A. salmonicida,* the *Ceu*I digestion fingerprint of the four *A. hydrophila* strains also showed extensive polymorphism (Fig. 24C, lanes 4-7). *A. hydrophila* Ah30 (lane 6) and Ah55 (lane 7) yielded ten fragments each, TF7 (lane 4) yielded eight fragments while A300 (lane 5) yielded seven fragments. The *Ceu*I digestion fingerprint of TF7 and Ah300 were practically identical, with the exception of the additional fragment produced from the TF7 genome.

Figure 24. Comparison of the *Ceu*I genomic digestion fingerprint of *Aeromonas* strains.
(A) Typical *A. salmonicida* strains, lanes 1, A449; 2, A450; 3, A488; 4, A505; 5, A202; 6, A251; 7, A440; 8, A438; 9, A447. (B) Atypical *A. salmonicida* strains, lanes 1, A419; 2, A402; 3, A400; 4, A491; 5, A600; 6, A477; 7, A588; 8, A461; 9, A522; 10, A480; 11, A475. (C) lanes 1, λ concatemer; 2, *A. sobria* biotype *veronii* strains As701 and 3, As702; 4, *A. hydrophila* strains TF7; 5, Ah 300; 6, Ah30; and 7, Ah55; 8, Atypical strains of *A. salmonicida* A523; 9, A601; 10, A602; 11, A462; and 12, A460; 13, λ concatemer. M_r in kb are indicated on the left. The three panels were separated using a pulse ramp of 5-90 s for 28 h at 185 V.

Construction of the *A. salmonicida* A449 *Ceu*I physical map

The *Ceu*I physical map of the chromosome of *A. salmonicida* A449 was constructed mainly using a combination of complete and partial digestions.

REs generally cleave DNA molecules at specific sites. When a DNA molecule has several of the cleavage site for a particular enzyme, the enzyme "randomly" cleaves at any of the sites present until all sites have been cleaved. If digestion were to be terminated before complete digestion is achieved, this "random" cleavage produces partial fragments (partials) which are composed of different combinations of portions of the original DNA molecule being digested.

As mentioned earlier, complete *Ceu*I digestion of the *A. salmonicida* A449 genome is routinely obtained after 4h digestion at 37°C (Fig. 22). Partial digestion experiments were set up such that digestion were terminated at 10, 20, 30, 40, 50, 60, 70, 80, and 90 min (data not shown). The partial digests were separated by PFGE beside an A449 *Ceu*I complete digest to help identify the partials in each lane. Termination of digestion after 40 min was identified as a good target for obtaining a suitable range of partials (Fig. 25). The partials were excised from the gel under low wavelength uv light to prevent cleavage of the DNA molecules. The partials within the pieces of agarose gel were subsequently digested to completion with *Ceu*I and then separated by PFGE. Initial analysis revealed that the amounts of the resultant fragments were often too small to be visualized using the uv light alone. Hence such digests were first end-labeled with (α^{32}P)dATP and (α^{32}P)dTTP using Klenow, and then separated as usual. After PGFE separation, the gels were dried and subjected to autoradiography. Figure 26 shows a representative result obtained when such dried gels containing the complete digests of partials were subjected to autoradiography.

Using this approach, several partial fragments were isolated and the constituent *Ceu*I-derived fragments determined (Table 9). By comparison and alignment of the fragments obtained from the complete digestion of the partials, the chromosomal order of the nine *Ceu*I-derived fragments of the *A. salmonicida* A449 genome were deduced as 1-5/6-3-7-9-8-5/6-4-2. Since Ceu#5 and Ceu#6 migrated as a doublet under the separation condition utilized in the partial digestion analysis, the identity of any fragment that migrated at this size needed to be confirmed.

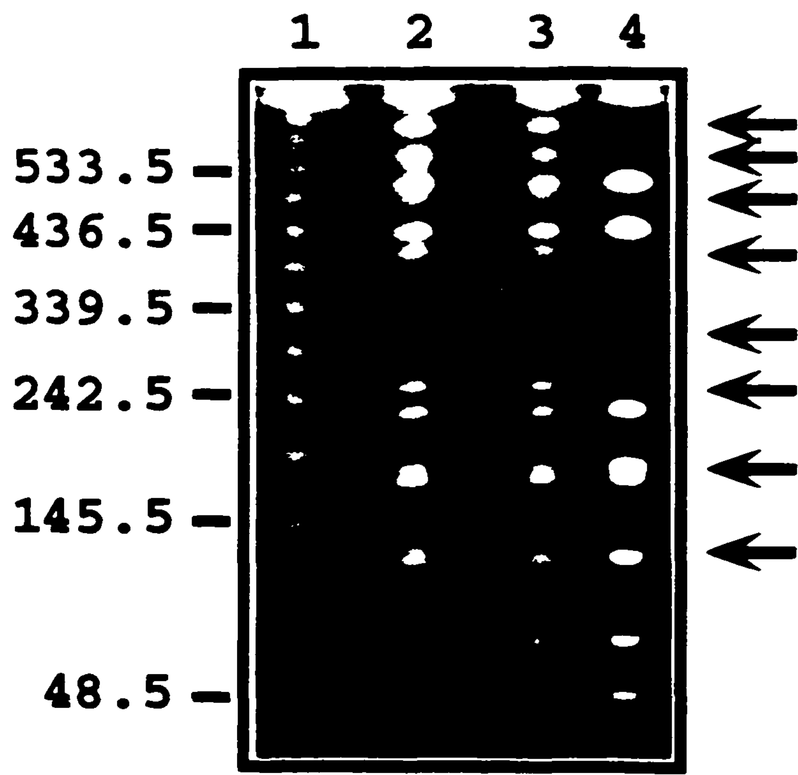


Figure 25. Partial *Ceu*I digestion analysis of the genome of *A. salmonicida* A449.
In the lanes with partial digests (lanes 2-3), digestion was terminated after 40 min and then analyzed beside a complete digest (lane 4) conducted for 4 h. Only the resolved partial fragments are indicated by arrow heads. λ concatemer M_r markers is shown in lane 1. M_r sizes are indicated on the left. The fragments were separated at 5-50 s pulse ramp for 20 h at 185 V using a 0.7% agarose gel.

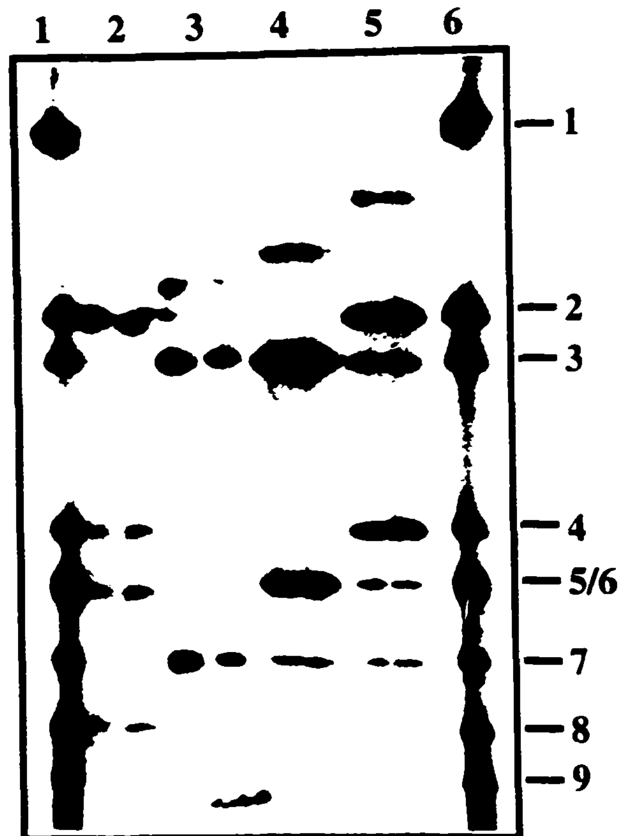


Figure 26. An autoradiograph showing the complete *CeuI* digests of several partials obtained from the *A. salmonicida* A449 chromosome.
The digests were first end-labeled using radioactive dNTPs and Klenow prior to being analyzed by PFGE. The partials (lanes 2-5) were analyzed beside the complete *CeuI* digests (lanes 1 and 6) to aid in identifying the constituent DNA fragments. The nine *CeuI*- derived fragments from the A449 chromosome are numbered on the right.

Partials	Constituent fragments	Deduced physical map
P1	8 + 9	9-8
P2	7 + 9	7-9-8
P3	3 + 7	3-7-9-8
P4	5/6 + 8 + 9	3-7-9-8-5/6
P5	8 + 5/6 + 4	3-7-9-8-5/6-4
P6	4 + 5/6	3-7-9-8-5/6-4
P7	3 + 5/6 + 7 + 9	5/6-3-7-9-8-5/6-4
P8	3 + 5/6	5/6-3-7-9-8-5/6-4
P9	2 + 4 + 5/6	5/6-3-7-9-8-5/6-4-2
P10	1 + 5/6	1-5/6-3-7-9-8-5/6-4-2

Table 9. The *Ceu*I-derived partial fragments (P1-P10) analyzed in this study.
The fragments obtained from each partial following complete digestion with *Ceu*I, and the deduced chromosomal order of the *Ceu*I-derived fragments of the *A. salmonicida* A449 chromosome.

Determination of the location of Ceu#5 and Ceu#6 in the deduced chromosomal order of the *Ceu*I-derived fragments

To confirm the location of Ceu#5 and Ceu#6 in the deduced chromosomal order of the *A. salmonicida* A449 *Ceu*I-derived fragments, *Pac*I-derived fragment #3 (Pac#3) was utilized. Cleavage of Pac#3 with *Ceu*I yielded 3 sub-fragments (data not shown). When separated beside an *A. salmonicida* A449 complete *Ceu*I digestion, one of the sub-fragments migrated as the same size as Ceu#4, suggesting that the other two sub-fragments were constituents of the *Ceu*I-derived fragments flanking Ceu#4 on the *A. salmonicida* A449 chromosome. Because these sub-fragments each contained part of an *rrn* operon of *A. salmonicida*, they were not used as probes in Southern blot analysis due to the fact they were potentially capable of hybridizing to all fragments containing the *rrn* operon. Rather a DNA fragment unique to each of the three sub-fragments was cloned and utilized as probe on A449 *Ceu*I genome digests. The probes hybridized to Ceu#2, Ceu#6 and Ceu#4. Thus Pac#3 revealed the order of the *Ceu*I-derived fragments in that region of the chromosome to be 6-4-2. These results therefore suggested that Ceu#5 was located between Ceu#1 and Ceu#3 on the deduced *Ceu*I cleavage map of the *A. salmonicida* A449 chromosome. That Ceu#5 was indeed located beside Ceu#1, and Ceu#6 beside Ceu#4 was confirmed by the fact that digestion of Ceu#6 with *Pac*I yielded two sub-fragments, one of which corresponded to the same size as that obtained by the *Ceu*I digestion of Pac#3 while Ceu#5 was not cleaved by *Pac*I (data not shown). Therefore the deduced chromosomal order of the nine *Ceu*I-derived fragments of *A. salmonicida* A449 genome was 1-5-3-7-9-8-6-4-2.

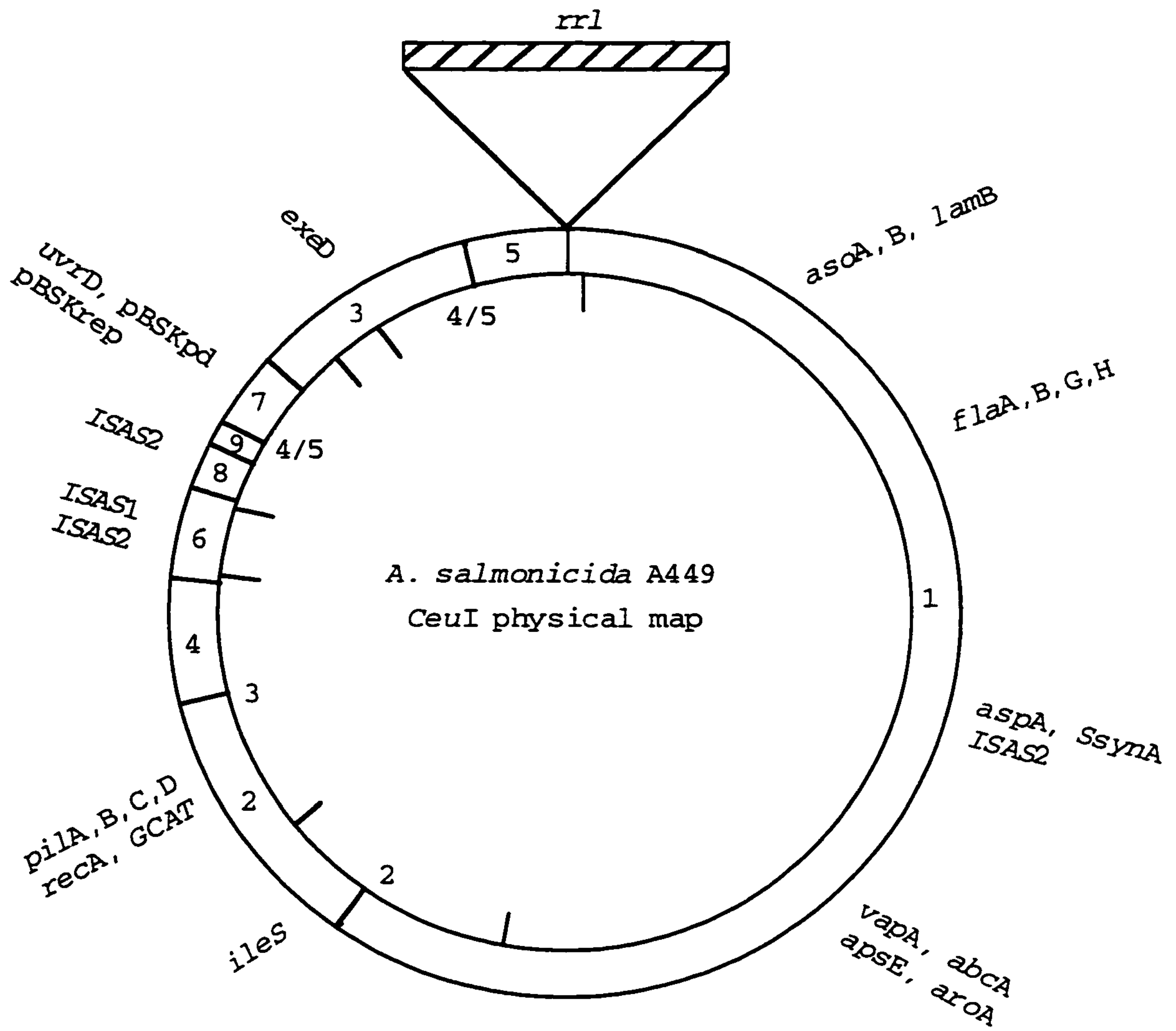
Further confirmation of the deduced chromosomal order of the *Ceu*I-derived fragments was obtained using information provided when the

Pac#4/5 doublet was digested with *Ceu*I. Digestion of Pac#4/5 with *Ceu*I yielded seven sub-fragments, four of which migrated at the size of Ceu#5, Ceu#7, Ceu#8, Ceu#9, while the remaining three did not migrate at the size of any *Ceu*I-derived fragments. Use of DNA fragments cloned from the three sub-fragments of Pac#4/5 which did not correspond to the size of any *Ceu*I-derived fragments showed that one of the fragments originated from Ceu#1, while the other two were localized to Ceu#3. These results therefore aligned one of the Pac4/5 doublets to the location of Ceu#1-5-3. The other member of the Pac4/5 doublet was aligned to the *Ceu*I physical map at the position of Ceu#8-9-7-3. Since both of the Pac#4/5 doublet hybridized to Ceu#3, it was further examined if the two fragments were physically linked. However, cleavage of Ceu#3 with *Pac*I yielded 3 sub-fragments showing that the Pac#4 and Pac#5 were not directly linked, rather they were separated by another *Pac*I-derived fragments which corresponded in size to the Pac#19/20 doublet.

Circularization of the *Ceu*I physical map

Since *B. burgdorferi* (56) and several *Streptomyces* spp. (195, 206) have been shown to possess linear chromosomes, we sought to determine the conformation of the *A. salmonicida* A449 chromosome. The *Pac*I-derived fragment #2 (Pac#2) was utilized to attain this goal. Digestion of Pac#2 with *Ceu*I yielded two sub-fragments (data not shown). When DNA fragments cloned from each of the two Pac#2 sub-fragments were used as probes on the *A. salmonicida* A449 *Ceu*I genomic digests, one hybridized to Ceu#1 while the other hybridized to Ceu#2. This showed that *A. salmonicida* A449 possessed a circular chromosome, and that Pac#2 was located at the junction between Ceu#1 and Ceu#2 (Fig. 27).

Figure 27. The circular *Ceu*I physical and genetic map of the *A. salmonicida* A449 chromosome.
The chromosomal order of the nine *Ceu*I-derived fragments were determined using a combination of partial and complete digestion. The approximate positions of the *Pac*I-derived fragments which were used in linking or confirming the position of the *Ceu*I- derived fragments are shown with bars inside the circle. The genes which localized to the various fragments are indicated. A single *rrl* gene is shown between the Ceu#5 and Ceu#1 fragments, however, such a gene exists at each of the junctions between adjacent *Ceu*I fragments.


rrl
5
asoA, B, lamB
exeD
uvrD, pBSKpd
pBSKrep
3
4/5
flaA, B, G, H
7
ISAS2
9
4/5
8
ISAS1
ISAS2
6
A. salmonicida A449
CeuI physical map
1
4
3
aspA, SsynA
ISAS2
pilA, B, C, D
recA, GCAT
2
2
ileS
vapA, abcA
apsE, aroA

Construction of the *Pme*I physical map

The *Pme*I physical map was deduced and aligned to the *Ceu*I map using several strategies. Firstly, by comparing the *Ceu*I- and *Pme*I-derived A449 chromosomal DNA fragments which had common genes (Table 7), nine of the eleven *Pme*I-derived fragments were aligned to their corresponding positions around the deduced *Ceu*I map (Fig. 28). The alignment also gave a good indication of the *Pme*I-derived fragments which were likely to be linked.

Isolation of Ceu#1 and digestion with *Pme*I yielded six sub-fragments (data not shown). Four of the six sub-fragments migrated at the size of Pme#2, #5, #9 and #10, which showed that these fragments were completely located within Ceu#1 fragment. Two of the six sub-fragments did not correspond to the size of any *Pme*I-derived A449 DNA fragments, suggesting that these two sub-fragments originated from the *Pme*I fragments located at the junction between Ceu#1-5 and Ceu#1-2. Use of the two sub-fragments as probes on *Pme*I digests revealed they constituted part of Pme#1 and Pme#3.

To confirm some of the linkages between adjacent *Pme*I fragments, the *Pac*I-derived fragments which contained *Pme*I sites (Pac#1, #2, #4/5, #7, #8/9 and #13) were utilized. Pac#1 was cleaved into 2 sub-fragments by *Pme*I and when used as probe, one sub-fragment hybridized to Pme#1 (and Ceu#1), and the other to Pme#2 (and Ceu#1). Thus Pac#1 showed that Pme#1 and Pme#2 were linked in the *Pme*I physical map, and also confirmed that the two fragments where aligned to the deduced *Ceu*I physical map at the location of Ceu#1. Similarly, Pac#2 linked Pme#3+4 (aligned to Ceu#2), Pac#4 and #5 linked Pme#1+11+7+6 (aligned to Ceu#1-5-3-7-9-8), Pac#7 linked Pme#2+10 (aligned to Ceu#1) and Pac#8/9 hybridized to Pme#5+9+10 (aligned to

Ceu#1). The linkage order deduced using this approach was as follows: 6-7-11-1-2-10-9-5 and 3-4.

Finally, partial *Pme*I digestion analysis of the *A. salmonicida* A449 genome yielded several partial fragments, two of which corresponded in size to Pme#6+8 and Pme#9+10. This provided further evidence for the linkage between Pme#10+9 and increased the deduced order of the *Pme*I-derived fragments to 8-6-7-11-1-2-10-9-5. Since Pme#8 hybridized to Ceu#6, and Pme#4 was localized to Ceu#6-4-2 by hybridization, this showed that the chromosomal order of the *Pme*I-derived fragments was 3-4-8-6-7-11-1-2-10-9-5. This accounted for the eleven *Pme*I-derived DNA fragments and the deduced linkage order was in agreement with the order predicted by comparison of *Pme*I- and *Ceu*I-derived DNA fragments which had common genes. Since the *A. salmonicida* A449 chromosome has been deduced to be circular, and the region remaining to be linked is located within a single fragment (Ceu#1), it follows that to complete the circle, Pme#5 and Pme#3 were linked (Fig. 28).

Further confirmation of the above deduced chromosomal order of the *Pme*I-derived fragments was provided by the following:

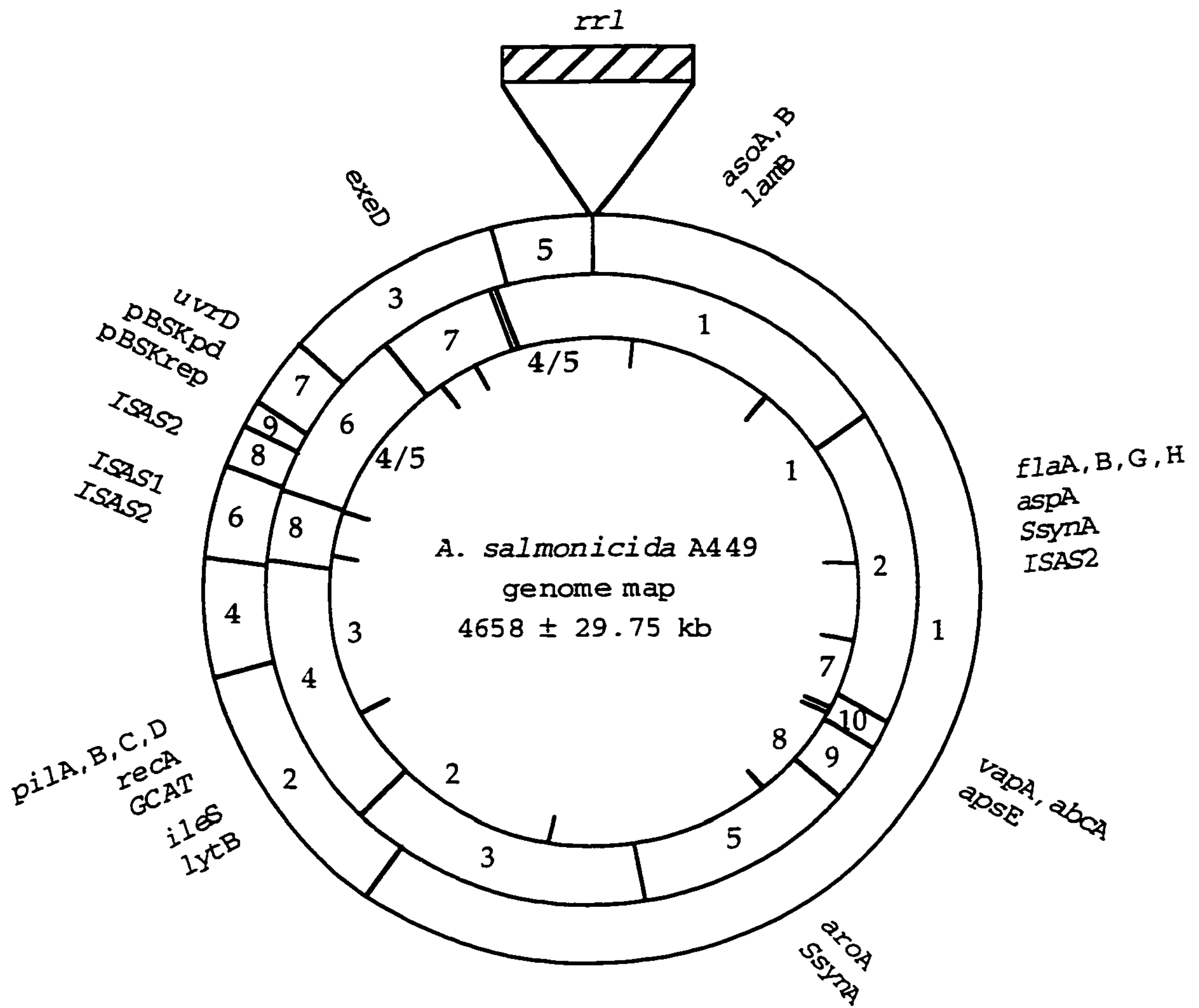
1. Pme#6 was localized to Ceu#8,#9,#7 and #3 by digestion with *Ceu*I followed by comparison to *Ceu*I digested A449 genomic DNA and by hybridization. Digestion of Ceu#3 by *Pme*1 yielded Pme#11 and #7 and two sub-fragments which were localized to Pme#1 and Pme#6 by hybridization.
2. Digestion of Pme#1 with *Ceu*I yielded Ceu#5 as well as two other sub-fragments. When the two sub-fragments were used as probes on *A. salmonicida* A449 *Ceu*I DNA digest, one was localized to Ceu#1 while the other localized to Ceu#3.

3. Digestion of Pac#4/5 with *Pme*I yielded five sub-fragments, one of which corresponded to Pme#11. The other four sub-fragments were localized to Pme#1, Pme#7 and Pme#6 by Southern hybridization.

4. The genes which hybridized to Ceu#1 (Table 7) also hybridized to either Pme#1, #2, #5, or #10.

5. Pme#4 was aligned to the *Ceu*I physical map at the position of Ceu#4-2 by Southern hybridization and by digestion with *Ceu*I followed by comparison with *Ceu*I digest of the *A. salmonicida* genomic DNA.

Figure 28. The physical and genetic map of the *A. salmonicida* A449 chromosome constructed using the REs *Ceu*I and *Pme*I. The approximate positions of the genes of *A. salmonicida* localized to the fragments produced by the two REs are shown. The *Pme*I map is represented by the inner complete circle and the *Ceu*I map is represented by the outer circle. The bars inside the inner circle represent the approximate positions of the *Pac*I derived *A. salmonicida* A449 genomic DNA fragments used to link or confirm the linkage between *Ceu*I- and *Pme*I-derived DNA fragments. The genome map was constructed using a combination of strategies such as partial and complete digestion analysis, alignment of fragments with common genes, and use of fragments obtained with one enzyme as probes on the fragments obtained with the other enzymes.


rrl
asoA,B
lamB
exeD
uvrD
pBSKpd
pBSKrep
ISAS2
ISAS1
ISAS2
flaA,B,G,H
aspA
SsynA
ISAS2
vapA, abcA
apsE
aroA
SsynA
pilA,B,C,D
recA
GCAT
ileS
lytB
A. salmonicida A449
genome map
4658 ± 29.75 kb
5
3
7
9
8
6
4
2
1
1
2
1
7
10
9
8
5
3
2
4
3
8
6
4/5
7
4/5
1

DISCUSSION

The extensive similarities observed between the genome maps of several *Escherichia* and *Salmonella* spp., initially led to the prediction that bacterial genomes would be very stable during evolution (181). However, as the genome maps of numerous unrelated organisms have been deduced and compared, it has become evident that bacterial genomes can be very complex, and are indeed more flexible than was originally believed (181). Considering the results obtained in this study, it would appear that the evolution of the chromosome of *A. salmonicida* A449 has not deviated significantly from those of *Escherichia* and *Salmonella*.

Using PFGE, the size of the *A. salmonicida* A449 chromosome has been estimated to be approximately 4,658±29.75 kb. At 4658 kb, the *A. salmonicida* A449 chromosome is similar in size to the genome of the *Escherichia* and *Salmonella* spp which have been deduced to range from 4000 kb to 4808 kb (211, 212, 326). Also, like *Escherichia* and *Salmonella* spp, *A. salmonicida* A449 possesses a single chromosome which is circular in structure.

There were extensive similarities in the fragment sizes and organization of the *Ceu*I physical maps of *A. salmonicida* A449, *Escherichia* and *Salmonella* (211). The number of *Ceu*I cleavage sites on the *A. salmonicida* A449 chromosome suggested the presence of nine copies of the 23S rRNA-encoding gene (*rrl*), and this was confirmed by Southern blot analysis using the *rrl* gene as probe. While *Ceu*I produced only seven fragments from the genomes of *Escherichia* and *Salmonella* spp (211), a large fragment greater than 2400 kb was produced from the genome of the three species. In addition, the 16S rRNA- and 5S rRNA-encoding genes were localized to all but the large Ceu#1 fragment of *A. salmonicida* A449. These results showed that the

rrn operons were all clustered within less than half of the genomes of *Aeromonas, Escherichia* and *Salmonella*. Clustering of the *rrn* operons have also been reported for *B. subtilis* (141). In *B. subtilis*, the origin of replication (OriC) was localized near the clustered *rrn* genes. The relationship of the OriC of *A. salmonicida* A449 relative to the clustered *rrn* operons is unknown.

The genes which encode proteins implicated in the pathogenesis of *A. salmonicida* A449 such as *asp*A, *aro*A, *vap*A and *GCAT*, were found to be widely dispersed around the circular chromosome. This information suggested that the construction of an attenuated *A. salmonicida* vaccine strain with mutation in several or all of these genes would have a very low probability of being complemented by incoming DNA carrying a homologue of one of these genes. The *exe* and *aps*E genes encoding proteins of the two protein secretion systems identified in *A. salmonicida* were also far apart on the chromosome, showing they are distinct in location in addition to function. While it was known that *abc*A was located immediately downstream of *vap*A, the location of the *aps* genes relative to the *vap*A-*abc*A loci was unknown. The results obtained from localization of these genes to the fragments generated by *Pme*I suggest that the *vap*A, *abc*A and *aps* genes are clustered because the three genes all localized to a 68 kb *Pme*I-derived fragment of the *A. salmonicida* A449 chromosome. The recently identified operons, one encoding the *fla* genes (363), and the other encoding the pilin genes (214), are widely separated on the *A. salmonicida* A449 chromosome. The two endogenous IS elements *ISAS*1 and *ISAS*2 of *A. salmonicida* (135), were clustered with the exception that one of the three copies of *ISAS*2 was localized to a site distant from the others. While the copy number of *ISAS*1 and *ISAS*2 in the *A. salmonicida* A499 chromosome were previously

unknown, this study has revealed that there are at least three copies of *ISAS2* and possibly only one of *ISAS1*.

Gustafson *et al.* showed that the attenuation of *A. salmonicida* by growth at high temperature was a result of insertion of *ISAS1* and *ISAS2* into *vapA* and its flanking DNA (135). In this regard, it is of interest to note the chromosomal relationship between *vapA* and the IS elements. Although one copy of *ISAS2* is located near the vicinity of *vapA*, two copies of ISAS2 and the only copy of ISAS1 are located approximately 2300 kb from *vapA*, yet, they both are capable of translocating into *vapA* and its flanking DNA. This suggests interesting mechanisms for endogenous mutation.

As previously discussed, the different phenotypic and genotypic methods which have been used in the taxonomy of *Aeromonas* have produced a lot of conflicting data (54, 55, 374). The results obtained in this study suggest that comparison of the *CeuI* digestion fingerprint of *Aeromonas* strains may play a significant role in alleviating the current uncertainties associated with classification within the genus. The nine typical strains of *A. salmonicida* analyzed in this study possessed a near constant *CeuI* cleavage fingerprint, confirming the homogeneity within this group as has been reported by others (30, 230, 260, 261). Because these nine typical strains were obtained from diverse geographical locations, these results provide further evidence that the genome of *A. salmonicida* is quite stable as has been suggested by Boyd *et al.*, and also supports the suggestion that the population structure of the typical strains is clonal (41).

In contrast to the typical strains, the *CeuI* cleavage fingerprint of the genomes of several atypical *A. salmonicida* strains showed quite an extensive variation, although there were clearly closer relationship between some strains than others. *A. salmonicida* A400, A402, A419, A491, and A601 all of

which were isolated from diseased goldfish from either Australia, USA or Europe had nine fragments of similar fingerprint following digestion with *Ceu*I. In addition, *A. salmonicida* A600 isolated in Germany had a *Ceu*I digestion fingerprint closely related to the five strains isolated from goldfish. These results also suggested a clonality for the goldfish isolates. These observations are in agreement with an earlier DNA-DNA reassociation studies conducted by Belland and Trust which reported as much as 99±3.0% homology between goldfish isolates from one location which had only an estimated 79±2.9% identity with atypical strains isolated from other diseases and locations (30). Also not surprisingly, another group of strains which appeared to be clustered were A460, A461 and A462 which were all isolated from a single disease outbreak in Atlantic salmon in Nova Scotia, Canada. The *Ceu*I cleavage fingerprint of the rest of the atypical strains examined in this study were highly variable and could not be grouped, suggesting they had deviated from the other atypical strains. A possible explanation for the differences observed within the atypical group of strains could be that host-specific selective pressures may have influenced the evolution of these strains, for example, such that goldfish isolates would evolve differently from other strains specific for other fish types, in the same way that typical strains which colonize salmonid fish appear to be highly related.

An earlier taxonomic study conducted by Belland and Trust (30) had suggested that atypical strains which were grouped under the subspecies *nova* had a potential for being subdivided. Indeed a new subspecies *smithia* was recently created from a cluster of strains belonging to the *nova* group (18). While firm conclusions cannot be made considering the limited number of strains analyzed in this study, the results we have obtained suggest that comparison of the *Ceu*I genomic cleavage fingerprint of atypical strains could

help identify groups of related strains and thus result in better classification of the atypical strains.

A surprising finding in this study was the variability in the number of fragments obtained from the *Ceu*I genomic digests of atypical strains when compared to the typical strains. While the genome of all the typical strains examined yielded nine fragments on digestion with *Ceu*I, the atypical strains yielded genomic fragments ranging from as low as three, and up to ten, in number. Some variability was also observed in the *A. hydrophila* genome, although the number of *Ceu*I-derived fragments varied between seven and ten. It is reasonable to think that such a wide variation in the number of the *rrl* gene would not normally occur within a particular species. Indeed such a variation, to our knowledge, has not been reported for any other species. It is therefore tempting to speculate that some of the strains currently classified as atypical *A. salmonicida* may not belong in the species. Because the representative *A. salmonicida* strains, such as A449, A450 and A251 had nine *Ceu*I sites, then it is reasonable to suggest that nine be taken as the actual number of copies of the *rrl* gene in this species. This would imply that all the strains which are currently regarded as "atypical" which contain ten, eight, seven and especially three *rrl* genes do not belong in the species *A. salmonicida*, and may have been wrongly classified. This could be a possible explanation for the difficulties which have been encountered in the classification of atypical *A. salmonicida* strains, as well as other strains of the genus *Aeromonas*.

Liu and Sanderson reported that genetic rearrangements as a result of homologous recombination in *rrn* genes are surprisingly common in bacteria (210), and indeed Sneath, on comparison of the published sequences of the 16S rRNA sequences of several strains of different *Aeromonas* species,

reported that rearrangements have occurred within the *rrs* genes of these strains (330). It is possible that homologous recombination events within the *rrn* genes could lead to horizontal transfer of large segments of the chromosome of one species or strain to another leading to acquisition of one or more extra *rrn* operons, or result to extensive deletions of portions of DNA within a particular strain leading to loss of one or several of the *rrn* operons. These two possibilities could explain the variations in the number of the *rrl* genes observed in the atypical strains. However, if this is the case, why was this not observed in the typical strains screened?

In terms of the genomic relationship between the three *Aeromonas* species examined in this study, it would appear that, at least at the level of the organization of the *rrl* genes around the chromosome, the *A. sobria* biotype *veronii* strains examined in this study were more closely related to *A. salmonicida* strains than were the four *A. hydrophila* strains.

As was discussed earlier, *A. salmonicida* possesses several endogenous plasmids (31, 331, 352). In *A. salmonicida* strain A449, four plasmids have been identified, three small plasmids of sizes 5.0, 5.2 and 5.4 kb and a large plasmid of approximately 145 kb (31). The results obtained in this study show that these endogenous plasmids did not interfere in the linkage of either the *Ceu*I-derived fragments or the *Pme*I-derived *A. salmonicida* A449 chromosomal DNA fragments. While the three small plasmids probably migrated off the gel under the PFGE conditions used in this study, the large plasmid might not. Indeed, a very faint band migrating at approximately 150 kb was sometimes seen in overloaded lanes containing the *A. salmonicida* A449 *Ceu*I genomic DNA digests, and could be the 145 kb plasmid of *A. salmonicida* A449. None of the genes used as probes in Southern blot analysis hybridized to the faint apparent plasmid band, and neither did any of the

fragments obtained during the *Ceu*I partial digestion analysis of A449 genome migrate at this size.

This study presents the first chromosomal map of an *Aeromonas* species to be determined. The deduced A449 chromosomal map provides the basic framework from which a more comprehensive map of *A. salmonicida* can be built over the years as more genes are characterized and localized, until such a time as when the whole genome can be sequenced. The information provided by the chromosomal map of *A. salmonicida* A449 should be useful for the rational development of a live attenuated vaccine strain of *A. salmonicida* to protect fish against furunculosis because well separated regions of the chromosome can be targeted for mutation. The deduced *A. salmonicida* A449 chromosomal map will also be useful for the comparison of the genomic organization of other *Aeromonas* species as more strain maps are determined. Finally, this study has identified a tool which could be powerful in the taxonomy of *Aeromonas*, either in confirming the current classification of strain or in recognizing new isolates.

CONCLUDING DISCUSSION

This study has significantly increased the state of our knowledge regarding the genetics of *A. salmonicida*, provided information which will be valuable in the rational development of a vaccine strain, and identified a tool that could make an important contribution to the taxonomy of *A. salmonicida* and other members of the genus *Aeromonas*.

The extrachromosomal DNA's of *A. salmonicida* were not considered in this study. Inclusion of the sizes of the four resident plasmids reported for *A. salmonicida* A449 would result to a genome size of approximately 4819.43 $\pm$ 29.0 kb. At this size, *A. salmonicida* A449 appears to have an average sized genome, considering that the largest bacterial genome size determined is 9454 kb (71) and the smallest is 600 kb (62).

Since 1980, fish farming has increased dramatically, and coupled to this expansion has been an increase in outbreaks of fish diseases, including furunculosis (17, 329). One goal of these studies had been to identify the location of the virulence factor-encoding genes on the *A. salmonicida* chromosome, information which we deem essential for the rational development of a live furunculosis vaccine. Live attenuated vaccines have been successfully used in several cases to protect against disease (57, 118, 218). For example, an attenuated *S. typhi* vaccine strain, Ty21a, developed by chemical mutagenesis in the 1970s (118), has been licensed for use in humans to prevent typhoid fever. However, because the Ty21a vaccine strain requires multiple doses to achieve acceptable immunogenicity, research is still in progress to construct an improved rationally developed *S. typhi* vaccine (339). In mice, a rationally constructed recombinant live *S. typhimurium* vaccine expressing immunogenic epitopes of tetanus toxin and glycoprotein D from

Herpes Simplex virus protected mice against salmonellosis and tetanus, or Herpes Simplex virus infections (57). Similarly, an attenuated *S. typhimurium* strain harbouring mutations in two genes, *cya* and *crp*, protects chickens against infection by the virulent strain (139). In *Salmonella* rational vaccine development, some of the other genes being targeted for mutations include those encoding enzymes involved in biosynthetic pathways, e.g. *aro*A, *aro*C, *pur*A, *pur*C, and regulatory proteins such as *pho*P, *omp*R and *cya* (61). A rationally constructed live attenuated *V. cholerae* vaccine, harbouring deletions eliminating all the currently known *Vibrio* toxin-encoding genes, is currently undergoing clinical trails in human (238). The BCG vaccine which is an attenuated derivative of *M. bovis*, a virulent strain very closely related to *M. tuberculosis*, is used to protect humans against tuberculosis (218). However, the BCG vaccine was obtained not by rational development but rather by spontaneous mutations which occurred during 230 serial passages in liquid culture. Although *M. bovis* BCG has successfully been used for over five decades, the attenuating mutations which arose during serial passage of the original BCG strain have never been identified (218). Considering the rigorous regulations surrounding the release of live organisms as vaccines today, it is questionable if the BCG vaccine would have received approval today because of its spontaneous origin. However, the BCG vaccine has never been known to revert to virulence in animals which is indicative that the attenuating mutations are stable deletions or multiple mutations which do not readily revert (218). The spontaneous vaccine strains of *A. salmonicida*, particularly the strains described by Thornton *et al.* (346), certainly show good protection against furunculosis. Like the BCG vaccine, the Thornton strain may well be an excellent furunculosis vaccine, but will there be a chance to prove it? Having said that, the current precautions surrounding the licensing

of live vaccines is a very reassuring guard against the potential catastrophes that could arise by the release of deadly virulent strains.

As a result of this study, it should be easier to construct a rational furunculosis live vaccine. The chromosomal map of *A. salmonicida* revealed that the genes encoding virulence determinants such as GCAT, AspA, AroA and VapA, the subunit for the A-layer, are good targets for mutation. For vaccine development, mutations caused by deletions are preferable, because insertion mutations, such as would be obtained by inserting an antibiotic-resistance cassette, would be undesirable. This is because the insertions involving the introduction of foreign DNA fragments can be unstable in the absence of selective pressure. Deletions eliminating all of the coding region for *vap*A, *asp*A, and *GCAT* could provide a good furunculosis vaccine. These genes have been chosen because they are widely dispersed on the chromosomal map, and the proteins they encode play confirmed roles in virulence. Mutations leading to loss of the A-layer have been shown to attenuate *A. salmonicida* (265). Secondly, the pathological effect of AspA on fish tissues has been demonstrated experimentally. AspA also plays a significant role in activating other virulence determinants of *A. salmonicida*, such as some hemolysins as well as pre-GCAT (90). Finally, GCAT was shown to possess a strong hemolytic activity against fish erythrocytes, and when in complex with LPS, the GCAT/LPS toxin complex was extremely potent in fish (201). An essential property of the vaccine must be the ability to survive long enough in fish to stimulate the production of protective and long lasting CMI. For ease of administration and cost, important concerns to fish farmers, an ideal furunculosis vaccine should be administered to fish by immersion or orally. The three possible vaccine types, e.g. purified antigens, bacterins or live cells can satisfy both of these two major concerns. However,

considering the results which have been reported in trials involving the three possible vaccine types, it appears that stimulation of CMI using live vaccines is the most efficient route to achieving long term protection in fish against furunculosis.

Mutation of *rec*A in the above hypothetical vaccine would be a very desirable addition. This is because a *rec*A phenotype would minimize the chances of reversion to virulence by homologous recombinations and facilitate licensing procedures. I would encourage construction of the above furunculosis vaccine even with an intact *rec*A for several reasons. Firstly, the wide separation of each of the attenuating mutations on the chromosome guarantees that the chances of a single step complementation by an incoming piece of DNA will be very low. In addition, there is little or no evidence that *A. salmonicida* is naturally transformable with respect to acquisition of linear pieces of DNA from dead cells or other strains. However, it must be pointed out that to my knowledge, this phenomenon has not been widely investigated in *A. salmonicida*. Some studies have reported that *A. salmonicida* R-plasmids can be transferred from one strain to another (12, 13). There is no evidence that these R-plasmids serve as shuttles for the transport of chromosomal DNA fragments.

Comparison of the pathogenic mechanisms of unrelated organisms have revealed common themes. Different determinants produced by a variety of organisms serve similar functions, for example, as adhesins, invasins, etc. The location of virulence genes within the genome of pathogens has recently attracted considerable attention. Pathogenicity islands, (PIs), which contain genes encoding proteins required for virulence have been identified in several bacterial pathogens (37, 270). PIs comprise more than 30 kb of DNA and are located either on large plasmids, phage genomes or the chromosome

(37). PIs may have been acquired by horizontal gene transfer from other species and are also easily lost via spontaneous deletions. In *Yersinia* spp., the majority of the determinants required for virulence are encoded on a 70 kb plasmid, and loss of the plasmid results to attenuation. These genes encode a set of proteins, some of which constitute part of a "contact regulated" type III secretion system and effector YOP proteins. The type III secretion system is utilized for the translocation of effector proteins through the zone of contact between the bacteria and the host cell, into the host cell cytosol. Once translocated into the host cell, the effector proteins carry out specific anti-host functions which ultimately compromise specific host cell functions in ways which are beneficial for the pathogen and detrimental to the host. In *Yersinia*, the effector YOP proteins interfere with the host signal transduction mechanisms so that phagocytosis and subsequent elimination are inhibited (73). In *S. typhimurium*, a 40 kb *spa* gene cluster encodes a variety of determinants that mediate the entry of the pathogen into the host cell (244), and the *spi* island contains a regulator protein and a type III secretion system essential for virulence in mice and survival in macrophages (270). In uropathogenic *E. coli*, two PIs have also been identified, one of which encodes two hemolysins which contribute to destruction of host cells and a Prf fimbria involved in adhesion (37, 300).

At present, relatively little is known about the pathogenic mechanisms of *A. salmonicida*. Only a limited number of genes encoding virulence determinants have been identified. Like other organisms, *A. salmonicida* may possess such features as PIs. However, the results obtained in this study did not reveal clustering of the genes encoding the known virulence factors to any particular region of the chromosome. While this suggests that *vap*A, *aro*A, *GCAT* and *asp*A do not belong to one particular PI, it does not preclude

that some of the genes could indeed belong to a yet to be identified PI together with other genes encoding virulence determinants or proteins of a type III secretion system. Because one feature of PIs is the ability to be spontaneously deleted leading to loss of virulence, it is possible that *A. salmonicida* could indeed possess such an island. It has been reported that *A. salmonicida* undergoes rearrangements which lead to loss of virulence (29, 135). Some of these attenuating events have resulted from mutagenesis of *vap*A by endogenous IS elements (135). Albeit unlikely, it is possible that loss of virulence could also be due to deletion of PIs. Because *A. salmonicida* is an invasive organism (111, 113), the possibility of there being a type III secretion system is reasonable.

There are several similarities between the flagellin export system and the type III secretion system. The proteins secreted via the two systems do not possess cleavable N- or C-terminal sequences. Rather, the exported proteins possess sequences which are recognized by the export machinery and are thus necessary for export. Analysis of YopE and YopH of *Yersinia*, two proteins secreted by the type III secretion system, revealed that the signal required for secretion through the bacterial cell membrane and into the eukaryotic plasma membrane occur in the N-terminal region (73, 332). The C-terminal regions of YopE and YopH contain the effector domain which exerts specific anti-host functions once translocated into the host cell cytosol (73). Similarly, the N-terminal region of the flagellin, a protein secreted by the flagellin export channel, is essential for export (133, 217). And like YopE and YopH, the N-terminal of the flagellin is not cleaved for export to occur. The C-terminal region of the flagellin is required for polymerization and assembly into the flagella filament (217).

There are important genes and operons in *A. salmonicida* yet to be identified and characterized, for example, the LPS genes. Because two distinct types of LPS have been reported in *A. salmonicida* (202, 345), there may be two sets of genes/operons encoding the two LPS types. This study revealed that *A. salmonicida* possesses the capability to synthesize and assemble flagella, and a *fla* operon containing at least four flagellar genes was identified and localized on the chromosomal map. In other motile organisms such as *Escherichia* and *Salmonella*, flagellar genes occur in three or four spatially distinct operons (217). This suggests that there should be two or three other *fla* operons on the *A. salmonicida* A449 chromosome. The location of other *fla* genes in *A. salmonicida* is currently unknown. The same can be said for the genes involved in genetic recombinations. The chromosomal location of the *A. salmonicida recA* has been identified. However, there are at least 25 genes whose products are involved in DNA repair and recombination (185), which are yet to be identified, characterized and localized.

Because of considerable phylogenetic relationships, *Aeromonas* and *Vibrio* shared a common family prior to the elevation of *Aeromonas* to an independent family. Indeed the deduced amino acid sequences of several *A. salmonicida* proteins obtained in this study showed the closest evolutionary relation to *Vibrio* and *Pseudomonas*. The chromosomal order of the four *fla* genes of *A. salmonicida* A449 characterized in this study was identical to those of *V. parahaemolyticus*. These findings suggested that there could be considerable similarities between the genome of the three genera. Comparisons of genomic relationships are made difficult by factors such as the use of different enzymes for mapping and placement of emphasis on genes unique to each genera. For example, it is difficult to compare the relation between *A. salmonicida* and the other two genera when the

emphasis in genetic map constructions is placed on cholera toxin genes in *V. cholera*, and alginate genes in *Pseudomonas*, both of which are absent in *A. salmonicida*. At present, a comparison of the chromosomal maps of *V. cholerae* and *A. salmonicida* is more revealing because both chromosomes have been mapped using *Ceu*I.

The *Ceu*I map of the approximately 3200 kb *V. cholera* chromosome was also deduced by partial and complete digestion (219). In contrast to *A. salmonicida*, *V. cholera* possessed seven *rrl* genes as is found in other Enterobacteriaceae and hence was cleaved into seven fragments by *Ceu*I. Based on the numbering of the *Ceu*I-derived fragments from the largest to the smallest, there are similarities in the chromosomal order of the *Ceu*I-derived fragments of *V. cholera* chromosome compared to those of *A. salmonicida*. For example, the order of the *Ceu*I-derived fragments of *V. cholera* were 1-2-4-6-5-7-3 while that of *A. salmonicida* was 1-2-4-6-8-9-7-3-5. Unfortunately, the genes which were localized on the *V. cholera* chromosome were different from those on the *A. salmonicida* chromosome, hence the chromosomal position of common genes, including the *fla* genes, cannot be compared at the present time.

The origin of *A. salmonicida* is debatable as circumstantial evidence supporting spread by importation from Europe to North America and vice versa abounds in the literature (17). The population structure of typical strains of *A. salmonicida* was found to be clonal in this study regardless of geographical origin. This is good news in terms of vaccine development because of the possibilities of a single vaccine being universally applicable. What is the origin of the atypical strains? Did they evolve by diverging from typical strains?

The clonality of the population structure of typical strains of *A. salmonicida* revealed by comparison of *Ceu*I digestion fingerprints was not surprising. Numerous evidence provided by other studies have alluded to this fact (30, 41, 261). Even in this study, *rec*A, *fla*A and *fla*B hybridized to fragments migrating at the same size in all the typical strains screened in contrast to atypical strains in which the fragment sizes varied. Other evidence which support clonality includes, antigenic conservation of the A-layer and LPS O-polysaccharide chains (84), plasmid profiles (261), multilocus enzyme electrophoresis (41) etc. The atypical strains are more complex to categorize because of variations within the population. Despite the obvious differences with the typical strains, some conservations exist between the two groups. For example, the conservation of antigenic properties of the A-layer, the LPS O-polysaccharide chains, and an extensive number of genes including *rec*A, *fla*A, *fla*B, *vap*A etc. Maintenance of the integrity of the surface structure of *A. salmonicida* is essential for disease (265, 346), hence conservation of the A-layer/LPS is both typical and atypical strains is not surprising.

In conclusion, future research on *A. salmonicida* could include genome sequencing projects to obtain the complete nucleotide sequence of the chromosome and extrachromosomal DNA. Once the genome sequence of *A. salmonicida* is available, functional analysis could ensue. This would involve making defined mutations in specific genes in order to determine the roles of the encoded proteins in the overall biology and pathogenesis of *A. salmonicida*. Putative virulence determinants can be identified by comparing the LD_{50} of isogenic mutants to that obtained by infection caused by the wild type strain. The task of assigning functional roles to genome sequences could be made easier by comparisons with sequences from other organisms. Once the deduced amino acid sequence of a gene shows significant homology to a

protein of known function from another organisms, experiments can easily be designed to confirm the predictions regarding the functions of the encoded proteins.

Until the complete genome sequence can be obtained, other research could be designed to investigate the possible occurrence of a type III secretion system in *A. salmonicida*. The type III secretion system have been shown to be crucial for pathogenesis in organisms in which they occur. If this system is present in *A. salmonicida*, identification of the virulence determinants exported via the system and their biological effect on host cell targets could reveal a great deal about the disease process, an area which sadly, is poorly understood at present. In this regard, increased emphasis should be placed in understanding the host-pathogen interactions. While simply searching for potential virulence factors in vitro can indeed be useful, it may be more relevant to conduct more studies investigating *A. salmonicida* in contact with fish tissues.

One hypothesis which arose in this study was that the observed differences between the typical and atypical *A. salmonicida* strains could be a function of the predilection for different species of fish. A study could be launched which is aimed at investigating the relationship between type of fish colonized by *A. salmonicida* strains and *Ceu*I digestion fingerprints relative to other strains. This could help reveal if indeed host-related selective pressures are influencing *A. salmonicida* evolution.

The *A. salmonicida recA* locus could be investigated further to understand the cause of the difficulty in isolating a mutant. If it is because of polar effects in essential gene(s) around the locus, mutations in *recA* could be designed to allow expression of upstream or downstream genes while eliminating RecA activity. Finally a rational vaccine could be constructed by

making defined deletion mutations in at least the three virulence factor-encoding genes suggested above.

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