

MOLECULAR ANALYSIS OF THE STRUCTURE AND EXPRESSION OF
THE *AEROMONAS SALMONICIDA* SURFACE LAYER PROTEIN GENE *vapA*

by

ACCEPTED

Shijian Chu

B. Med., Shanghai Medical University, 1982

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 thesis as conforming to the required standard

Dr. T. J. Trust, Supervisor

Dr. W. W. Kay, Departmental Member

Dr. R. W. Olafson, Departmental Member

Dr. P. J. Romaniuk, Departmental Member

Dr. M. J. Ashwood-Smith, Outside Member

Dr. W. J. Page, External Examiner

©SHIJIAN CHU, 1993

University of Victoria

All rights reserved. Dissertation may not be reproduced in whole or in part,
by photocopying or other means, without the permission of the author.

Supervisor: Dr. Trevor J. Trust

ABSTRACT

Aeromonas salmonicida is a Gram negative rod shaped bacterium capable of causing furunculosis in salmonid fish and other chronic and inflammatory diseases in goldfish, carp and also salmonids. The surface layer of *A. salmonicida*, the A-layer, has been demonstrated to be a major virulence factor for the organism, and its subunit A-protein has been purified and its structural gene *vapA* has been cloned.

The *vapA* gene from *A. salmonicida* strain A450 was subcloned (pSC150) and expressed in *Escherichia coli*. Its DNA sequence was then determined to consist of 1,506 bp encoding a 502-amino acid residue protein, containing a 21-residue signal peptide and a mature protein of 50,778 Dalton. The A-protein assembled on the cell surface in the form of an S-layer was refractile to trypsin cleavage while trypsin digestion of the purified mature protein revealed a highly resistant 39,400 Dalton N-terminal fragment and a 16,700 Dalton C-terminal fragment with moderate resistance. These trypsin-resistant fragments may form distinct structural domains, consistent with three-dimensional ultrastructural observations.

The plasmid pSC150 contained 62 bp of *Aeromonas* DNA in front of the *vapA* structural gene. A promoter (P2) was predicted in this region which showed sequence homology to the *E. coli* σ^{70} promoter. However, primer extension in the wild type strain A449 showed a transcriptional start site 181 bp upstream from the gene, and thus, another promoter (P1) was shown to be the major promoter. The DNA sequence coding for the untranslated leader mRNA contained two stem-loop structures, a putative small open reading frame spanning the stem-loop structures, and a palindromic sequence which

overlaps the predicted ribosome binding site. Northern analyses of A449 *vapA* mRNA showed that incubation at 15°C produced the highest level of the transcript, and the transcript half-life was 22 min in cells grown at 15°C compared to 11 min in cells grown at 20°C. DNA gyrase inhibitors nalidixic acid and novobiocin significantly reduced the *vapA* transcript level.

A. salmonicida 30°C mutants were found to produce significantly reduced levels of A-protein and some of them were shown to have the native insertion elements, ISA1 and ISA2, inserted in the *vapA* area. These insertion elements have been cloned and sequenced, and also identified in the wild type strains A449 and A450. ISA2 was shown to have sequence similarity to other bacterial insertion elements.

Plasmid encoded *vapA* expression in *E. coli* was also affected by a downstream gene *abcA*, which, when deleted from the clone, significantly reduced *vapA* expression. This reduction could be complemented by the *abcA* gene carried on a second plasmid. In addition, the lipopolysaccharide (LPS) O-chain deficient phenotype of A449 mutant strain TM4, which has the *abcA* gene interrupted by ISA1, was also complemented by *abcA*. DNA sequence analysis showed that the *abcA* gene coded for a 308 amino acid residue protein, which was confirmed by in vivo and in vitro expression and gene fusion with *lacZ*, and was localized in the inner membrane fraction of *E. coli*. At the N-terminal part of the protein, the predicted sequence of AbcA displayed high homology with a bacterial transport protein super family, including a well conserved nucleotide binding sequence. This binding sequence was shown by site-directed mutagenesis to be required for LPS O-chain complementation in TM4. ATP binding activity was confirmed in the purified AbcA-LacZ fusion protein. A leucine zipper-basic region sequence with predominantly α -helical conformation was predicted further

downstream, with leucine residues in four of the five heptad repeats and a valine residue in the remaining heptad repeat.

Examiners:

Dr. T. J. Trust, Supervisor

Dr. W. W. Kay, Departmental Member

Dr. R. W. Olafson, Departmental Member

Dr. P. J. Romaniuk, Departmental Member

Dr. M. J. Ashwood-Smith, Outside Member

Dr. W. J. Page, External Examiner
(University of Alberta)

TABLE OF CONTENTS

ABSTRACT.....	ii
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
LIST OF ABBREVIATIONS.....	xiii
ACKNOWLEDGEMENTS.....	xv
INTRODUCTION.....	1
Bacterial S-layers.....	1
Biological functions of S-layers.....	1
S-layer proteins.....	4
S-layer protein genes.....	5
S-layer protein gene promoters.....	7
<i>Aeromonas salmonicida</i>	8
The genus <i>Aeromonas</i>	8
The species.....	9
Pathogenesis.....	10
A-layer.....	13
Morphology.....	13
Subunits.....	15
A-layer formation and anchoring.....	16
Virulence.....	17
Other biological functions.....	18
Other <i>Aeromonas</i> S-layers.....	20
Lipopolysaccharides (LPS).....	21
Purpose of this dissertation.....	22
MATERIALS AND METHODS.....	25
Bacterial strains, vectors and media.....	25
DNA techniques.....	25
DNA preparation.....	25
<i>Aeromonas</i> chromosomal DNA.....	25
Plasmid DNA preparation.....	28
M13 DNA preparation.....	29
Oligonucleotide preparation.....	30
Molecular cloning.....	30
Preparation of <i>E. coli</i> competent cells.....	30
DNA restriction digestion.....	31
DNA ligation.....	31
Electroporation.....	32
M13 cloning.....	32
Southern analysis.....	33
Non-radioactive labeled DNA.....	33
DNA fragments radiolabeled by nick translation.....	34
End-labeled oligonucleotides.....	34
Polymerase chain reaction (PCR).....	35
DNA sequencing.....	35
Manual sequencing.....	36

Automated sequencing.....	36
Site-directed mutagenesis	37
Computer analysis.....	38
RNA preparation and detection.....	38
RNA preparation.....	38
Northern hybridization.....	39
RNA stability assay.....	39
Primer extension experiments.....	39
Detection and purification of proteins.....	40
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining	40
Immunogold electron microscopy assay	41
Cell fractionation.....	41
Culture supernatant.....	41
Periplasm.....	42
Cytoplasm.....	42
Inner membrane and outer membrane.....	43
Western blot.....	43
Silver stain for proteins.....	44
Protein preparation for N-terminal sequencing.....	44
T7 polymerase in vivo transcription.....	45
In vitro gene expression.....	46
Purification of the AbcA-LacZ fusion protein.....	47
β -galactosidase assay.....	48
Nucleotide affinity chromatography.....	48
LPS detection.....	49
Silver stain for LPS.....	49
RESULTS.....	51
I. Structure of <i>vapA</i> gene and its product, A-protein.....	51
Subcloning and sequencing of the <i>A. salmonicida</i> A449 A-protein gene (<i>vapA</i>).....	51
Subcloning of the <i>vapA</i> gene from <i>A. salmonicida</i> A450.....	54
DNA sequencing of the A450 <i>vapA</i> gene.....	55
Conservation of the <i>vapA</i> gene	62
A-protein structure.....	63
Cellular localization of A-protein in <i>E. coli</i>	67
A-protein conformation in periplasm.....	67
II. Characterization of the <i>vapA</i> gene promoter area.....	73
Subcloning and sequencing of the flanking region in front of the <i>vapA</i> gene.....	73
Primer extension analysis.....	77
<i>vapA</i> transcript size	79
Growth phase dependence of <i>vapA</i> transcription	79
Effects of growth temperature on <i>vapA</i> transcription	82
<i>vapA</i> transcript stability.....	82
Variation of <i>vapA</i> transcript levels in other growth conditions.....	85
Effects of nalidixic acid and novobiocin on <i>vapA</i> transcription.....	85

III. The <i>vapA</i> gene in some <i>A. salmonicida</i> 30°C mutants is interrupted by insertion elements	89
DNA rearrangements in 30°C mutants.....	89
Identification of two insertions in the <i>vapA</i> area in <i>A. salmonicida</i> 30°C mutants.....	93
Cloning and sequencing of ISA2	95
Effect of insertion elements in the <i>vapA</i> promoter region on A-protein production.....	102
IV. Characterization of a gene downstream from <i>vapA</i>	103
A-protein expression in <i>E. coli</i> affected by a downstream <i>Aeromonas</i> DNA sequence.....	103
<i>vapA</i> expression in <i>E. coli</i> can be complemented by the downstream sequence.....	103
DNA sequence analysis of the downstream region	105
Predicted AbcA protein structure.....	113
Identification and localization of AbcA protein	115
Analysis of <i>abcA</i> expression.....	118
Purification of the AbcA- β -galactosidase fusion protein and identification of ATP binding activity.....	122
<i>abcA</i> gene complements LPS O-chain expression in TM4.....	124
Site-directed mutagenesis in the P-loop of AbcA	126
DISCUSSION	129
I. Structure of <i>vapA</i> and analysis of its promoters.....	129
II. <i>Aeromonas</i> insertion elements.....	140
III. The <i>abcA</i> gene.....	143
IV. Summary	149
REFERENCES	151
APPENDIXES.....	172
I. Entire DNA sequence determined in this study.....	172
II. List of oligonucleotides used in this study	175

LIST OF TABLES

Table 1.	<i>Aeromonas</i> strains used in this study.....	26
Table 2.	Vectors used in this study.....	27
Table 3.	Codon usage for the <i>A. salmonicida</i> A450 <i>vapA</i> gene, and <i>A. hydrophila</i> genes coding for exported proteins	60
Table 4.	<i>vapA</i> transcript level affected by pH.....	86
Table 5.	<i>vapA</i> transcript level affected by Ca ⁺⁺ addition and depletion.....	86
Table 6.	<i>vapA</i> transcript level affected by Fe ⁺⁺⁺ depletion.....	87
Table 7.	<i>vapA</i> transcript level affected by Mg ⁺⁺ addition.....	87
Table 8.	<i>vapA</i> transcript level affected by oxygen.....	87
Table 9.	<i>vapA</i> transcript level affected by nalidixic acid.....	88
Table 10.	<i>vapA</i> transcript level affected by novobiocin.....	88
Table 11.	Results of DNA hybridization of <i>A. salmonicida</i> 30°C mutants.....	96
Table 12.	Codon usage for the <i>A. salmonicida</i> A450 <i>abcA</i> gene and <i>vapA</i> gene	111

LIST OF FIGURES

Figure 1.	Schematic illustrations of the bacterial envelopes containing S-layers.....	2
Figure 2.	Electron micrographs of <i>Aeromonas</i> A-layer	14
Figure 3.	Physical map of the cloned <i>A. salmonicida</i> chromosomal DNAs containing the <i>vapA</i> gene	52
Figure 4.	Western blot of A-protein subclones in pTZ18R	56
Figure 5.	Nucleotide sequence of the <i>vapA</i> gene and flanking DNA from <i>A. salmonicida</i> strains A449 and A450, and the translated amino acid sequence	58
Figure 6.	Southern blot analysis of <i>Bam</i> HI digested chromosomal DNAs from <i>A. salmonicida</i> strains probed with pSC650	64
Figure 7.	Kyte-Doolittle hydrophilicity plot of the A-protein	64
Figure 8.	SDS-PAGE analysis of <i>A. salmonicida</i> A450 A-protein structure by TPCK-trypsin cleavage and CNBr hydrolysis	66
Figure 9.	Schematic maps of <i>A. salmonicida</i> A450 A-protein secondary structure, CNBr hydrolysis fragments and TPCK-trypsin fragments	68
Figure 10.	Western immunoblot analysis of 7.5% SDS-PAGE showing the cellular localization of A-protein coded by pSC150 in <i>E. coli</i> DH5 α	69
Figure 11.	Western blot analysis of 12% SDS-PAGE of TPCK-trypsin partially digested periplasmic A-protein from <i>A. salmonicida</i> and <i>E. coli</i> strains	71
Figure 12.	DNA sequence of the 5' region of the <i>vapA</i> gene of A450, the immediate upstream flanking DNA, and the predicted	

	amino acid sequence	74
Figure 13.	Primer extension of the <i>vapA</i> gene in A449 and A450	78
Figure 14.	Northern hybridization of wild type and mutant <i>Aeromonas</i> strains probed with TJ41	80
Figure 15.	<i>vapA</i> transcript dynamics during cell growth at 15°C	81
Figure 16.	<i>vapA</i> transcript level at various temperatures measured by Northern hybridization	83
Figure 17.	<i>vapA</i> transcript stability assay	84
Figure 18.	Electron micrographs of immunogold labeling of A450 and A450-3 cells	90
Figure 19.	Southern hybridization of A450 and A450-3 chromosomal DNAs probed with oligonucleotide TJ44	91
Figure 20.	Comparison of physical maps of A450 and A450-3 chromosomes	92
Figure 21.	Western blot of whole cell lysate of <i>A. salmonicida</i> 30°C mutants with rabbit anti-A-protein antiserum	94
Figure 22.	<i>A. salmonicida</i> ISA2 sequence	97
Figure 23.	Amino acid sequence alignment of the predicted ISA2 encoded protein and other proteins coded by prokaryotic insertion elements	100
Figure 24.	Restriction maps of inserts in each subclone used for <i>abcA</i> subcloning and complementation	104
Figure 25.	Western blot detection of A-protein and densitometric analysis of the blot showing the complementation in <i>E. coli</i> by <i>abcA</i>	106
Figure 26.	A-protein production from <i>E. coli</i> strains with and without <i>abcA</i> gene detected by in vitro transcription-translation	107

Figure 27.	Nucleotide sequence of the <i>abcA</i> gene and flanking DNA from <i>A. salmonicida</i> strain A450, and translated amino acid sequence	108
Figure 28.	Schematic map of deduced sequence of AbcA	114
Figure 29.	α -helix structure of the leucine zipper region between L211 to L239 viewing from one end of the helix	116
Figure 30.	Alignment of the AbcA protein of <i>A. salmonicida</i> with other ABC-transporter proteins	117
Figure 31.	In vivo plasmid expression and localization of AbcA protein from pSC161 using the T7 polymerase expression system	119
Figure 32.	Expression, localization and purification of AbcA- β -galactosidase fusion protein as shown by SDS-PAGE and Western blot analysis using monoclonal antibody against <i>E. coli</i> β -galactosidase	121
Figure 33.	Nucleotide binding ability of the purified AbcA- β -galactosidase fusion protein	123
Figure 34.	Silver stained SDS-PAGE gel of LPS samples	125
Figure 35.	Sequences indicating mutation sites in the <i>abcA</i> gene P-loop constructed by site-directed mutagenesis	127
Figure 36.	Phylogenetic tree of putative transposases from ISA2 and other related bacterial insertion elements by GeneWorks	144

LIST OF ABBREVIATIONS

A600	Absorbance at 600 nm
ATP	Adenosine triphosphate
bp	Base pair
cAMP	Cyclic adenosine monophosphate
cfu	Colony forming unit
CRP	cAMP receptor protein
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid
h	Hour
kb	Kilobase
Kd	Kilodalton
LB	Luria-Bertani broth
L-agar	Luria-Bertani agar
LPS	Lipopolysaccharide
M	Molar
mg	milligram
μ g	microgram
min	Minute
ml	milliliter
μ l	microliter
mM	millimolar
Mr	Molecular weight

ng	nanogram
ORF	Open reading frame
PBS	Phosphate buffered saline
PEG	polyethylene glycol
pg	picogram
pI	Isoelectric point
pmol	picomolar
PSI	Pounds per square inch
rbs	ribosome binding sequence
sec	Second
SD	Shine Dalgarno ribosomal binding sequence
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPK buffer	The 10 X SPK buffer contains 200 mM Tris Cl (pH 8.0), 50 mM MgCl ₂ , 5 mM DTT, 1 mM EDTA, 500 mM KCl, and 50% glycerol.
TE buffer	10 mM of Tris and 1 mM of EDTA, pH 8.0
Tris	Tris (hydroxymethyl) aminomethane
X-gal	5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside

ACKNOWLEDGEMENTS

I am grateful to my supervisor Dr. T. J. Trust for his constant inspiration, guidance and encouragement. I thank all the members in Dr. Trust's lab, past and present, for their valuable help and support in my experiments. I also thank my family for their support and understanding during my program.

This study was supported by University of Victoria Fellowship and research grants to Dr. T. J. Trust.

INTRODUCTION

Bacterial S-layers

S-layers, or paracrystalline surface protein arrays, are regular two-dimensional assemblies of protein or glycoprotein monomers that constitute the outer layer of the cell envelope of a wide variety of bacteria (143, 191) (Fig. 1). The subunits in these S-layer lattices are held together and onto the underlying surface by non-covalent forces (142). S-layers were relatively unknown three decades ago. The first S-layer to be described was by Houwink in a study of a *Spirillum* species by electron microscopy in 1953 (94). Today, S-layers have been identified on hundreds of bacteria and archaea (143, 192, 193) and studies on S-layers have expanded from simple morphological analysis to considerations of their biochemistry, immunochemistry, molecular biology and their role in pathogenesis.

For comparative and classification purposes, S-layers have been classified according to their space groups, unit cell size, and the position of their protomers and pores relative to the symmetry elements (186). In theory, six distinct arrangements can be found for a P6 crystal, three for a P4, and one only for P3, P2 and P1 crystals. So far, P6, P4 and P2 symmetry lattices have been identified. Although P1 and P3 lattices are theoretically possible, they are relatively rare if they exist at all.

Biological functions of S-layers

Although a wide range of S-layers have been identified morphologically, further detailed studies on the biological functions of S-layers are still at an early stage. Up to now, bacterial S-layers have been

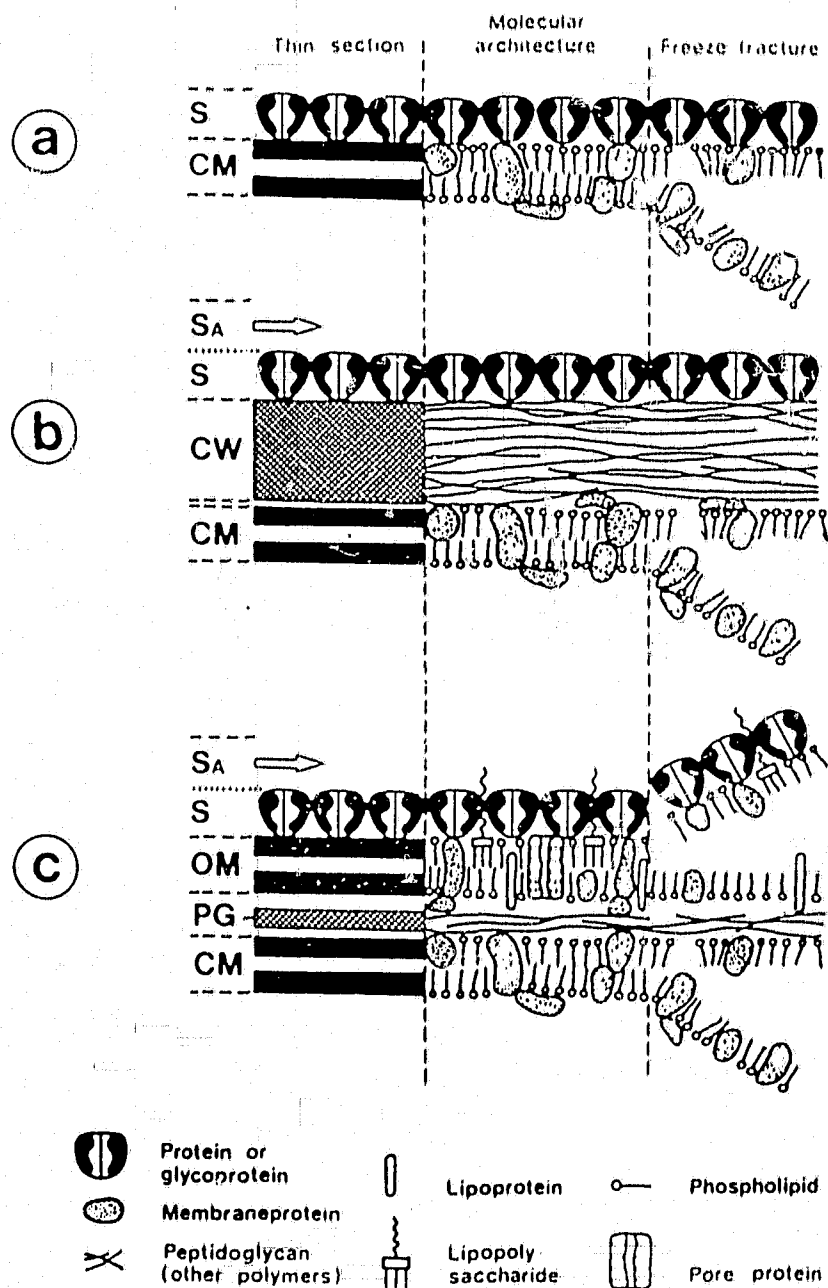


Fig. 1. Schematic illustrations of the bacterial envelopes containing S-layers. (a) Archaea. (b) Gram positive bacteria and some archaea possess a pseudomurein layer. (c) Gram negative bacteria. CM: cytoplasmic membrane; CW: rigid cell wall layer made up of peptidoglycan; OM: outer membrane; PG: peptidoglycan layer; S: the surface layer. SA: additional S-layers. From Sleytr and Messner (192).

shown to function in maintaining cell shape, blocking entrance of large size molecules, preventing binding of certain harmful molecules to the cell membranes, binding bacterial phages, and mediating cell adhesion. In some cases S-layers determine and maintain cell shape or contribute to envelope rigidity (192). This was best illustrated in *Halobacterium* strains where glycosylated S-layers are the exclusive cell wall component (141). Upon blocking the glycosylation by exposing growing cells to bacitracin, the cell shape changed from rods to spheres (232).

The physical characteristics of the protein meshwork determines the permeability of a particular S-layer. Ultrastructural studies have suggested the existence of channels of 2 to 6 nm diameter (143). This pore size would not significantly affect the permeation of smaller nutrients and degradation products, but could block lytic enzymes and bacteriophage particles from the environment which could be harmful to the cells (191, 192). As a protective coat, for example, the *Campylobacter fetus* S-layer prevents C3b binding to the bacterial cells (27) and renders the cells serum and phagocytosis resistant (26). S-layers have also been shown to protect cells from bacteriophages (105) and from predation by *Bdellovibrio bacteriovorus* (120), and also function as bacteriophage receptors (50, 95). From available data, it also appears that S-layers can provide organisms with advantages in cell adhesion and surface recognition (192). S-layers mediating cell adhesion have been demonstrated among bacterial cells (auto-agglutination) and between bacterial cells and epidermal surfaces (55).

The bacterial S-layer is also a promising tool for a variety of biotechnological applications. For example, with well defined molecular weight cut-off values (10,000-15,000 (143)), S-layers can be used as ultrafiltration membranes. In contrast to the amorphous structure and a

pore-size distribution of regular ultrafiltration membranes, S-layers provide a crystalline structure and uniform pore sizes. In addition, functional groups on the surface of the S-layers such as carboxyl, amino and hydroxyl groups can be modified to suit various applications. These structural features could be also useful for the immobilization of macromolecules, a process which traditionally employs amorphous polymers with a random structure and binding sites. In addition to these potential industrial applications, S-layers also have potential in biomedical research. Because of their abundant production and their outside location, S-layers could become ideal carriers for other proteins. Indeed as early as 1989, Yamagata, Udaka and their group reported that the human epidermal growth factor (hEGF) gene could be fused to *B. brevis* 47 "middle wall" S-layer protein gene and successfully expressed in *B. brevis* 47 (235). The protein was produced efficiently in a large amount (0.24 g/liter of culture), and the mature hEGF protein was correctly cleaved from the bacterial middle wall protein leader sequence and secreted.

S-layer proteins

S-layers are usually composed of a single protein species and are among the most abundant of all prokaryotic cellular proteins. Thus far, a number of bacterial S-layer proteins have been purified and biochemically characterized. The molecular weights of these proteins are in the range of 40 to 220 Kd (194). Some of the S-layer proteins are glycosylated, with a remarkable structural diversity of the carbohydrate chains even among strains of the same species (142). S-layers composed of two protein subunits, although rare, have been reported (86). S-layer proteins share a number of common features. For example, they contain 40 - 50% of hydrophobic amino acids, more acidic amino acid residues rather than basic residues (193), and a very low content of

Cys and Met residues. In terms of amino acid sequence, similar primary sequences can be seen among strains within the same genus such as *Methanothermus* (*M. fervidus* and *M. sociabilis*) (30), *Caulobacter* strains (shown by Southern hybridization) (195), *Rickettsia* (*R. prowazekii* and *R. typhi*) (33), and *Bacillus brevis* (*B. brevis* HPD31 hexagonal array protein HWP and *B. brevis* 47 middle wall protein MWP) (48). However, S-layer proteins from unrelated bacteria usually do not display sequence homology, except for a few examples such as the homology seen between the N-terminal regions of the *Acetogenium kivui* S-layer protein and *B. brevis* 47 middle wall protein (165).

S-layer protein genes

In contrast to their relatively simplicity with which S-layer proteins can be isolated, cloning and expression of S-layer protein genes has often proven to be difficult. In some cases, S-layer genes have had to be cloned on overlapping fragments (165). Partly due to these difficulties, and also due to the late start of genetic research on S-layers, studies of S-layer structural genes are still at a quite preliminary stage. Udaka and their coworkers cloned the first S-layer protein gene from *Bacillus brevis* 47 in 1984 (217). However they were not able to clone the promoter region into *E. coli*, and were subsequently forced to use *Bacillus subtilis* as the host cell (234). Since this first cloning of an S-protein gene, a number of S-protein genes have been cloned and sequenced. So far, the S-layer protein genes being sequenced include *Bacillus brevis* 47 (48, 214, 216), *Rickettsia rickettsii* (76), *Bacillus sphaericus* (29), *Acetogenium kivui* (166), *Bacillus brevis* HPD31 (48), *Halobacterium halobium* (128), *Haloferax volcanii* (201), *Methanothermus fervidus* and *Methanothermus sociabilis* (30), *Deinococcus radiodurans* (164, 166), *Campylobacter fetus* (25), *Rickettsia prowazekii* (33), *Thermus thermophilus* (56), *Lactobacillus brevis* (222) and *Caulobacter crescentus* (75). From

these studies it appears that S-layer proteins are usually encoded by single copy genes (195) and transcribed as monocistronic units (59, 222). In the case of *C. fetus*, where S-layer protein antigenic variability is displayed, there appear to be several copies of the gene, only one of which is expressed at a time (M. Blaser, personal communication). *B. brevis* is another exception. This organism has a polycistronic operon (*cwp* operon) since two different S-layer protein genes coding for outer and middle wall proteins (OWP and MWP) are expressed in this species (236). Although OWP and MWP are cotranscribed from the same operon, transcriptional terminator-like sequences were found following each of these genes (216). The monocistronic nature of other S-layer genes is also supported by the fact that transcriptional terminator-like sequences have been found immediately following the genes (25, 29, 48, 75, 128, 166, 222). For example, *T. thermophilus* HB8 S-layer gene has an 11-base-long inverted repeat with a predicted free energy value of -24.2 kcal/mol followed by a T-rich stretch 15 nucleotides downstream from the translational stop codon (56).

In most of the S-layer genes, a region coding for a protein leader sequence has been identified at the 5' end. In the case of *B. brevis* 47 middle wall protein gene, two tandemly located translation initiation sites were found (3). Translation initiation from these two sites resulted in two different leader sequences with the length of 54 residues and 23 residues respectively. However, the sequence of the mature protein remained the same since the precursors were cleaved at the same site. In the longer precursor, the initiation codon TTG was found to be efficiently used to code for a Met residue. The tandem translation initiation sites were also found in other *B. brevis* strains (48). Several S-layer proteins do not have a cleaved leader sequence. Examples include *Campylobacter fetus* strain 84-32 (23D) (25),

Caulobacter crescentus (59, 75), and *Rickettsia prowazekii* (33). In the latter case, the C-terminal region of the protein was proposed to have a potential transmembrane sequence which was cleaved after translocation.

S-layer protein gene promoters

As S-layer proteins are abundantly produced, S-layer protein gene promoters are generally considered to be efficient promoters. However, only a few S-layer protein gene promoters have been identified. In many cases, only a single promoter appears to be present (56, 59, 218). In other cases, S-layer protein genes have been shown to have more than one promoter (2, 222). In the case of *B. brevis* 47 which contains two S-layer protein genes coding for OWP and MWP, Adachi, Udaka and their coworkers found several tandemly arranged promoters in the 5' region of the cotranscriptional unit in the *cwp* operon (2). Two of the most active of these promoters, P2 and P3, displayed different transcriptional activities at different stages of cell growth. This promoter containing sequence also contains a 12-nucleotide palindromic sequence downstream from an infrequently used promoter P1 (234). This complex structure in the promoter region was found to be highly conserved among HWP (S-layer protein) genes from *B. brevis* strains HPD31, HPD52 and HP033, suggesting that the synthesis of the S-layer proteins is intricately regulated (48). In the case of *Lactobacillus brevis*, two promoters direct transcription of the S-layer protein gene (222). These two promoters function with roughly equal efficiency in exponential growth phase. In the case of *C. crescentus* which undergoes cell differentiation, S-layer gene transcription appeared not to be subject to developmental regulation (59). In the case of cloned S-layer protein gene promoters, the *T. thermophilus* HB8 S-layer gene promoter was recognized by *E. coli* RNA polymerase (56), while the *C.*

crescentus promoter was not recognized in *E. coli* although the sequence of the promoter was reasonably similar to the *E. coli* σ^{70} promoter consensus sequence (59). Fisher et al. proposed that transcription of the adjacent region or auxiliary transcriptional factors may have some effects on the transcription of the *Caulobacter* S-layer gene (59).

Aeromonas salmonicida

The genus *Aeromonas*

Aeromonads are Gram negative rod shaped bacteria that are native to aquatic and soil environments world wide (32). Many of them also cause diseases among both warm- and cold-blooded animals (106, 209). The genus *Aeromonas* was classified in the family *Vibrionaceae* in the latest Bergey's Manual (172), however a new family *Aeromonadaceae* has also been proposed (39). Using DNA-DNA reassociation kinetics and multilocus enzyme electrophoresis analysis, Janda et al. have identified at least 12 genospecies or hybridization groups (HG1-12) in this family: HG1 - *Aeromonas hydrophila*, HG2 - unnamed, HG3 - *Aeromonas salmonicida*, HG4 - *Aeromonas caviae*, HG5 - *Aeromonas media*, HG6 - *Aeromonas eucrenophila*, HG7 - *Aeromonas sobria*, HG8 - *Aeromonas veronii* biotype *sobria*, HG9 - *Aeromonas jandaei*, HG10 - *Aeromonas veronii*, HG11 - unnamed, and HG12 - *Aeromonas schubertii* (107).

With the exception of HG3 - *A. salmonicida*, the aeromonads are mesophilic and motile. A number of the motile aeromonads are associated with human infections such as septicemia, meningitis, osteomyelitis, gastroenteritis, and wound infections among pediatric and adult populations, both immunocompromised and otherwise healthy individuals (61, 224). The five implicated in human diseases are: *A. hydrophila*, *A. veronii* biotype *sobria*, *A. caviae*, *A. veronii* and *A. schubertii* (34). *A. hydrophila* causes fish diseases as

well (149). The psychrophilic non-motile group HG3 - *A. salmonicida* has also been shown to cause fish diseases (105, 209). A common feature shared by disease isolates in both mesophilic and psychrophilic groups is the presence of an S-layer (108, 111, 116, 219). In the case of *A. salmonicida* the S-layer has been demonstrated to be an important virulence factor (105).

The species

The first definitive isolation of *Aeromonas salmonicida* was from brown trout in Germany by Emmerich and Weibel in 1894 (51). Initially, it was thought to be the causative agent for fish furunculosis in hatcheries only. But studies by Plehn showed that the disease was widely prevalent in Bavarian trout strains (171). At the same time, a number of investigations showed that the disease was widely spread out in many European countries (63, 144, 170). The first description of its occurrence in the Americas was by Marsh in Michigan hatcheries in 1902 (134). It was later found in wild salmonids in B.C. (47) and also other regions in Americas. Now, the occurrence of *A. salmonicida* has been reported virtually all over the world, including Australia and Asia.

When *A. salmonicida* was first isolated, it was called *Bacterium salmonicida* (51). In 1953, Griffin *et al* (82) proposed that the species *B. salmonicida* be included in the Genus *Aeromonas* as *Aeromonas salmonicida*. Since then, although arguments have persisted on the classification of the species, the organism has remained in Genus *Aeromonas*. Bergey's Manual describes three subspecies of *A. salmonicida* (172). Subspecies *salmonicida* produces a brown pigment on media containing 0.1% tyrosine or phenylalanine, does not produce indole, and does hydrolyze esculin and ferment mannitol. Subspecies *achromogenes* does not produce the brown

pigment, may produce indole, and does not hydrolyze esculin and ferment mannitol. Subspecies *masoucida* does not produce the brown pigment, does produce indole, hydrolyze esculin, and ferment mannitol (172). For working purposes, the subspecies *salmonicida* and *achromogenes* are termed "typical" *A. salmonicida* strains and cause furunculosis in salmonids. However, there have been a number of other reports concerning "atypical" *A. salmonicida* strains causing diseases in other fishes such as goldfish, minnows, carp, as well as salmonids. This group of strains usually exhibits fastidious nutritional requirements, slow growth and distinctive biochemical properties (103). The diseases are often chronic and inflammatory, and can involve surface ulceration and erythrodermatitis (28, 89, 160, 190, 197). In the late 70's, McCarthy undertook a comprehensive analysis in which 25 "atypical" strains were compared to 145 other bacteria, primarily "typical" strains of *A. salmonicida* and various representatives of the motile Aeromonads (138). From his phenotypic and genotypic analysis, he proposed that the species *A. salmonicida* include three subspecies. The first subspecies comprised the "typical" strains of *A. salmonicida* isolated from salmonid fish species. The second subspecies was also restricted to isolates from salmonid fish and retained the name *achromogenes* by historical precedent. The third subspecies proposed was subspecies *nova*, which was retained for *A. salmonicida* isolates from non-salmonid sources. A DNA-DNA hybridization study by Belland and Trust (22) confirmed McCarthy's proposal.

Pathogenesis

A. salmonicida produces fish diseases in freshwater, estuarine, and marine environments (209). The diseases caused by this organism in the sea are often related to the establishment of an asymptomatic carrier state during

the fishes' early growth in fresh water. The latent infection may not be detectable by culture methods, so corticosteroid injections and heat stress are commonly employed to promote acute infection (31). Studies using this technique have shown that latent infection with *A. salmonicida* can be common in salmonids. Furthermore, strains isolated from carrier fish show no reduced virulence. The organism appears to be carried in the kidney and later becomes active when fish go to sea or are stressed in some fashion (209). Although the latent state of the disease is not clearly understood, McIntosh and Austin suggested that L-forms may play a role (139). After Atlantic salmon were injected with a suspension of *A. salmonicida* L-forms, no clinical signs of disease were observed, and parental type or L-form colonies were not recovered. However, microscopic examination revealed possible L-form cells remaining within the fish tissues, particularly in the kidney (139).

There have been a number of virulence factors reported for *A. salmonicida*. Among them, the best described is the surface protein array of *A. salmonicida* known as A-layer, which will be discussed in detail later in this dissertation. Other virulence factors include proteases, glycerophospholipid-cholesterol acyltransferase (GCAT), as well as leukocytolytic and hemolytic activities. The proteinases and hemolysins identified include a 70 Kd serine proteinase, a 56 Kd hemolysin (66), a 25.9 Kd H-lysin (broad-spectrum hemolysis) (206), a T-lysin (lysis of trout erythrocytes) (205), and a 200 Kd salmolysin (lysis of salmonid erythrocytes) (154).

When injected intramuscularly, the 70 Kd serine proteinase produced a lesion histologically similar to natural infections (65). The gene (*aspA*) has been cloned and sequenced. The predicted amino acid sequence indicated that the protein is of 64,173 Dalton, and has a 24-amino acid residue signal peptide and the NGTS consensus sequence of serine proteinases (231). Further

evidence for a role for protease as a virulence factor in the pathogenesis of fish furunculosis has been provided by Sakai (181). Sakai found that although the protease-deficient mutant NTG-1, which was induced with N-methyl-N'-nitro-N-nitrosoguanidine, remained autoagglutinative, hemagglutinative, serum resistant, adhesive, hemolysin positive, and leukocytolysin positive, its LD₅₀ in sockeye salmon and rainbow trout was increased to more than 10⁸ as opposed to 10⁴-10⁵ of its virulent parent strain A-7301. NTG-1 strains were also eliminated from rainbow trout in a short time. In other studies, Lee and Ellis reported that purified *A. salmonicida* extracellular protease was lethal to Atlantic salmon (*Salmo salar* L.) when intraperitoneally injected (129). The LD₅₀ was 2,400 ng/g fish.

In the case of hemolytic activities, Titball and Munn purified the H-lysin and showed that it had horse erythrocyte lysis activity and GCAT activity. It also exhibited cytotoxicity with rainbow trout gonad cells and rainbow trout leukocytes. However, when the H-lysin was injected intravenously into rainbow trout, no pathological effect was observed (206). Fyfe et al. showed that the 56 Kd hemolysin had hemolytic activity with trout erythrocytes, although they did not test for lysis of horse erythrocytes (66). In other studies, Nomura et al. purified the heat labile salmolysin from the culture supernatant and showed that it was a glycoprotein which lacked protease activity, but was lethal to rainbow trout when injected intramuscularly (154).

A GCAT/LPS complex was isolated and purified from *A. salmonicida* extracellular products (130). This complex was lethal for Atlantic salmon (*Salmo salar* L.) by injection of 0.045 mg per g of body weight. The virulence mechanisms of this 2,000 Kd complex may include its hemolytic, leukocytolytic and other cytotoxic activities. The LPS was found to play an

active role in the complex. The specific hemolytic activity and lethal toxicity of the complex was about eight times higher than free GCAT. Also, the complex was more resistant to proteolytic and heat inactivation than free GCAT (130). Lee and Ellis also found an additive relationship between purified protease and the hemolysin complex when injected in Atlantic salmon by the intraperitoneal route (129).

Thornton et al reported two attenuated mutants that possessed A-layer and were avirulent to salmonid fish (204). These mutants were deficient in aerobic metabolism. In addition, although the cell surface composition of these mutants were very similar to wild type strains, their A-layer architecture was drastically altered. Electromicroscopic examination indicated that the A-layer in these strains might be multilayered or aggregated rather than a monomolecular layer. This may indicate that the native organization of the A-layer is essential for virulence.

A-layer

A. salmonicida has the capacity to produce a paracrystalline surface-protein array (Fig. 2) (111, 219), known as the A-layer, which appears to be essential to virulence (105). Its location external to the outer membrane was demonstrated by thin section electromicroscopy (105, 210) and cell surface labeling using ^{125}I DISA (diazotized (^{125}I) iodosulfanilic acid) (111).

Morphology

The ultrastructure of the A-layer is best observed when the layer has sloughed off the cell surface during preparation of samples for negative staining and electron microscopy. Early studies showed that A-layer displayed P4 symmetry (105, 210). Two tetragonal patterns, type I and type II, were subsequently observed by image enhancement techniques in

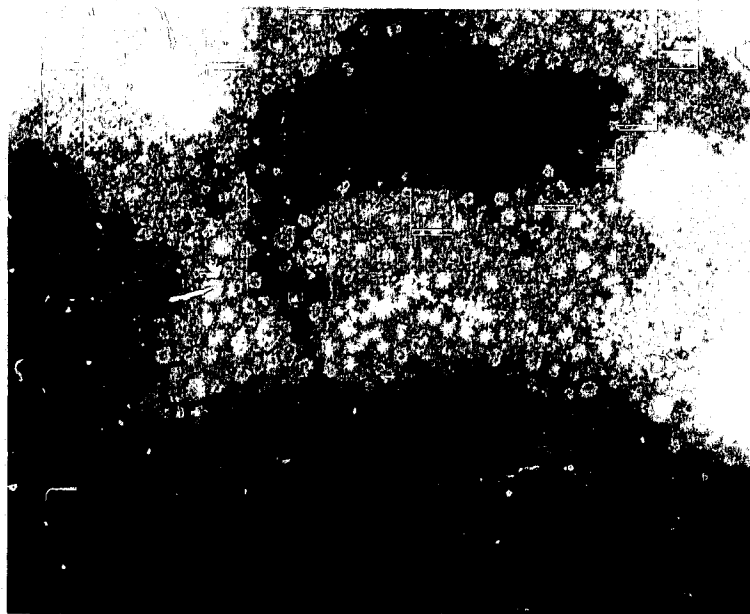
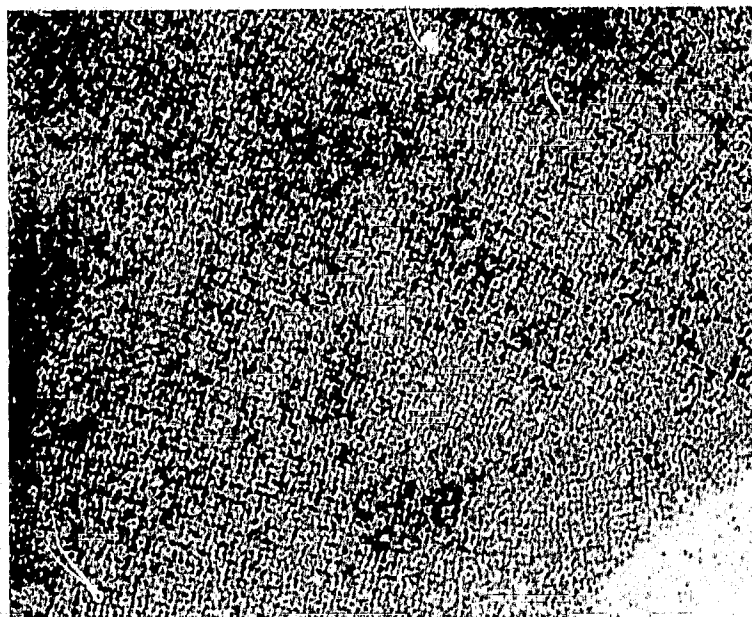
A.**B.**

Fig. 2. A: Negatively stained electron micrograph of A450 cell and sloughed A-layer sheets (X 150,000). **B:** Negatively stained electron micrograph of a sloughed A-layer sheet from A450 (X337,000) (Courtesy of R. Garduño).

two-dimensional (2-D) mass distribution projections of negatively stained A-layer preparations (198). Dooley et al. later showed that the type I pattern was the result of two layers in a back to back arrangement, while projection through the single layer gave the type II pattern (44). Using computer-simulated superimposition, Garduño and Kay recently confirmed that the type I patterns were indeed formed from superimposed A-layers during preparation for negative staining (70). Three dimensional reconstruction of the A-layer (44) showed a lattice constant of 12.5 nm, and a major tetragon and a minor tetragon were observed at a resolution of 1.6 nm. The major tetragon contains the four major domains of four subunits, and forms a large depression towards the inside of the layer. The minor tetragon is located toward the outside of the layer and has been proposed to provide connectivity within the layer. This structure provides the surface of the layer with a certain amount of three dimensional architecture. Normal organization of the A-layer seems to require the presence of Ca^{++} , since Ca^{++} limitation resulted in a sequence of structural rearrangements in the A-layer (72).

Subunits

The A-protein has been identified as the protein subunit of the A-layer and has been isolated, purified, and characterized biochemically (111, 169). It is the major protein component in outer membrane fractions of A-layer producing strains. The apparent molecular weight of A450 A-protein is 48,000 to 53,000 as determined by SDS-PAGE. Early studies showed that A-protein had several isoelectric forms with pIs of 4.8-5.3, however, later studies showed that the native protein on the surface of mid-exponential phase cells had a single pI corresponding to the most basic value in the previously observed pI range (167). Amino acid composition studies showed that A-protein contained 45% non polar residues (Val, Met, Ile, Leu, Ala, Phe, Trp, and Pro)

and the N-terminal sequence was shown to have very few charged residues. Other studies showed that A-proteins from various *A. salmonicida* strains had conserved biochemical properties such as molecular weight, amino acid composition, N-terminal sequence, pI, chymotrypsin digestion sensitivity and immunological reactivity (167).

A-layer formation and anchoring

In a transposon mutagenesis study using *A. salmonicida* A449, Belland and Trust demonstrated that two Tn5 insertion mutants, TM1 and TM2, accumulated the majority of their A-protein in the periplasm (20), suggesting that A-protein was translocated through the periplasmic space, and that at least one gene product was required for A-protein export. Preliminary studies indicate that the Tn5 insertions in mutants TM1 and TM2 are in different loci. The location of the Tn5 insertion in TM2 has been recently mapped relative to the A-protein gene (155), and the DNA sequence of the region revealed an open reading frame coding for a putative protein with high sequence similarity to another bacterial transport protein (Noonan, personal communication).

Another Tn5 insertion mutant *A. salmonicida* A449-TM5 excreted A-protein into the culture supernatant in quantities approaching 1 mg A-protein per ml (20). When the LPS of this mutant was examined by silver staining of SDS-PAGE, O-polysaccharide chains were shown to be absent. Electron microscopy showed however that A-protein had assembled into sheets. These findings suggest that the anchoring of A-layer to the cell surface requires intact LPS O-chains. This hypothesis was supported by the evidence that many O-polysaccharide chains penetrated the A-layer and were exposed on the cell surface (36) and that A-protein could be reattached to the *A. salmonicida* cell surface as long as the cell possessed LPS O-chains (167).

Virulence

Among all of the bacterial S-layers studied to date, the *A. salmonicida* A-layer is the best described in terms of biological activities. Early studies by Ishiguro and colleagues demonstrated the importance of the A-layer in the virulence of *A. salmonicida* (105). Most cells in a culture grown at higher than optimum temperatures ($>26^{\circ}\text{C}$) would die, and the remaining cells were A⁻ and avirulent. The LD₅₀ for coho salmon by the intraperitoneal route was increased from 8×10^3 cfu per 7 g fish for the wild type strain to 1×10^8 cfu per 7 g fish for the attenuated strains. In another experiment the virulence of the single-site Tn5 insertion periplasmic accumulating mutant TM4, i.e., an isogenic A-layer mutant (20), was compared with its parent strain. While the LD₅₀ for the parent A449 strain was $<10^3$ per fish, the mutant was avirulent (209).

The role of A-layer in virulence was further illustrated by following the fate of injected A⁺ and A⁻ cells in fish tissues (209). Intramuscular injection of 10^4 virulent A⁺ cells into fish was followed by a very early bacteremia and extremely rapid accumulation of organisms in the spleen, kidney, and liver. The viable A⁺ cells in each organ multiplied to very high levels, the tissues became necrotic after 72 h, and all fish died 72-96 h post-infection. Septicemia was first seen 24 h post-infection. Injection of an identical dose of high-growth temperature derived A⁻ cells was also followed by an initial accumulation in the spleen, kidney, liver, and heart. By 48 h post-infection, however, the fish had cleared itself of viable A⁻ cells, and all fish survived. When fish were injected with as many as 10^6 A⁻ cells, the cells persisted in the organs in high numbers for several days, but were rapidly cleared after 96 h; all fish survived. In neither experiment with A⁻ cells were bacteria recovered from the blood (209).

The role of the A-layer in pathogenesis was also investigated in serum resistance experiments (150). With both non immune and immune fish and rabbit serum, Munn and coworkers compared wild type *A. salmonicida* strains with high temperature mutants and spontaneous mutants that lack A-layer or both A-layer and LPS O-chains. While A⁺ wild type strains showed resistance to all sera applied, A⁻ strains, regardless of their LPS, demonstrated significantly less resistance. This indicated that A-layer contributed protection against the bactericidal activities of both immune and non immune sera.

Other biological functions

The A-layer has been demonstrated to bind to a wide variety of substrates including macrophages (211) and a number of molecules including Congo red (104), porphyrins (113), immunoglobulin (168), fibronectin and laminin (41), and collagen type IV (212).

Interaction between *Aeromonas* cells and macrophages has been well documented with a number of cell lines including resident peritoneal marine macrophages, rainbow trout head kidney tissue macrophages (211), mammalian cell line BHK-21, salmonid cell line RTG-2 (157, 158), peritoneal elicited-exudate cells from rainbow trout and coho or masu salmon (180), elicited or activated peritoneal macrophages from brook trout (156), rainbow trout blood leukocytes (110), salmonid cell lines (182), murine macrophage cell line P388D1 (71) and rainbow trout macrophages (69). Recent studies by Garduño and Kay using murine macrophages and rainbow trout macrophages showed that only A⁺ bacterial cells were readily internalized in phosphate-buffered saline in the absence of opsonins (69, 71). In addition, A-layer coated latex beads were more efficiently phagocytosed than A-protein coated beads, while uncoated beads were not taken up by macrophages,

indicating that A-layer mediated the phagocytosis of both A⁺ cells and A-layer coated beads.

Although the A-layer appears to mediate phagocytosis, it is probably not cytotoxic. While A⁺ cells were shown to be more cytotoxic to macrophages than A⁻ cells, A-protein and A-layer coated latex beads failed to cause morphological changes in macrophages when phagocytosed (71).

The A-protein subunit has been shown to bind Congo red (104). On Congo red containing solid culture media, red-colored A-layer⁺ colonies can be readily distinguished from white or light orange-colored A-layer⁻ colonies. Two classes of binding have been observed. When high concentrations of Congo red were used, a nonspecific hydrophobic interaction between Congo red and A-protein was demonstrated at a dye-to-protein molar ratio of about 8 - 30. This nonspecific binding could be enhanced by higher salt concentrations (104, 113). Specific binding was observed at low Congo red concentrations and this binding was competitively inhibited by protoporphyrin IX and hemin and vice versa (113). Based on this competitive inhibition and the similar structures of Congo red and porphyrins, it was hypothesized that a hydrophobic porphyrin binding domain exists in the A-protein which could act as a nucleation site for further binding at higher Congo red concentrations. The porphyrin binding may represent an initial stage in iron transport.

A⁺ cells of *A. salmonicida* also bind rabbit IgG and human IgM specifically (168). In contrast to *Staphylococcus aureus* protein A, which binds immunoglobulin Fc fragments, binding of native A-layer to immunoglobulins required structurally intact IgG. In addition, a special molecular arrangement of A-protein monomers in a native A-layer was required for the binding since purified A-protein bound IgG only weakly and

reconstituted array on A-layer negative cell surfaces did not bind IgG. The immunoglobulin binding by A-layer was shown to be saturable, but there was no single easily definable binding site on the A-protein monomers. Indeed one IgG molecule was estimated to bind four or five A-protein monomers, leading to the speculation that a four fold symmetric pit in the A-layer formed by four monomers may act as a specific trap for immunoglobulin molecules. This immunoglobulin binding activity may serve as a mechanism to shield the bacterium from host immune defenses (168).

While immunoglobulin binding required an intact supramolecular array and could not be inhibited by A-protein, the binding of the extracellular matrix proteins laminin and fibronectin was significantly inhibited by A-protein and its 37.6K N-terminal major trypsin-resistant domain. This suggests that the binding sites for these matrix proteins are localized in this segment of A-protein (41). Since fibronectin is present in body fluids including blood plasma, it was suggested that binding of this protein to the surface of the bacterium via the A-layer could block the host's immune response to the bacterium by sterically masking immunogenic epitopes. The binding might also facilitate adhesion of the bacterium to host cells such as macrophages. In addition, laminin is a major component on basement membranes, and the binding of this glycoprotein might facilitate binding of *A. salmonicida* to basement membranes in ulcerated regions. Another basement membrane protein, collagen type IV, has also been reported to bind to the A-layer, and the binding of this protein was rapid, specific, saturable, and essentially irreversible. This might add to the ability of the bacterium to bind the basement membranes of ulcerated regions (212).

Other *Aeromonas* S-layers

The S-layers of mesophilic aeromonads have been shown to have very similar morphology to the A-layer of *A. salmonicida* (116, 151). The best described is that of the *A. hydrophila* strain TF7. This S-layer shows a lattice constant of 12.2 nm and two structural units, a major tetragon and a lesser tetragon (151, Dooley, 1989 #91). It has an apparent molecular weight of 52,000, and a measured pI of 4.6 (43), similar to *A. salmonicida* A-protein. However, differences exist in the primary structures and antigenicity of these S-layer proteins (119). Also, in contrast to A-protein which is antigenically conserved, the S-layer proteins from mesophilic aeromonads displayed considerable antigenic variability (119). Furthermore, unlike A-layer, the *A. hydrophila* TF7 S-layer apparently does not possess a wide range of binding activities, and its role in fish pathogenesis remains undefined. Recently, however, a Tn5 transposon insertion mutant was isolated in *A. hydrophila* TF7 which produced a truncated S-layer protein, which did not form an S-layer (202). This mutant was shown to be more sensitive to immune trout serum and have a 5-fold higher LD₅₀ in rainbow trout compared to the wild type strain, indicating a possible role of the S-layer in pathogenesis (Thomas, personal communication).

Lipopolysaccharides (LPS)

Early reports suggested that *A. salmonicida* LPS had no toxic effects on salmonid fish through injection of up to 0.714 g/kg of fish (161, 230). However, serum resistance experiments have shown that the LPS is a virulence factor (150). When A⁻ O⁺ (A-layer negative, O-polysaccharide chains positive) and A⁻ O⁻ mutants were examined in serum killing experiments, O-chains were shown to contribute to the serum resistance of *A.*

salmonicida, especially in normal sera. Comparison with A⁺ and A⁻ strains suggested that O-chains were the major contributors to serum resistance.

The structural morphology of LPS from various strains of *A. salmonicida* has been analyzed by intrinsic ³²P-radiolabeling and silver staining by Chart et al. (36). The high-molecular-weight fraction of LPS was resolved into a small number of distinct bands, indicating a very high degree of homogeneity in O-polysaccharide chain length. These LPS O-chains from diverse strains are antigenically cross-reactive. Their monosaccharide composition is also very similar (36). When compared with S-layer producing mesophilic aeromonads, Dooley et al. observed that O-polysaccharide chains were longer in *A. salmonicida*, and there were common epitopes and species-specific epitopes in the two species (45). Chart et al. also showed that some LPS O-polysaccharide penetrated to the outer surface of the A-layer (36). These exposed O-polysaccharide still functioned as phage 55R-1 receptors, although the phage adsorption to an A⁺ strain was slower than that to an A⁻ strain. This was also confirmed by positive reaction of anti-O-polysaccharide monoclonal antibody with A⁺ cells (36). A similar arrangement of LPS and S-layer was also observed in *A. hydrophila* (45). Because of their surface exposure, the O-chains were strongly immunogenic and appeared to be antigenically conserved (36). However, while both LPS and A-protein promoted antibody response in fish (35, 161), neither served as a protective antigen (209).

Purpose of this dissertation

Infectious diseases of fish are a world wide problem. Today, as the world population increases rapidly and people are more and more concerned about the preservation of our limited natural resources, more fish farms are

required to provide daily source of fish for the ever increasing world demand. As a result, infectious diseases in fish farms become a major problem in this intensive culture practice, and often lead to serious economic consequences. Because of this economic significance, the control of fish disease is urgently needed.

Aeromonas salmonicida is a fish pathogen of major importance. Its "typical" and "atypical" strains cause diseases in various fishes, and have been isolated world wide. Although a number of virulence factors have been suggested for *A. salmonicida*, the best described virulence factor is the surface protein layer A-layer. The A-layer of *A. salmonicida* has been shown to be involved in fish pathogenesis using an isogenic mutant in serum resistance and fish injection experiments. While detailed structures and biological functions of most other *A. salmonicida* virulence factors are still undefined, studies of the *A. salmonicida* A-layer have revealed a number of biological activities which are relevant to fish pathogenesis. In addition, this surface layer has provided a good example for the study of bacterial surface layers which have been found in hundreds of bacteria and archaea. The S-layers comprise up to 15% of the cellular protein and can be efficiently exported through cell membranes and assembled to cover the entire cell surface. The S-layers usually constitute the outermost layer of the cell envelope and directly interact with various molecules, cells, and environmental conditions, as well as the underneath cell surface. Indeed, *A. salmonicida* A-layer is one of the best studied bacterial S-layers with known three dimensional ultrastructure and a number of defined biological functions. Therefore, the impact of the study on *A. salmonicida* A-layer is two-fold, it involves economically important fish pathogenesis and the biologically important bacterial S-layers.

Molecular biological characterization of the gene for the A-layer subunit A-protein is essential for understanding the gene structure, expression and regulation. It will also provide information on the protein's primary and tertiary structure, biological activity, and location of their corresponding domains. However, prior to this study, most of the work on A-protein dealt with biochemical, structural and functional characterizations. A few pioneer genetic studies by Bellanc (20, 21) and Phipps (167) not only reflected the needs, but also provided a solid ground, for further investigations. Following the cloning of the *A. salmonicida* A-protein gene, it became possible to carry on the detailed characterization and analysis of the gene. This study was therefore aimed at understanding the structure, expression and regulation of the A-protein and its gene at the molecular genetic level. The results described in this dissertation are organized into four major groups, i.e., structural characterization of the A-protein gene, transcriptional studies on the A-protein gene expression, native insertion elements which affect the A-protein gene expression in *A. salmonicida*, and a structural and functional description of a downstream gene which may play a role in A-protein gene expression.

MATERIALS AND METHODS

Bacterial strains, vectors and media

A. salmonicida strains from the culture collection of T. J. Trust are listed in Table 1. *E. coli* strains used were DH5 α for recombinant plasmids (87) and JM109 for M13 recombinants (237). All strains were grown in L-broth or on L-agar (145). Wild type *A. salmonicida* strains were grown at 20°C. Strain A450-3 and other 30°C mutant *A. salmonicida* strains were grown at 20°C or 30°C. *E. coli* strains with plasmids were grown at 37°C with antibiotics. Antibiotics used included ampicillin (50 mg/L), kanamycin (50 mg/L) and streptomycin (100 mg/L). When required, CaCl₂ or MgCl₂ (1 - 30 mM) in the presence and absence of ethylene glycol-bis (β -aminoethyl ether) N-N'-N'-N'-tetraacetic acid (EGTA, 5 and 10 mM, Sigma Chemical Co., St. Louis, MO), or FeCl₃ (0.01 - 1.00 mM) in the presence and absence of 2, 2'-dipyridyl (0.01 and 0.10 mM, Sigma), was added. Vectors used including plasmid and phage vectors are listed in Table 2. When anaerobic incubation was required, a BBL GasPak was used in a BBL GasPak jar (BBL Microbiology Systems, Cockeysville, MD) with shaking.

DNA techniques

DNA preparation

Aeromonas chromosomal DNA

A 50 ml-culture of *Aeromonas* cells was centrifuged at 5,000 X g for 10 min and the cell pellet was washed once in 20 ml of saline-EDTA (0.75% NaCl (v/v) and 5 mM EDTA). The cells were resuspended in 4.25 ml of saline-EDTA and SDS was added to a final concentration of 3% (w/v). This cell suspension was then heated to 50°C for 25 min with occasional

Table 1. *Aeromonas* strains used in this study

Species	Strain	Source	S-layer
<i>A. salmonicida</i> typicals	A185	Canada	+
	A202	Japan	-
	A203	Japan	-
	A251	Atlantic salmon, U.K.	-
	A362	Atlantic salmon, U.K.	+
	A395	U.K.	-
	A440	Brook trout, U.S.A.	-
	A447	U.K.	-
	A449	Brown trout, France	+
	A450	Brown trout, France	+
	A451	Rainbow trout, France	+
	A488	Brook trout, U.S.A.	+
	A500	Atlantic salmon, U.K.	+
	A591	Canada	+
atypicals	A206	Japan	-
	A400	Goldfish, Australia	+
	A401	Goldfish, Australia	-
	A419	Goldfish, U.S.A.	+
	A438	Coho salmon, Canada	-
	A460	Atlantic salmon	+
	A461	Atlantic salmon, Canada	+
	A477	Carp, The Netherlands	+
	A480	Carp, The Netherlands	+
mutants	TM1	Tn5 insertion mutant of A449	-
	TM4	Tn5 insertion mutant of A449	-
	CEG4	30°C mutant of A449	-
	CEG6	30°C mutant of A450	-
	CEG7	30°C mutant of A450	-
	CEG22	30°C mutant of A449	-
	CEG24	30°C mutant of A449	-
	CEG27	30°C mutant of A450	-
	CEG28	30°C mutant of A450	-
	CEG29	30°C mutant of A450	-
	A450-2	30°C mutant of A450	-
	A450-3	30°C mutant of A450	-
<i>A. hydrophila</i>	TF7	Trout, Canada	+
	A274	Sloth, Australia	+
<i>A. veronii</i> biotype sobria	A700	Human diarrhea	+
	A702	Human diarrhea	+

Table 2. Vectors used in this study

Name	Type	Reference
pUC18/19	Plasmid	(237)
pTZ18R/19R	Plasmid	(140)
pMC1871	Plasmid	(188)
pMMB67EH/HE	Plasmid	(64)
pDSK19	Plasmid	(114)
pLG338	Plasmid	(199)
pSU2718	Plasmid	(135)
M13mp18/19	Phage	(220)
λ gt11	Phage	(238)

swirling and cooled in tap water. A 1.25 ml volume of 5 M sodium perchlorate was added and the solution was swirled for a few seconds. After adding 6 ml of chloroform and hand-shaking for 15 min, the aqueous layer was separated from the organic phase by centrifugation at 10,000 X g at 4°C for 15 min. This chloroform extraction was repeated once more. Two volumes of cold ethanol were added 1/4 at a time while stirring and spooling with a glass rod. Excess ethanol was removed and the DNA was dissolved in minimum amount of 0.1 X SSC. The solution was then adjusted to 1.0 X SSC and treated with RNase at a concentration of 20 µg/ml, chloroform-extracted once with gentle shaking, and ethanol precipitated.

Plasmid DNA preparation

Sambrook's method

Plasmids were prepared according to Sambrook's method (183). Cells in overnight cultures were collected by desk top centrifugation (Eppendorf Centrifuge 5415C, Brinkmann, Westbury, N.Y.) at 2940 X g for 2 min and the cell pellets were resuspended in solution I (50 mM glucose, 25 mM TrisHCl (pH 8.0) and 10 mM EDTA (pH 8.0)). Two volumes of solution II (0.2 N NaOH and 1% (w/v) SDS) were then added and the tubes were gently mixed and incubated on ice for 5 min. A 1.5 volume of solution III (3 M sodium acetate (pH 4.8)) was then added, and after mixing, the tubes were incubated on ice for 10 min. The mixture was then centrifuged at 16,000 X g on an Eppendorf centrifuge for 10 min. The supernatant was extracted with an equal volume of phenol-chloroform (1:1 mix), followed by extraction with an equal volume of chloroform. An equal volume of isopropanol was then added, mixed and incubated on ice for 10 min to precipitate DNA. After 10

min Eppendorf centrifugation (16,000 X g), the DNA pellet was washed once with 70% (v/v) ethanol and dried under vacuum.

Riggs' method

For screening of large number of plasmids, a quick method was used to prepare DNA (177). Cells in 1 ml overnight cultures were pelleted by centrifugation at 2940 X g for 2 min. The cell pellets were resuspended by vortexing in a lysis buffer containing 8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50 mM EDTA, and 10 mM TrisHCl (pH 8.0). The tubes were boiled in a boiling water bath for 45 sec, and centrifuged for 15 min at 16,000 X g. Supernatants were collected and a 0.1 volume of 3 M sodium acetate (pH 4.8) and one volume of isopropanol were added. The tubes were kept on ice for 10 min and centrifuged at 16,000 X g for 10 min afterwards. The pellets were washed once with 70% (v/v) ethanol, dried under vacuum and dissolved in 30 μ l of TE buffer (10 mM of Tris and 1 mM of EDTA, pH 8.0) for restriction digestion and gel electrophoresis.

M13 DNA preparation

Two phage plaques were picked from a plate to inoculate 2 ml of a log-phase culture of *E. coli* JM109, and the culture was incubated at 37°C for 2 h with shaking. This culture was then used to inoculate 45 ml of L-broth and incubated for 6 h. For double stranded DNA preparations, the cell pellet was separated from the supernatant by centrifugation at 4,000 X g for 5 min. Phage particles in the supernatant were precipitated by addition of a 1/20 volume of 40% (w/v) polyethylene glycol (PEG, Mr=8,000) and a 1/20 volume of 4 M NaCl. The solution was left at 4°C overnight and the phage particles collected by centrifugation at 10,000 X g for 30 min at 4°C. The tube wall was carefully dried with a piece of Kimwipe and the pellet was resuspended in 0.4 ml of

distilled water. Phage DNA was then extracted 3 times by equal volumes of phenol, phenol-CHCl₃ (1:1 mix) and CHCl₃ respectively, and ethanol-precipitated (183).

Oligonucleotide preparation

Oligonucleotides were either ordered from the Regional DNA synthesis Laboratory (University of Calgary, Calgary, Alberta, Canada) or synthesized on a PCR-MATE EP DNA Synthesizer, Model 391 (Applied Biosystems, Foster City, CA), on a 40 nM scale.

When synthesized on the PCR-MATE EP DNA synthesizer, the beads with oligonucleotide product were washed off from the synthesizing column by concentrated ammonium hydroxide and collected in an Eppendorf tube and then capped tightly. After incubation at room temperature for 2 h with occasional vortexing, the tube was spun for 30 sec in a desk top centrifuge at 16,000 X g. The supernatant was carefully transferred to a fresh 1.5 ml screw-capped tube and incubated at 55°C for more than 5 h, or overnight. The tube was cooled down on ice before opening. A 100 µl volume of the ammonium hydroxide solution was transferred to a fresh tube (the remainder was stored in at -20°C for future use), dried in a SpeedVac Concentrator and redissolved in 100 µl of distilled water. The solution was measured at 260 nm and the oligonucleotide concentration was calculated as: $\text{DNA (ng/}\mu\text{l)} = A_{260} \times 50 \times \text{dilution factor}$. The preparation was stored at -20°C.

Melting temperatures of the synthesized oligonucleotides were calculated as $T_m = 69.3 + \{41 \times (G+C) - 650\} / n$, where n is the number of bases in the oligonucleotides.

Molecular cloning

Preparation of *E. coli* competent cells

One ml of L-broth was inoculated with a single colony and incubated at 37°C overnight with shaking. This culture was used to inoculate 100 ml of fresh L-broth and incubated at 37°C for 2.5 h with vigorous shaking. The cell density was about 0.45-0.55 at A_{600nm}. The culture was cooled on ice and all further procedures were performed at 4°C or on ice. The culture was centrifuged at 4,000 X g for 5 min and the pellet was resuspended in 20 ml of ice cold CM1 buffer (10 mM sodium acetate (pH 5.6) 50 mM MnCl₂ and 5 mM NaCl) followed by 20 min incubation on ice. Then the cells were centrifuged again as above and the pellet resuspended in 2 ml of ice cold CM2 buffer (10 mM sodium acetate (pH 5.6) 5% (v/v) glycerol, 70 mM CaCl₂ and 5 mM MnCl₂). The cell suspension was divided into 40 µl aliquots and stored at -70°C.

DNA restriction digestion

One-pho-all plus buffer from Pharmacia (Uppsala, Sweden) was used for most restriction digestions including double digestions. Reactions were usually incubated at optimum temperature for longer than 2 h. For double digestions where One-pho-all buffer was not suitable, 1X SPK buffer was used, except for reactions containing *Sma*I which does not work with the SPK buffer. The 10 X SPK buffer contains 200 mM TrisHCl (pH 8.0), 50 mM MgCl₂, 5 mM DTT (dithiothreitol), 1 mM EDTA, 500 mM KCl, and 50% (v/v) glycerol.

DNA ligation

Ligation reactions were carried out as previously reported with some modifications (183). DNA restriction fragments were separated by agarose gel electrophoresis and the fragments to be cloned were excised and frozen with 200 µl of phenol. Then the gel slices were crushed by a sterile needle while

frozen, thawed and centrifuged at 16,000 X g for 15 min. The upper phase was extracted with an equal volume of 1:1 phenol-chloroform, followed by an equal volume of chloroform, and the DNA was precipitated as described in the plasmid preparation section. Vector DNA was digested with appropriate restriction enzymes and added to a reaction mix containing the purified DNA fragment, 30 mM TrisHCl (pH 7.8), 10 mM DTT, 1 mM ATP and 2 units T4 DNA ligase (Pharmacia). To reduce self-ligation, if the vector DNA was digested by a single enzyme, the digested vector DNA was treated with 2 units of alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany), and phenol-chloroform-extracted and isopropanol-precipitated as described in the plasmid preparation section.

Electroporation

Aeromonas cells were grown in L-broth at 20°C with vigorous shaking to a density of 0.8 at A_{600nm}. The culture was chilled on ice for 15 min and the cells collected by centrifugation at 4,000 X g for 15 min. The cell pellet was then washed three times with ice cold 15% (v/v) glycerol in equal volume, half volume and 0.02 volume respectively. The cells were then resuspended in 0.002 volume of 10% (v/v) glycerol and frozen at -70°C in 40 µl aliquots.

Plasmid DNA used for electroporation was dissolved in distilled water in concentrations higher than 0.1 mg/ml. A BioRad Gene Pulser electroporation unit (Hercules, California) was used at settings of: 25 µFD capacitance, 200 ohms resistance and 2.5 volts with 2 mm-gap cuvettes. After pulsing, the cells were immediately resuspended in 1 ml of ice cold L-broth followed by shaking at 20°C for 3 h. The cells were then plated on selective culture plates.

M13 cloning

The replication form of M13 DNA (double stranded) was digested and ligated with the DNA restriction fragment to be inserted at 15°C overnight. After adding 40 µl of competent *E. coli* JM109 cells and incubation on ice for 30 min, the ligation mix was heat-shocked at 42°C for 45 seconds, cooled on ice and mixed with 0.1 ml of log-phase *E. coli* JM109 cells, 40 µl of 20 mg/ml (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) X-gal, and 3 ml of 50°C soft L-agar (0.6%, w/v). This mixture was quickly poured onto an L-agar plate. The plate was incubated for more than 8 h and white plaques picked to amplify for DNA preparation.

Southern analysis

Non-radioactive labeled DNA

The reagents and procedures were provided in a non-radioactive DNA labeling and detection kit (Boehringer Mannheim GmbH). The kit uses random hexanucleotides, incorporated with a nucleotide analog, digoxigenin-11-dUTP, to label DNA probes. After hybridization with the labeled probes, the digoxigenin is detected by an antibody-enzyme conjugate, anti-digoxigenin-alkaline phosphatase, and visualized by an enzyme-linked color reaction as described in immunoblot. Digested DNA in agarose gels was transblotted to nitrocellulose membranes with 10 X SSC (183). After baking at 80°C for 1 h, the membranes were prehybridized in a buffer containing 5 X SSC, 0.1% (w/v) N-lauroylsarcosine and 1% (w/v) blocking reagent (provided in the kit), and then hybridized with the boiled labelled probe in the same buffer. Prehybridization was at 62°C for 3h, and hybridization was at 62°C for 12 h. The membranes were washed in 2 X SSC with 0.1% SDS (w/v) twice (5 min each at room temperature) followed by two 10 min washes in 0.1 X SSC

with 0.1% SDS (w/v) at 37°C. Bound probe was detected using the reagents and procedures of the kit manufacturer.

DNA fragments radiolabeled by nick translation

A nick translation kit (Amersham) was used for DNA labeling. The labeling reaction included 1 µg of DNA, 6.6 µl each of 300 µM dCTP, dGTP and dTTP, 10 µl of (α -³²P)dATP (100 µCi, Amersham) and 10 µl of enzyme solution (containing 5 units of DNA polymerase I and 100 pg of DNase I) in a total volume of 100 µl. After incubation at 15°C for 2 h, 80 µl of 0.5 M EDTA was added and the reaction was heated in a boiling water bath for 5 min. The total volume of the labeling reaction was added into the prehybridization solution which had been incubated with a transblotted membrane at 65°C for at least 2 h. The prehybridization solution included 3 ml of 20 X SSC, 2 ml of 50 X Denhardt's reagent (183), 0.25 ml of 10 mg/ml tRNA (Boehringer Mannheim GmbH), 0.5 ml of 10% (w/v) SDS, 20 µl of 0.5M EDTA in a total volume of 10 ml. The hybridization reaction was carried out at 65°C overnight. After washing as described in the Non-radioactive labeling section, the wet membrane was wrapped in plastic wrap and autoradiographed with Kodak X-OMAT AR film overnight.

End-labeled oligonucleotides

To label oligonucleotides, 1 µg of synthesized oligonucleotides was mixed with 8 µl of γ -³²P-dATP (80 µCi, Amersham), 10 units (1 µl) of polynucleotide kinase (Pharmacia), 0.1M Tris (pH 8.0), 5 mM dithiothreitol (DTT), and 10 mM MgCl₂ in 20 µl volume. After incubation at 37°C for 30 min, 1 µl of 0.5 M EDTA was added and heated at 65°C for 5 min. The mix was diluted with TE buffer (pH 8.0) to a final volume of 100 µl and kept at -20°C. Half of the volume was used for each Southern hybridization. DNA

fragments were transblotted from agarose gel to nitrocellulose membrane as described above. Prehybridization and hybridization were carried out at 37°C for 4 and 12 h respectively. Prehybridization and hybridization solution contains 10% (w/v) PEG, 1M NaCl and 1% (w/v) SDS. The membranes were then washed and autoradiographed as described in the nick-translation section.

Polymerase chain reaction (PCR)

PCR amplification of chromosomal DNA was performed under the following conditions. A 10 µl reaction volume contained 1 µl 10 X PCR buffer (500mM KCl, 100mM Tris-Cl (pH 8.3)), 1 µl 25 mM MgCl₂, 1 µl 10mM dNTP mix (Perkin Elmer Cetus, Rexdale, Ontario) 1 µl each primer (5µM), 4 µl sterile distilled water, 0.5 units AmpliTaq (Perkin Elmer Cetus), and 1 µl of purified chromosomal DNA (50 ng/µl). Amplification was carried out using capillary tubes in a 1605 Air Thermo-Cycler from Idaho Technology (Idaho Falls, Idaho). The samples were subjected to at least 30 cycles of 1 sec denaturation at 94°C, 5 sec primer annealing at 51°C, and extension at 73°C. The duration of extension depended on the length of the fragment to be amplified. PCR generated DNA products were detected by gel electrophoresis. Following PCR amplification, the 10 µl reaction mix was subjected to 1% (w/v) agarose gel or 8% (w/v) polyacrylamide electrophoresis with *Hinf*I digested pBR322 DNA as molecular weight markers. DNA fragments were visualized by UV fluorescence after ethidium bromide staining.

DNA sequencing

DNA sequencing was done by both manual and automated methods. Sequencing probes used included commercially available oligonucleotides (Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta,

Canada) and synthesized primers (methods described in oligonucleotide preparation section).

Manual sequencing

DNA templates were prepared as described in the sections of plasmid DNA preparation and M13 DNA preparation, except that an RNase (Boehringer Mannheim GmbH) treatment step was added before phenol-chloroform extraction when preparing plasmid templates. Procedures and most of the reagents for manual sequencing were from a Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, Ohio). Radiolabeled nucleotide ^{35}S -dATP was purchased from New England Nuclear (Wilmington, DE). DNA template, primer and sequencing buffer (from the kit) were mixed and heated to 65°C for 2 min and then slowly cooled to lower than 35°C . The reaction mix was divided and each quarter was added into a termination mixture (A, C, G, and T, from the kit). Labeling mix and Sequenase enzyme were then added into each tube, as well as ^{35}S -dATP. After 5 min at room temperature, stop solutions were added and the samples were denatured at 75°C for 2 min and loaded onto the gel. The gel electrophoresis was carried out at 1,500 volts until the dye had traveled a desired distance. The gel was then transferred to a piece of Whatman paper, dried and autoradiographed.

Automated sequencing

Sequencing reactions of plasmid template DNA were carried out using a dye terminator sequencing kit (Applied Biosystems, Inc (ABI), Mississauga, Ontario, Canada) and a Perkin Elmer Cetus Model 480 thermal cycler following the protocol and cycling parameters suggested by ABI. DNA template preparation procedures suggested by the kit manufacturer were

used. When PCR fragments were used as DNA templates, PCR products from the 1605 Air Thermo-Cycler (Idaho Technology) were purified by agarose gel electrophoresis and QIAGEN agarose gel extraction kit (QIAGEN, Chatsworth, CA). For each dye-terminator sequencing reaction, 1 μ g of double stranded DNA template (in the case of PCR fragment, 250 ng of DNA), 3.2 pmol of primer and 9.5 μ l of reaction pre-mix (from the kit) were mixed and the reaction was carried out in the Perkin Elmer Thermal Cycler. The reaction was then extracted twice with a phenol-chloroform-distilled water mix (68:14:18), isopropanol-precipitated and dried. Before loading, the reaction was resuspended in 4.5 μ l of a buffer containing 5 volumes of deionized formamide and 1 volume of 50 mM EDTA (pH 8.0), and denatured at 90°C for 2 min. Sequence data was analyzed using the ABI SeqEd program.

Site-directed mutagenesis

Site-directed mutagenesis was performed using a Transformer Mutagenesis Kit from Clontech (Clontech Laboratories, Palo Alto, CA). A selection primer, which replaces the *EcoRI* site in the pTZ18R polycloning site with an *EcoRV* site, and a mutagenic primer, which changes the bases in the desired sequence, were synthesized and phosphorylated at the 5' end. The mutant strand was synthesized by incubation of the denatured template DNA, the selection primer and the mutagenic primer, DNA polymerase and DNA ligase. An repair-deficient *E. coli* strain BMH 71-18 *mutS* (Clontech Laboratories) was transformed by the reaction mix which was *EcoRI*-digested. The *EcoRI* digestion eliminated transformation by most parental plasmids which would be linearized. DNA pool from the transformed cells was then digested by *EcoRI* again and used for the second transformation. Single colonies were isolated and screened by DNA sequencing.

Computer analysis

DNA Strider release 1.1 (Institut de Recherche Fondamentale, CEA, France), GeneWorks release 1.02 (Intelligenetics Inc.) and PC Gene release 6.01 (Intelligenetics Inc. and Genofit, SA) were used for DNA and protein sequence analysis. Protein sequence similarity searches were performed in GenPept and Swiss-prot data bases through Genbank by the FASTA protocol of Pearson and Lipman (163).

RNA preparation and detection

RNA preparation

Aeromonas RNA's were prepared following the procedure described by Reddy *et al.* (174). A 20 ml volume of overnight bacterial culture was centrifuged (4,000 X g, 10 min) and the cell pellet resuspended in 10 ml protoplasting buffer containing 15mM TrisHCl (pH 8.0), 0.45M sucrose, 8mM EDTA and 0.5 mg/ml lysozyme (Boehringer Mannheim GmbH). After incubation on ice for 15 min, the mixture was centrifuged at 2,000 X g for 5 min. The pellet was resuspended in 0.5 ml lysing buffer composed of 30 mM TrisHCl (pH 7.5), 100 mM NaCl, 1 mM sodium citrate, 1.5% (w/v) SDS and 3% (w/v) diethylpyrocarbonate (DEPC, Sigma). The mixture was incubated at 37°C for 5 min, chilled on ice, and 250 µl saturated NaCl was added. After an additional 10 min on ice, the mixture was centrifuged at 12,000 X g for 15 min, the supernatant was transferred to a fresh tube and RNA was precipitated by adding 1 ml ethanol at -20°C. The next day, the RNA was collected after washing with 70% ethanol, dried and resuspended in DEPC treated distilled water (DEPC was added to a final concentration of 0.2% (v/v) and the water was shaken vigorously for a few minutes followed by autoclaving).

Northern hybridization

Sample RNA was dissolved in 5 μ l of 25 mM EDTA and 0.1% (w/v) SDS and 15 μ l of electrophoresis buffer, containing 0.02 M MOPS, 5 mM sodium acetate and 1 mM EDTA (pH 7.0), was added. The sample was then heated at 65°C for 15 min. After chilling, 1 μ l of 1 mg/ml ethidium bromide was added. The sample was loaded onto a 1% (w/v) agarose gel prepared in the electrophoresis buffer plus 5.1% (v/v) formaldehyde. After electrophoresis at 100 V at room temperature for 1 h, RNA in the gel was transblotted to a piece of nitrocellulose membrane in 10 X SSC by capillary action. The RNA on the membrane was fixed by baking at 80°C for 2 h. Prehybridization and hybridization were done at 42°C according to methods described earlier.

RNA stability assay

Rifampicin was added to cell cultures at the concentration of 0.2 mg/ml. Cells were collected by centrifugation (3,000 X g, 2 min) at 0 min and at 5 min intervals up to 35 min after the addition of rifampicin and kept on ice. The subsequent RNA preparation steps were strictly kept at below 4°C. Following the conventional Northern hybridization and overnight exposure to Kodak X-OMAT AR film, bands were quantified on a personal densitometer with software ImageQuant v3.2 (Molecular Dynamics, Sunnyvale, CA). Obtained values were plotted on a semi-log background on Cricket Graph (version 1.3.1, Malvern, PA) and the half life was measured.

Primer extension experiments

RNA was extracted as described and was treated with RNase-free DNase (Promega, Madison, Wis.) for 10 min at 37°C, phenol extracted, and ethanol precipitated. Oligonucleotide primers were labelled with (γ -³²P) ATP

(du Pont-NEN Research Products) as described in the Southern analysis section. Primer extension analysis of transcripts was performed as described by Fouser and Friesen (60). Briefly, 50 ng of ^{32}P -labelled primer was mixed with 12 μg of RNA in 10 μl of 10 mM TrisHCl (pH 8.3) and 250 mM KCl. After heating at 80°C for 5 min, the primer was annealed at 50°C for 45 min. Then, 2.4 μl of mixed dNTP's (2.5 mM each), 3 μl of cDNA buffer (35 mM Tris (pH 7.5), 8 mM MgCl_2 , 1.7 mM DTT, 50 $\mu\text{g}/\text{ml}$ actinomycin D), and 20 units AMV reverse transcriptase (Pharmacia) were added. Following incubation at 50°C for 45 min, 5 μl of sequencing loading buffer was added and the sample was heated for 3 min in a boiling water bath. Ten μl of each extension reaction was run on an acrylamide sequencing gel in parallel with a DNA sequence ladder primed with the same oligonucleotide used in the primer extension reaction.

Detection and purification of proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining

Polyacrylamide gel electrophoresis was as originally described by Laemmli (125). Before preparing the gel, several stock solutions were made including separating gel 4 X buffer (18.17 g Tris base and 0.4 g SDS in 100 ml, pH 8.8), 30% acrylamide solution (30 g acrylamide and 0.8 g bisacrylamide in 100 ml), and stacking gel 4 X buffer (6.06 g Tris base and 0.4 g SDS in 100 ml, pH 6.8). A 12.5% separating mini gel was made from 2.5 ml of distilled water, 30 ml of 30% acrylamide solution, 1.9 ml of separating gel 4 X buffer, 112 μl of 10% ammonium persulfate and 5 μl of TEMED (N, N, N', N'-tetramethylethylenediamine). A stacking mini gel was made from 1 ml of distilled water, 300 μl of 30% acrylamide solution, 444 μl of stacking gel 4 X

buffer, 28 μ l of 10% ammonium persulfate and 5 μ l of TEMED. One liter of electrophoresis buffer was composed of 3 g Tris-base, 14 g glycine and 10 ml of 10% (w/v) SDS. Before loading, protein samples were mixed with 2 X loading buffer and boiled for 5 min. Each 10 ml of the 2 X loading buffer contained 2 ml of glycerol, 2 ml of 10% (w/v) SDS, 0.25 mg of bromophenol blue, 2.5 ml of stacking gel 4 X buffer and 0.5 ml of β -mercaptoethanol. Gels were run at 100 volts for 15 min then increased to 200 volts. After electrophoresis, gels were stained for 20 min in staining solution containing (per liter) 250 ml of isopropanol, 100 ml of glacial acetic acid and 2.5 g of Coomassie brilliant blue R250, followed by destaining in 10% glacial acetic acid and 20% ethanol.

Immunogold electron microscopy assay

Grids were floated on bacterial drops for 2 min and self-dried. The grids were then laid on drops of a blocking buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) BSA and 0.05% (v/v) Tween 20) for 15 min. Anti-A-protein antiserum (kindly provided by Dr. W. W. Kay) was diluted 1/10 with the blocking buffer and reacted with the grids for 60 min. After three 5-minute washes (10 mM TrisHCl (pH 8.0) and 150 mM NaCl), the grids were laid on drops of 15 nm gold labeled protein A (Amersham, Arlington Heights, IL) and diluted 1/50 with the blocking buffer. After 60 min incubation, the grids were washed three times and stained with 2% (w/v) ammonium molybdate.

Cell fractionation

Culture supernatant

Overnight cultures of both *A. salmonicida* and *E. coli* were centrifuged at 5,000 X g for 30 min. The supernatant was collected and proteins were precipitated by addition of three volumes of ice cold acetone. After being kept

on ice for 30 min, the solution was centrifuged at 5,000 X g and the pellet was dried in a SpeedVac.

Periplasm

Cell pellet was washed twice in 0.33 M Tris buffer (pH 7.3) and suspended in 10 ml of 0.15 M TrisHCl (pH 7.3), 2 mM EDTA, and 20% (w/v) sucrose. Cells were then shaken for 10 min at room temperature and collected by centrifugation at 8,000 X g for 15 min. The pellet was rapidly suspended in 2 ml of ice cold, deionized distilled water containing 1 mM MgCl₂ and the cells were stirred on ice for 10 min prior to centrifugation at 12,000 X g for 15 min. The supernatant was used as the periplasmic protein fraction (20).

An alternative method for periplasmic fraction was from Ames and coworkers (11). Bacterial cells were collected from 2 ml overnight cultures by centrifugation at 1,100 X g for 10 min. After decanting the supernatant, the cell pellet was resuspended by brief vortexing in the residual medium and 20 ml of chloroform was then added. The tubes were vortexed briefly and maintained at room temperature for 15 min. Then, 0.2 ml of 0.01 M Tris (pH 8.0) was added. The cells were separated by centrifugation at 6,000 X g for 20 min. The supernatant fractions were taken as periplasmic fractions.

Cytoplasm

Following the first periplasm preparation method described above, the cell pellet was resuspended in 10 ml of 20 mM TrisHCl (pH 7.3). Cells were frozen once before going through a French Pressure Cell twice at 1,200 pounds per square inch (PSI). Unbroken cells were removed by centrifugation at 5,000 X g for 30 min. The membrane fraction was collected by centrifugation at 31,000 X g for 30 min and washed once with Tris buffer (pH 7.3). Cytoplasmic

proteins in the supernatant were concentrated by adding three volumes of cold acetone followed by incubation on ice for 30 min, centrifugation at 15,000 X g for 30 min, and vacuum-dried.

Inner membrane and outer membrane

Separation of inner membrane and outer membrane was done by sarcosyl differential solubilization (58). The collected membrane preparation was resuspended to 1 mg protein/ml with the Tris buffer (pH 7.3). After addition of 20 μ l sarcosyl (30% solution, GRACE, Nashua, NH)/ml, the suspension was incubated at room temperature for 30 min with shaking. Outer membrane was collected by centrifugation at 40,000 X g for 30 min at 4°C. The pellet was washed three times with the Tris buffer (pH 7.3), and the supernatant was used as the inner membrane fraction.

Western blot

Proteins in SDS-PAGE gels were electroblotted to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) at 12 volts for 30 min (207). The membranes were blocked in Tris-buffered saline (TBS, 10 mM TrisHCl (pH 8.0) and 150 mM NaCl) containing 2% (w/v) skim milk powder (Oxoid, Basingstoke, Hampshire, England) for 10 min, followed by 30 min incubation with appropriately diluted antibody in the same solution. After washing 3 times in the TBS containing 0.5% (v/v) Tween-20 (Bio Rad), the membranes were reacted with 1:5,000 diluted alkaline phosphatase conjugated goat-anti-rabbit or goat-anti-mouse IgG (CALTAG Laboratories, San Francisco, CA) in TBS for 30 min. After 3 additional washes, the protein bands on the membranes were visualized by reacting with 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim GmbH) and nitro blue tetrazolium (Sigma) (88).

Silver stain for proteins

The method was as previously reported by Tsai and Frasch (213). Conventional SDS-PAGE gels were soaked in 10% (v/v) unbuffered glutaraldehyde at room temperature for 30 min followed by extensive washing with distilled water at room temperature for 1 h. The gels were then stained in a fresh silver nitrate staining solution for 10 min. To prepare 100 ml of this solution, 1.4 ml of fresh NH_4OH was added to 21 ml of 0.36% (w/v) NaOH. To this solution, 4 ml of 19.4% (w/v) AgNO_3 (made by adding 2 g of AgNO_3 to 10 ml of distilled water) was added slowly while stirring. After staining, the gels were washed in distilled water for 2 min and transferred to a freshly prepared solution containing 50 mg/L citric acid and 0.5/1,000 (v/v) of 37% formaldehyde. Color development was stopped by washing with distilled water and the gels were dried.

Protein preparation for N-terminal sequencing

ProBlott membrane (Applied Biosystems) was used for transblotting and procedures recommended by the manufacturer were followed. Before transblotting, the conventional SDS-PAGE gel was soaked for 5 min in 1 X 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer which was diluted from 10 X CAPS buffer in 10% (v/v) methanol. To make 1 liter of 10 X CAPS, 22.13 g of CAPS was dissolved in 900 ml of distilled water, titrated to pH 11.0 with 2N NaOH and the final volume adjusted to one liter with distilled water. ProBlott membrane was wet in 100% methanol for 2 sec and then soaked in 1 X CAPS buffer before use. Electrotransblotting was carried out as described in the Western blot section except that the ProBlott membrane was used. The membrane was then rinsed with distilled water, soaked in methanol for 2 seconds, stained in 0.1% (w/v) Coomassie Blue R-250 (in 40%

(v/v) methanol and 1% (v/v) acetic acid) for 1 min, and destained in 50% (v/v) methanol. When protein bands appeared on the membrane, the membrane was rinsed extensively with distilled water and the bands excised before the membrane dried completely. The excised bands were air dried and stored at -20°C.

T7 polymerase *in vivo* transcription

The procedure was based on Rosenberg's method (179) with modifications. Cells containing pGP1-2 and other plasmids were grown in LB with kanamycin (50 µg/ml) and other appropriate antibiotics such as ampicillin (50 µg/ml) for 3 h at 30°C shaking. A 1.5 ml volume of the culture was harvested and washed in M9 medium. The pellet was resuspended in 3 ml of M9 medium (one liter M9 medium contained Na₂HPO₄·7H₂O 12.8g, KH₂PO₄ 3.0g, NaCl 0.5g, NH₄Cl 1.0g and glucose 20.0g, pH 7.5) (183) and 0.09 ml of methionine assay medium (DIFCO, Detroit, Michigan) was added. Cells were incubated at 30°C for 1 h, followed by 42°C for 45 min. After the first 30 min at 42°C, rifampicin (Sigma) was added to a final concentration of 1 mg/ml. At the end of the 42°C incubation, 1 ml of the culture was incubated for additional 45 min at 30°C before the cells were pulse labelled for 5 min with 10 µCi ³⁵S methionine. Cells were sedimented and resuspended in 30 µl SDS-PAGE loading buffer.

For cell fractionation, the pellets from six 1 ml aliquots of labeled cells were resuspended in 500 µl of a sucrose buffer (20% (w/v) sucrose in 10 mM EDTA and 100 mM TrisHCl (pH8.0) with 10 µl of a 5 mg/ml lysozyme solution. The cells were kept on ice for 10 min, and centrifuged for 2 min at 15,000 X g to separate the periplasmic protein supernatant from the spheroplast pellet. The spheroplasts were washed in 0.5 ml of the sucrose

buffer and resuspended in 50 μ l of 20% (w/v) sucrose in 10 mM EDTA, 10 mM MgCl_2 and 100 mM TrisHCl (pH 8.0) containing 50 μ g/ml DNase. After the addition of 200 μ l of ice-cold distilled water, the spheroplasts were lysed by four cycles of freeze-thaw and membranes were sedimented (10 min, 15,000 X g). All fractions were then precipitated with a 10% volume of 50% (w/v) trichloroacetic acid (TCA), acetone washed, dried and resuspended in SDS-PAGE loading buffer.

A preparative procedure for the protein products was also carried out without ^{35}S methionine labeling. Cells containing two plasmids were grown at 30°C in 2 X LB containing 2% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% (w/v) NaCl with appropriate antibiotics. When the culture reached an A_{600} of 1.5, the temperature was raised to 42°C for 45 min. Rifampicin was added to a final concentration of 1 mg/ml after 30 min incubation. After an additional 15 min incubation, the temperature was shifted to 37°C for 2 h, and the cells were harvested by centrifugation (4,000 X g, 10 min).

In vitro gene expression

A prokaryotic DNA-directed translation kit (Amersham Canada Ltd., Oakville, Ontario) was used for coupled in vitro transcription and translation (239). Plasmid DNA or DNA fragments were used as templates. Equal amounts of DNA template (5 μ g) were used in each transcription-translation reaction. The reactions were carried out according to the protocol provided by the manufacturer. The reaction mix, which contained template DNA, 7.5 μ l of supplement solution, 3 μ l of amino acids solution minus methionine, 20 μ Ci of ^{35}S methionine and 5 μ l of *E. coli* S-30 extract, was incubated at 37°C for 60 min. After adding 5 μ l of methionine chase solution, the reaction was incubated for an additional 5 min at 37°C. The reaction was then stopped by

placing it in an ice bath. A negative control was performed in parallel without adding the template DNA. One fifth of the reaction mixture was loaded onto a 12.5% SDS-PAGE gel, and after electrophoresis the gel was dried and exposed to Kodak X-OMAT AR film. Prestained low-range molecular weight standards (BioRad Laboratories, Richmond, CA) were used to facilitate estimations of apparent molecular weight.

Purification of the AbcA-LacZ fusion protein

One liter of pSC161-1/*E. coli* DH5 α culture was incubated overnight and cells collected by centrifugation at 4,000 X g for 10 min at 4°C. The cell pellet was resuspended in 20 ml of a 20 mM TrisHCl buffer (pH 7.5) including DNase (2.5 mg/ml). After one cycle of freeze and thaw, the cell suspension was passed through a French Press Cell twice at 1,200 psi. Unlysed cells and membrane debris were removed by centrifugation at 31,000 X g for 30 min. Saturated ammonium sulphate solution was added to the supernatant to 10% saturation. The solution was then stirred at 4°C for 30 min and centrifuged for 30 min at 31,000 X g. The protein precipitate was discarded and the supernatant was brought to 40% saturation with ammonium sulphate. Following stirring at 4°C for 30 min and centrifugation, the 10 - 40% ammonium sulfate protein pellet was collected and resuspended in 1 ml of TBSN buffer (50 mM Tris (pH 7.5), 150 mM NaCl, and 0.2% (v/v) NP-40). After dialysis against the same buffer with 3 changes of 1 liter volume each for 6 h, this preparation was passed through an immunoaffinity column twice, which contained 1 ml of ProtoSorb *lacZ* matrix, agarose beads bound with anti- β -galactosidase monoclonal antibody (Promega, Madison, WI). The column was washed with four bed volumes of TBSN buffer, and the bound proteins were eluted at a flow rate of 5 ml/h by three bed volumes of 0.1 M

NaHCO₃/Na₂CO₃ (pH 10.8) followed by 1 ml of TBS buffer (50 mM Tris (pH 7.5), and 150 mM NaCl). The majority of the fusion protein was found in the first two bed volumes of eluent. The first six fractions (0.5 ml each) were pooled and concentrated 20 fold in a Microsep Microconcentrator (molecular weight cutoff 30.0 Kd, Filtron Technology Corporation, Northborough, Mass). The purity of this preparation was checked by SDS-polyacrylamide gel stained by Coomassie blue.

β-galactosidase assay

The β-galactosidase activities were measured as previously described by Sambrook *et al.* (183) with some modifications. A 1 ml volume of overnight culture in L-broth was washed once with an A medium (including K₂HPO₄ 10.5 g, KH₂PO₄ 4.5 g, (NH₄)₂SO₄ 1.0 g, and Na citrate 2H₂O 0.5 g in 1 liter volume), then resuspended in 1 ml of the A medium. 0.1 ml of this cell suspension was added to 0.9 ml of a Z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄, and 0.05 M β-mercaptoethanol, pH 7.0). Two drops of chloroform and one drop of 0.1% (w/v) SDS were added, and the tube vigorously vortexed for 10 sec. Before adding 0.2 ml of the substrate o-nitrophenyl-β-D-galactopyranoside (4 mg/ml in A medium), the tube was equilibrated at 28°C for 5 min. When a yellow color developed, the reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃. It was then measured for absorbance at 420 nm and 550 nm. The cell suspension in A medium was measured at 600 nm. The units of β-galactosidase activity were calculated as described by Sambrook *et al.* (183): $\{A_{420} - (1.75 \times A_{550})\} \times 1000 / (t \times 0.1 \times A_{600})$, where t=time in min.

Nucleotide affinity chromatography

The entire affinity chromatography procedure was carried out at 4°C. A MOPS (3-(N-morpholino)propanesulfonic acid) buffer was prepared according to Black and Hruby (24) with some modifications. The buffer contained 10 mM MOPS (pH 7.2), 1 mM EDTA, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF). A 1 ml volume of ATP affinity column matrix (Sigma) was swollen in the MOPS buffer overnight at 4°C, and washed with more than ten bed volumes of the MOPS buffer. The protein preparation was dissolved in the same buffer and applied to the column at a flow rate of 0.1 ml/min. After washing the column with more than 10 bed volumes of the MOPS buffer, the protein was eluted with 1 ml volumes of buffer containing increasing concentrations of ATP, *i.e.*, 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM, 2.0 mM and 40 mM, 1 ml of each. Fractions of 1 ml were collected.

LPS detection

Silver stain for LPS

Silver staining of LPS was performed as described previously (92, 213). Overnight cell cultures of 1 ml volume were collected by centrifugation (4,000 X g, 10 min), resuspended in 0.1 ml SDS-PAGE loading buffer and boiled for 5 min. Equal volume of proteinase K (1 mg/ml) was added, incubated for 1 h at 60°C, and 5 µl was loaded onto a conventional SDS-PAGE gel. After electrophoresis, the gel was fixed in a fixing solution (40% ethanol and 5% acetic acid (v/v)) for at least 1 h with gentle shaking. Then the gel was incubated in an oxidizing solution (the fixing solution plus 0.7% (w/v) periodic acid) for 5 min. After three 10-min washes in distilled water, the gel was stained for 10 min with vigorous shaking. To make the stain, 2 ml of concentrated NH₄OH was added to 28 ml of 0.1 M NaOH. Then, 5 ml of 20%

(w/v) $\text{Ag}(\text{NO}_3)_2$ was slowly added while stirring. The final volume was adjusted to 150 ml with distilled water. The stain was kept in the dark at room temperature for a maximum of one month. The gel was washed three more times with distilled water and developed in a solution containing 50 mg citric acid and 0.5 ml of 37% formaldehyde per liter. The color development was stopped by washing in distilled water.

RESULTS

I. Structure of *vapA* gene and its product, A-protein

Subcloning and sequencing of the *A. salmonicida* A449 A-protein gene (*vapA*)

The A-layer subunit gene *vapA* from wild type *A. salmonicida* strain A449 was previously cloned into the phage vector λ gt11 (21). The clone could initially express A-protein as detected by immunoblot analysis. The apparent Mr of the protein expressed was indistinguishable from A-protein purified from A449 cells. However, plating of the recombinant phage on *E. coli* strain Y1090 always produced plaques unable to react with antibodies to A-protein. This instability increased with storage. After conversion to lysogeny in *E. coli* strain Y1089 and subculturing, immunologically detectable A-protein was no longer produced. Despite the phenotypical change, the *vapA* gene in the lysogenic recombinant phage λ 10g could still be identified by using two oligonucleotide probes to the N-terminal amino acid sequence (112). This suggested that although the *vapA* gene in λ 10g had been changed, the clone still retained part of the original insert. This was confirmed by subcloning of a 0.75 kb *Bam*HI-*Kpn*I fragment from λ 10g into pLG338 (pRJ5) and M13 (pSC149) (Fig. 3) and DNA sequencing. The sequence showed part of an open reading frame with the predicted amino acid sequence the same as previously determined N-terminal sequence of purified A-protein (169). This finding further localized the A-protein gene coding region.

A 1.5 kb *Pvu*II-*Pvu*II fragment from λ 10g was then subcloned into *Sma*I digested pTZ18R generating pSC249. This fragment was believed to code for

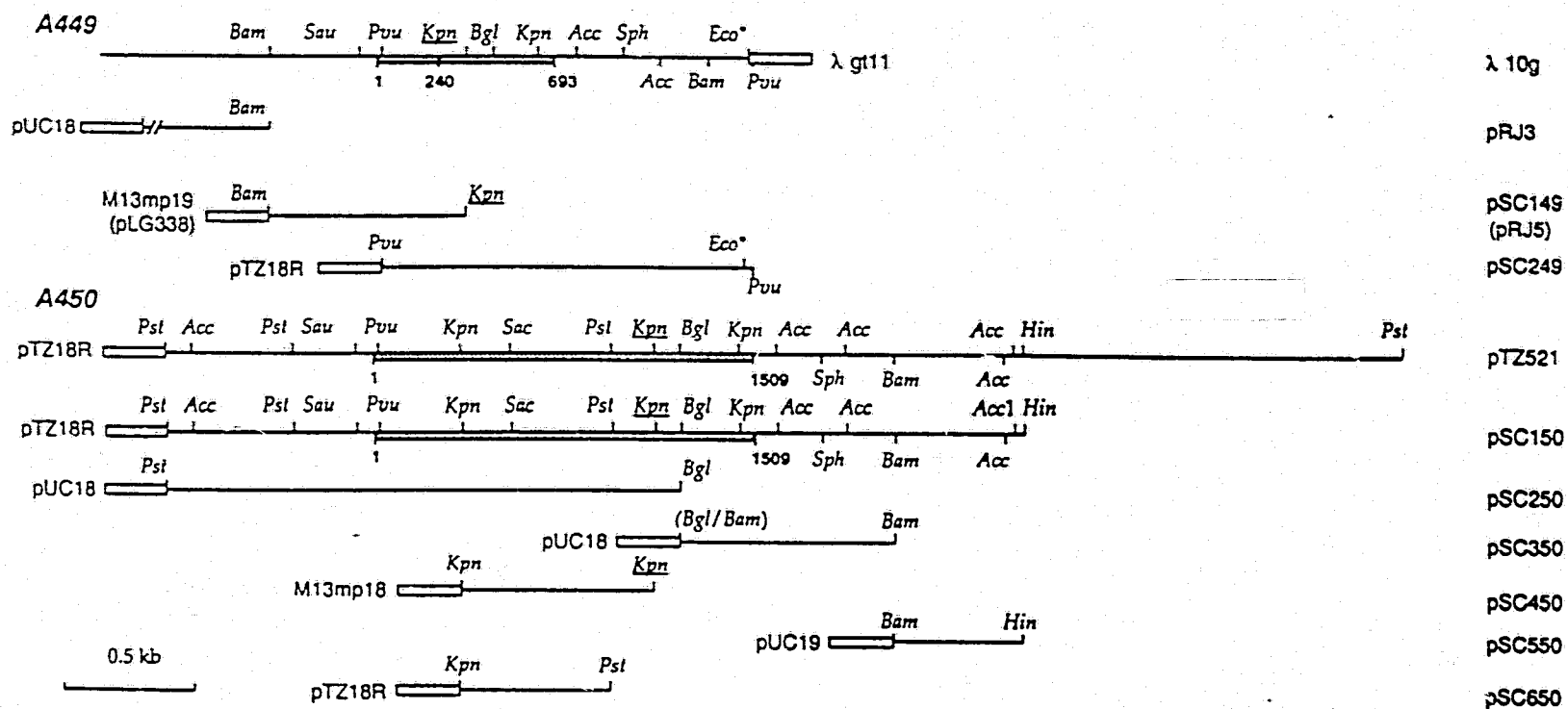


Fig. 3. Physical map of the cloned *A. salmonicida* chromosomal DNAs containing the *vapA* gene (cross-hatched box), and phage/plasmids used in this study. Nucleotide positions in the gene are indicated, and vectors (shaded box) carrying *A. salmonicida* DNA are identified, as are the restriction sites used for subcloning. The recombinant phage/plasmid designation is shown on the right. An identical *KpnI* site in the deleted gene of strain A449 and the complete gene of strain A450 is underlined. *Acc*=*AccI*, *Bam*=*BamHI*, *Bgl*=*BglII*, *Eco**=*EcoRI**, *Hin*=*HindIII*, *Kpn*=*KpnI*, *Pvu*=*PvuII*, *Pst*=*PstI*, *Sac*=*SacI*, *Sau*=*Sau3A*, and *Sph*=*SphI*.

an area near the N-terminal of A-protein because it reacted with the 17-mer oligonucleotide probe TJT18 (from base 226 in the *vapA* gene), and the *PvuII* site is only 22 bases downstream from the beginning of the gene. pSC249 was also subcloned into M13 vector for sequencing. Subsequent DNA sequencing revealed that the open reading frame with the known N-terminal sequence was interrupted by a number of stop codons. Possible misreading of the DNA sequence was ruled out because stop codons appeared in all three reading frames. Furthermore, cysteine residues appeared in all reading frames, which was not consistent with the amino acid composition of the A-protein. These results suggested that a DNA rearrangement had occurred in the clone. This necessitated recloning of the A-protein gene, which was performed by B. Phipps (167).

Subcloning of the *vapA* gene from *A. salmonicida* A450

The *vapA* gene of A450 was previously cloned on a 27 kb *Sau3A* insert into a broad host range cosmid pLA2917 (7) generating pBP501 (167). When screened by colony immunoblot, clones containing this plasmid reacted weakly with anti A-protein immunoglobulin. A 9.4 kb *SalI* fragment of pBP501 was then subcloned back into pLA2971 to yield pBP512. A-protein expression by subclone pBP512 was less than that of pBP501.

To further localize the coding region for A-protein, pBP512 DNA was digested by *PstI*, and ligated with *PstI* digested pTZ18R. After transformation into *E. coli* DH5 α , clones were screened for ampicillin resistance and *lacZ* gene complementation. One of 40 white colonies showed a strong reaction with anti-A-protein immunoglobulin, and Western blot analysis showed that the molecular weight of the A-protein produced was indistinguishable from that of the wild type protein (Fig. 4). This clone, pTZ521, contained a 4.6 kb insert

(Fig. 3) and, in contrast to clone λ 10g, A-protein production was stable during storage and subculture. When the 4.6 kb insert containing A-protein gene was ligated into pTZ19R where the inverted polycloning site positioned the T7 promoter downstream from the insert rather than upstream as was the case in pTZ521, there was no alteration in the level of A-protein produced. This suggested that A-protein synthesis was from a promoter on the insert.

The beginning of the *vapA* gene in the pTZ521 insert was localized near the 5' end of the insert. Further restriction mapping of the insert in pTZ521 identified a 1.3 kb *Hind*III fragment at the 3' end of the 4.6 kb insert (Fig. 3), removal of which did not affect *vapA* expression in *E. coli*. The plasmid obtained after removal of this *Hind*III fragment, pSC150, contained the smallest insert still capable of producing approximately the same level of A-protein as that of pTZ521 in *E. coli*. Attempts to subclone smaller fragments containing the entire *vapA* gene and capable of good expression of A-protein were uniformly unsuccessful, although subclones containing various regions of the gene were obtained and were stable.

DNA sequencing of the A450 *vapA* gene

The *Aeromonas* DNA insert in pSC150 was subcloned into other vectors for sequencing. pSC250 contained a 2 kb *Pst*I-*Bgl*II fragment in pUC18. pSC350 contained the middle region of the pSC150 insert, a 0.85 kb *Bgl*II-*Bam*HI fragment, in pUC18. pSC550 carried the 3' end of the pSC150 insert, a 0.55 kb *Bam*HI-*Hind*III fragment, in pUC19. The middle region of the pSC150 insert was also subcloned into M13mp18 and pTZ18R in a 0.7 kb *Kpn*I-*Kpn*I fragment (pSC450) and a 0.5 kb *Kpn*I-*Pst*I fragment (pSC650), respectively. For DNA sequencing, the primers used were synthesized oligonucleotides. Universal



Fig. 4. Western blot of A-protein subclones in pTZ18R. Lane 1: whole cell lysate of pTZ521/DH5 α ; lane 2: purified A-protein. Marker on right indicates the position of wild type A-protein. The antiserum used was rabbit anti-purified A-protein diluted 1:1,000.

primer was also used when the priming site was appropriately located for sequencing.

The entire *Aeromonas* DNA insert in pSC150 was sequenced in both directions (Fig. 5). The insert contained 3372 base pairs, and only one single complete open reading frame (ORF) greater than 1 kb was found in all three possible reading frames. This ORF was 1506 base pairs long, beginning at an ATG codon and terminated by a TAA stop codon, and was named *vapA* for "virulence array protein". The ORF contained an overall G+C content of 48%, lower than the overall 55 +/- 1.8% reported for the *A. salmonicida* genome (22). This was the first *A. salmonicida* gene to be sequenced, and the codon usage for *vapA* showed significant differences from that of *A. hydrophila* genes encoding exported protein (78, 97, 203) (Table 3). This was clearly apparent for Ile, Val, Ser, Pro, Thr, Ala, Gln, Glu, Arg, and Gly. Codon usage for the *A. salmonicida* gene was more in accord with *E. coli* nonregulatory genes, with optimal codons being preferred with Phe, Leu, Ile, Val, Pro, Thr, Ala, Tyr, Asn, Glu, Arg, and Gly (101). For amino acids contained in the protein, seven codons were not used. Three of these, i.e., AUA for Ile, UCA for Ser, and CCC for Pro, corresponded to rare codons in *E. coli*. The other four codons not used were UCG for Ser, ACG for Thr, and UGU and UGC for Cys (There was no Cys residue in A-protein).

The *vapA* gene contains a pair of direct 21-base pair repeat, GAAATCCAAGGTGANNGCCAA separated by 795 bases, beginning at nucleotides 220 and 1036 in the structural gene (Fig. 5). Upstream from the gene, there is an optimally positioned presumptive Shine-Dalgarno ribosomal-binding site AGGA. Possible control elements in the 5'-untranslated region of the A-protein gene included a predicted -10 region

CCTATTTTCCGGGAACACCGGTGTTTGTGTTCCGTGCTCAGTCACCGAAGTTGATCAACGGATAGCTTCAACCCATTGTTATATATCAATAAACTTCCTTAAGGAAAAAGT 449
 GCGTGGCAATCATCTGTTCAATCATCGGAAGCAAGCTCATCTGCTCTTGTATCATCAACGGATAGCTTCAACCCATTGTTATATATCAATAAACTTCCTTAAGGAAAAAGT 450
 1
 ATGTTTAAAGACTTTGATTCCAGCTGCCATTGTCGTCGGTTCCTGTCACCTGCGTTTCTGATGTCGTCGATTAGCCCGAAGCAGAACACTTTGTTACTACCTCCCTCCGATCTGTA 449
 ATGTTTAAAGACTTTGATTCCAGCTGCCATTGTCGTCGGTTCCTGTCACCTGCGTTTCTGATGTCGTCGATTAGCCCGAAGCAGAACACTTTGTTACTACCTCCCTCCGATCTGTA 450
 Leader peptide
 MetPheLysLysThrLeuIleAlaAlaIleValValGlySerAlaAlaProAlaPheAlaAspValValIleSerProAsnAspAsnThrPheValThrThrSerLeuAlaSerVal
 121
 ACTAAGCAGCCGGTACTGCACTTCTCCACTGCTCAACAAAACCTGACCCCTAACTTCAGTGAAGTTGGTGACCTTAAGAACAACGGTTTCATTGCTGCAAAATCCAAAGTCAAGCCCAA 240
 ACTAAGCAGCCGGTACTGCACTTCTCCACTGCTCAACAAAACCTGACCCCTAACTTCAGTGAAGTTGGTGACCTTAAGAACAACGGTTTCATTGCTGCAAAATCCAAAGTCAAGCCCAA 449
 ThrLysGlnProValLeuAspPheSerThrAlaGlnGlnAsnLeuThrLeuAsnPheSerGluValGlyAspLeuLysAsnAsnGlyPheIleValLeuGluIleGlnGlyGluGlyGln 450
 241
 TTCAACGACGCGGAAATCCGTGAGTGGCTGTCACACCGTTTCTGGCGTAGCCCGTTACCGGTCTGCTGTTAACCCGAATGATCATGTAATTTTCCCAATAGCGGTCAAGTTAATGAC 450
 PheAsnAspAlaGluIleArgGlnTrpLeuSerAsnGlyPheTrpArgArgPhePheThrGlyLeuLeuValAsnProAsnAspHisGlyAsnPheAlaAsnSerGlyGluValAsnAsp
 361
 GTTCGGAAGTTCTTTAAGATTATTTCCGACGGTACCCAGCTGACCATCGTTCACACTATCGACAGCAATGGCAAGCGTCTCGCTCTTCTGCTTCTGATGATAGAAGACAGCAATCAAT 450
 ValArgLysPhePheLysIleIleSerAspGlyThrGlnLeuThrIleValHisThrIleAspSerAsnGlyLysArgLeuArgLeuAlaLeuAlaSerAspValGluGluThrIleAsn
 481
 TTTCGTGATGCAGAGTTGAGCTGAAGCTGAACCTTAGCTAACCAAGCCTTTAAGCTGACCTCGGTTCTCAAGCTACATAGCTCTGACCGCAGGAGCTCTGTCGAACGCTTCTTACACT 450
 PheAlaAspAlaGluValGluLeuLysLeuAsnLeuAlaAsnGlnAlaPheLysLeuThrSerGlySerGlnGlyThrValAlaLeuThrAlaGlyAlaLeuTrpAsnAlaSerTyrThr
 601
 CCTGATCCGGTCTGCTACCAAGCCGCTGTTCAACTCGGTAACTGCTTCCACTTCAGTTTCACTAACCGCTGTAAGCTACCGCTCTGCTTCCGAAAGTTTCTTCAAACTTAATATCGGT 450
 AlaAspProValAlaThrLysProLeuPheLysLeuGlyLysLeuPheGlnLeuSerLeuThrAsnAlaGlyLysAlaThrAlaLeuValSerGluGlyPheLeuLysLeuAsnIleGly
 721
 GATTCATATTTCTGCTACTGATTTCGGGATTACCAAGCTTACTACTAACCAAGCTGACCAAGCTGACCAAGCTGACCTGCTGATGTTTCTGCCCTCAAGAAAGATGCC 450
 AspAlaAsnIleSerAlaThrAspPheAlaIleThrAsnValThrThrAsnGlnThrIleGlnArgAspLysValAsnLeuThrLeuThrGlyAspValSerAlaPheLysLysAspAla
 841
 AACCGTAACTTGGTAAACAAAGCTGGCTAGCATCGGTGGAAGCTGCTGCTGATGCTCAATCTGCTACAGCTGCTTCCGGTCTGCGCAACATCGCCCGGTGGCTTCAAAATGCTCTG 450
 AsnGlyAsnLeuValAsnLysAlaGlyAlaSerIleGlyTrpLysAlaAlaAlaAspGlyGlnSerAlaThrAlaValLeuGlyAlaGlyAsnMetAlaGlyGlyValGlnAsnAlaLeu
 961
 1057
 GCTGCTTTGCTACACTGTAAGTTCGTCAGATAAAGCTGTTCCGGTCTGCTGTTAACTTCAATGTTAAGGCTGAAATCCAAAGCTCATAGCCAAAGCTACCTATACTTCAAGGAC 449
 AlaAlaPheGlyThrLeuTyrValAlaAlaAspAsnThrValProValProAlaValAsnPheAsnValLysAlaGluIleGlnGlyAspSerGlnAlaThrTyrAsnTyrPheLysAsp 450
 1081
 GAGCTGGCTGATCTCTCATCTCCACCCGTCATGGTATGAAGTTTGACACAATTACTACTGGTACCACCTTCTGCGCAACCTCATCCACATTGCTGATGTATCAATCTCTGCTACTGAA 449
 GAGCTGGCTGATCTCTCATCTCCACCCGTCATGGTATGAAGTTTGACACAATTACTACTGGTACCACCTTCTGCGCAACCTCATCCACATTGCTGATGTATCAATCTCTGCTACTGAA 450
 GluLeuAlaAspLeuPheIleLeuThrArgAspGlyMetLysPheAspThrIleThrThrGlyThrThrSerAlaAsnLeuIleIleIleArgAspValSerAsnIleLeuProThrGlu
 1201
 GGTGGCAAGATCTTCTGAATCACTCACTGAATATGCAGATCATGCTGCCAATGCTGCTGCTGAAGCTACTGTTATGCTTACCCGTAAGCACTGCTGTTACCTCTCCCAAGCGGTGCTGCA 449
 CGTGGCAAGATCTTCTGAATCACTCACTGAATATGCAGATCATGCTGCCAATGCTGCTGCTGAAGCTACTGTTATGCTTACCCGTAAGCACTGCTGTTACCTCTCCCAAGCGGTGCTGCA 450
 GlyGlyLysIlePheValThrIleThrGluTyrAlaAspHisAlaAlaAsnGlyArgGlyGluGlyThrValLeuValThrArgLysAlaLeuSerValThrLeuProSerGlyGlyAla
 1321
 GTCACTCTGAAGCCTGCTGATGTTGCTGCTGACCTTGGTCTCTATCACTGCTGCGCGTCAGGCTCGCTTCTGTTGAAGTTGAAACCAATCAGCGTCAAGTACCTGTTAAGAAATCC 449
 GTCACTCTGAAGCCTGCTGATGTTGCTGCTGACCTTGGTCTCTATCACTGCTGCGCGTCAGGCTCGCTTCTGTTGAAGTTGAAACCAATCAGCGTCAAGTACCTGTTAAGAAATCC 450
 ValThrLeuLysProAlaAspValAlaAlaAspValGlyAlaSerIleThrAlaGlyArgGlnAlaArgPheLeuPheGluValGluThrAsnGlnGlyGluValAlaValLysLysSer
 1441
 1509
 AATGCTGAAGCGCTGGATATTCAGAATGGTACCCCGGACAGCACCGCTGCTGATTTCACTCTGTAAGAGCGAATCTAGTCGATAAAGCGCGCCGAGCGCCCTTTTAAATTAATGTA 449
 AATGCTGAAGCGCTGGATATTCAGAATGGTACCCCGGACAGCACCGCTGCTGATTTCACTCTGTAAGAGCGAATCTAGTCGATAAAGCGCGCCGAGCGCCCTTTTAAATTAATGTA 450
 AsnAlaGluGlyValAspIleGlnAsnGlyThrArgGlyThrAlaProLeuValAspPheThrLeu
 1561
 AAATAAGCAGTTTCATTGTTCCCTCACTCATCAATCTCTTCAATTTAGCGAGGTAGACAAGCTCTGCGGTGCTGCTGACACAGCCCTTATTCAGTTGCTATTATTGTTATACTCT 449
 AAATAAGCAGTTTCATTGTTCCCTCACTCATCAATCTCTTCAATTTAGCGAGGTAGACAAGCTCTGCGGTGCTGCTGACACAGCCCTTATTCAGTTGCTATTATTGTTATACTCT 450

Fig. 5. Nucleotide sequence of the *vapA* gene and flanking DNA from *A. salmonicida* strains A449 and A450 (numbers on right), and translated amino acid sequence. The numbers above indicate nucleotide positions. Direct repeat sequences in the *vapA* gene are double underlined, putative Shine-Dalgarno (SD) ribosomal-binding site, and -10 and -35 RNA polymerase-binding site in front of gene are indicated, and the putative terminator palindrome is indicated by a dotted underline. Vector nucleotide sequence in the region 5' to the *vapA* gene of strain A450 is shown in italics and dotted underline. The translated signal sequence is indicated. Partial amino acid sequences confirmed by Edman degradation are underlined. Oligo nucleotide CG3 is indicated.

Table 3. Codon usage for the *A. salmonicida* A450 *vapA* gene, and *A. hydrophila* genes coding for exported proteins

Amino acid	Codon	Codon usage	% codon usage		Amino acid	Codon	Codon usage	% codon usage	
			<i>A. salmonicida</i>	<i>A. hydrophila</i> ^a				<i>A. salmonicida</i>	<i>A. hydrophila</i>
Phe	UUU	10	36	21	Ala	GCA ^b	10	16	5
	UUC ^b	18	64	79		GCG ^b	4	6	24
Leu	UUA ^c	1	2	4	Tyr	UAU	2	40	50
	UUG ^c	8	17	2		UAC ^b	3	60	50
	CUU ^c	5	11	4	His	CAU	2	50	70
	CUC ^c	4	9	20		CAC	2	50	30
	CUA ^c	0	0	2	Gln	CAA	11	58	27
	CUG ^b	28	61	68		CAG ^b	8	42	73
Ile	AUU	11	42	7	Asn	AAU	14	36	18
	AUC ^b	15	58	69		AAC ^b	25	64	82
	AUA ^c	0	0	25	Lys	AAA ^b	8	30	16
Met	AUG	3	100	100		AAG	19	70	84
Val	GUU ^b	24	57	2	Asp	GAU	19	63	38
	GUC	3	7	46		GAC	11	37	62
	GUA ^b	10	24	5	Glu	GAA ^b	15	79	24
	GUG ^b	5	12	47		GAG	42	17	6
Ser	UCU	11	41	4	Cys	UGU	0	0	50
	UCC	8	30	31		UGC	0	0	50
	UCA ^c	0	0	5	Trp	UGG	4	100	100
	UCG	0	0	9					
	AGU	2	7	9	Arg	CGU ^b	10	72	18
	AGC	6	22	39		CGC ^b	2	14	54
Pro	CCU ^c	4	31	10		CGA	0	0	8
	CCC ^c	0	0	42		CGG	1	7	12
	CCA ^b	1	8	10		AGA ^c	0	0	5
	CCG ^b	8	62	39		AGG ^c	1	7	3
Thr	ACU ^b	23	48	7	Gly	GGU ^b	35	80	8
	ACC ^b	19	40	75		GGC ^b	7	16	68
	ACA ^c	6	12	9		GGA ^c	1	2	8
	ACG	0	0	9		GGG ^c	1	2	16
Ala	GCU ^b	42	66	11					
	GCC	8	13	60					

a Data from (78, 97, 203), **b** codons corresponding to the most abundant tRNA species in *E. coli* (101), **c** rare codons whose corresponding tRNA species in *E. coli* occur with an abundance of 0.3 or less on a scale of 0 to 1.0 (102) and whose percentage use is approximately 10% or less.

promoter sequence with a typical consensus hexamer TATAAT and a poorly conserved -35 region (NNGANA) ending 18 nucleotides further upstream. The 3' region contained a possible terminator structure beginning 17 bases after the termination codon with a GC-rich region of dyad symmetry followed by a cluster of thymine residues (Fig. 5).

Alignment of the sequence obtained from λ 10g for the A-protein gene of strain A449 identified the position of the deletion suffered in this clone which resulted in loss of the ability to produce A-protein. The deletion was of 816 base pairs between the pair of directed repeats (Fig. 5). Other than this deletion, the sequence of DNA from the two strains was identical within the ORFs and the 483 bases of the A449 DNA sequenced downstream. The sequence of the 62 bases immediately in front of the two genes were also identical.

The other difference between the A449 and A450 clones existed in the sequence further upstream (Fig. 5). Since the A450 gene was cloned using a *Sau*3A partial digestion and the sequence of a *Sau*3A site (GATC) was found at the point where the difference occurred, we suspected that this upstream sequence in pSC150 was from the cloning vector. This was proven to be true and will be discussed later in this thesis.

Conservation of the *vapA* gene

Southern blot analysis using the 0.5 kb insert in pSC650 as a probe with chromosomal DNA isolated from ten A-layer producing strains of *A. salmonicida*, representing isolates from diverse species of fish, diverse geographical regions, from diseases with different pathogenesis, and representing the three subspecies of *A. salmonicida*, showed that the gene was carried on a 2.5 kb *Bam*HI restriction fragment in each strain (Fig. 6). No

hybridization was seen with chromosomal DNA's of the four other tetragonal S-layer producing strains of *A. hydrophila* and *A. veronii* biotype *sobria*. Identical results were obtained with the oligonucleotide probe TJ19 which is from the 3' end of the *vapA* gene.

A-protein structure

The *vapA* gene encodes a protein of 502 residues as shown in Fig. 5. Comparison of the predicted sequence with the N-terminal sequence of the mature protein shows the presence of a classical 21-residue amino-terminal signal peptide which is highly hydrophobic (Fig. 7). The predicted amino acid sequence was confirmed in various regions of the mature protein by automated Edman degradation of peptides produced by CNBr hydrolysis of purified A450 A-protein, TPCK-trypsin, and endoproteinase Glu C cleavage. The confirmed residues underlined in Fig. 5 represent 31% of the sequence of the mature A-protein. The primary amino acid sequence was searched in data bases GenPept, SWISS-PROT and PIR (163) and no significant homology was found with other proteins, indicating that A-protein was unique to *A. salmonicida* strains.

The predicted Mr of the mature peptide at 50,778 and the amino acid composition were consistent with that determined previously. In overall composition, the predicted mature A-protein contained 45.1% nonpolar/hydrophobic amino acid residues (Val, Met, Ile, Leu, Ala, Phe, Trp, and Pro). The Kyte and Doolittle (124) hydropathic index using an interval of nine amino acids gave an average hydrophobicity score of -0.45 (the average for soluble protein is -0.4). The A-protein also contained 37.2% polar, 10.2% acidic, and 8.9% basic amino acids and displayed an overall

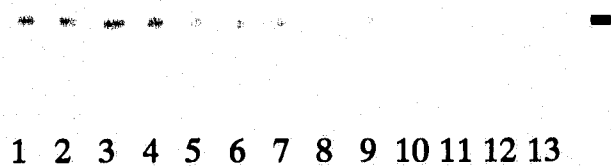


Fig. 6. Southern blot analysis of *Bam*HI digested chromosomal DNAs from *A. salmonicida* strains. From lane 1 to lane 13: A449, A450, A362, A500, A400, A477, A419, A480, A461, TF7, A274, A700 and A702. The probe used was pSC650.

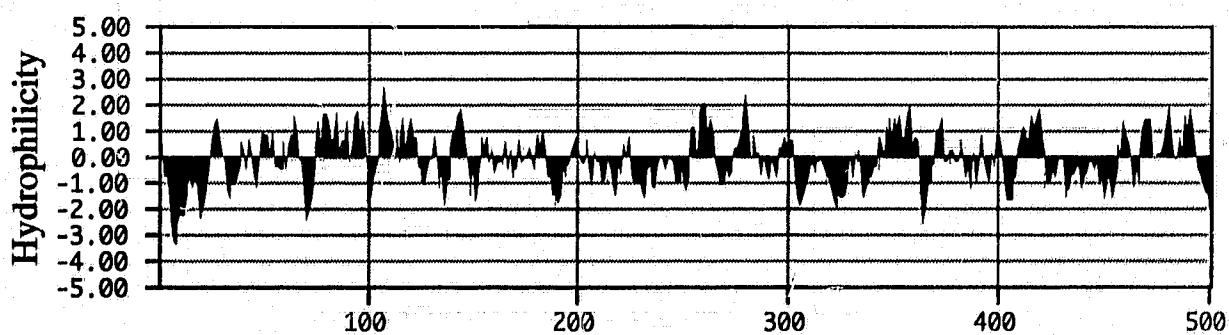


Fig. 7. Kyte-Doolittle hydrophilicity plot of the A-protein. The protein residue numbers are labeled below the plot.

negative charge. The predicted overall pI was 4.79 compared to the measured pI of 4.8-5.3 (167).

The secondary structure of processed A-protein predicted by combining the methods of Chou and Fasman (37) and Garnier et al. (73) (Fig. 9) was compared with the secondary structure previously measured by circular dichroism studies in the far UV (169). The predicted β -structure content of 26.2% was consistent with the measured 19-28%. The predicted α -structure content of 24.5% was significantly higher than the 14% content measured for A-protein under non-denaturing conditions, but was similar to the 29% content obtained in the presence of 0.12% SDS. This α -helix content of A-protein was confined to the sequence after amino acid residue 125. The protein also contained a small content of turns (6.7%) which were distributed evenly among the N-terminal 164 and C-terminal 146 amino acids, but were not present in the central region of the sequence (Fig. 9).

The peptide cleavage studies used to confirm the predicted amino acid sequence of A-protein also provided information on the conformation of A-protein (Fig. 8). When assembled into native A-layer on the surface of *A. salmonicida* cells, A-protein was resistant to TPCK-trypsin, even when cells were treated with a trypsin/cell wet weight ratio as high as 1:100. However, when purified A-protein was treated with TPCK-trypsin at trypsin/protein ratios between 1:200 and 1:10, SDS-PAGE showed that A-protein was rapidly degraded to a major peptide of approximate Mr 39,400, via a series of intermediates in the approximate Mr range 48,000-40,000, and a second major peptide of approximate Mr 16,700, via a Mr 21,300 peptide which was unstable at a ratio of 1:100. The Mr 16,700 peptide was ultimately digested at trypsin/protein ratios of >1:20, but the Mr 39,400 peptide was resistant to

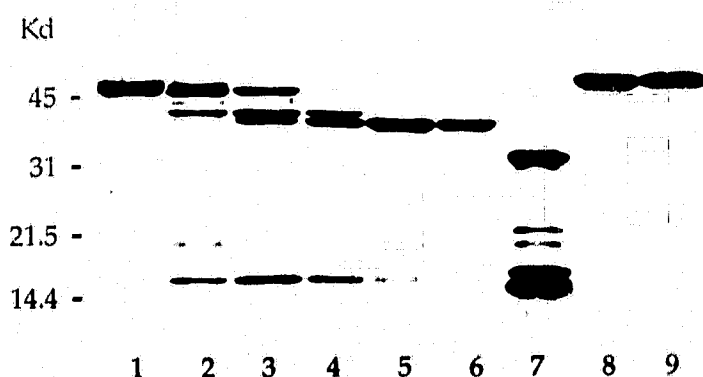


Fig. 8. SDS-PAGE analysis stained by Coomassie Blue of *A. salmonicida* A450 A-protein structure by TPCK-trypsin cleavage and CNBr hydrolysis. A-protein (lane 1), A-protein treated at trypsin/protein ratios of 1:200 (lane 2), 1:100 (lane 3), 1:50 (lane 4), 1:20 (lane 5), 1:10 (lane 6), A-protein treated twice with CNBr (lane 7). Also shown is A-protein in glycine extracts of cells before (lane 8) and after treatment (lane 9) with a trypsin/whole cell lysate protein ratio of 1:500. In contrast to the cleavage seen in the case of isolated A-protein, no cleavage is seen when A-protein is assembled as A-layer on the cell surface. Mr markers (X 1,000) are indicated at right.

further degradation even on prolonged incubation with the highest concentration of trypsin tested (1:5). Automated Edman degradation of intermediate Mr 48,000 and 40,000 peptides and the Mr 39,400 trypsin-resistant peptide showed the same N-terminal sequence as the mature protein. The first 25 residues of the Mr 21,300 peptide corresponded to the deduced A-protein sequence beginning at residue 275, while the first 22 residues of the Mr 16,700 peptide corresponded to the deduced sequence beginning at amino acid 324. Hydrolysis of A-protein by CNBr also resulted in two major peptides, the Mr 31,000 and 13,400 cleavage products. The position of these various sites is shown in Fig. 9.

Cellular localization of A-protein in *E. coli*

E. coli cells carrying plasmid pSC150 produced A-protein during growth at both 20 and 37°C. However, A-protein production in *E. coli* from the cloned *A. salmonicida* DNA was markedly lower than that of wild type *A. salmonicida* and detection required Western blot immunoassay. Comparison of growth curves of *E. coli* cells carrying plasmid pTZ18R with and without the A-protein gene insert showed that production of even this small amount of A-protein delayed the growth of *E. coli*. At 37°C the delay in reaching the mid-exponential phase of growth was approximately 2 h. Cell fractionation experiments showed that *E. coli* was capable of exporting A-protein to the periplasm (Fig. 10). This was shown using two procedures for the isolation of periplasmic proteins. A-protein was also detected in the cytoplasmic membrane, and cytosol fractions, but was not detected in the outer membrane fraction or the culture supernatant. Some proteolytic degradation of A-protein was apparent in the cytoplasmic fraction.

A-protein conformation in periplasm

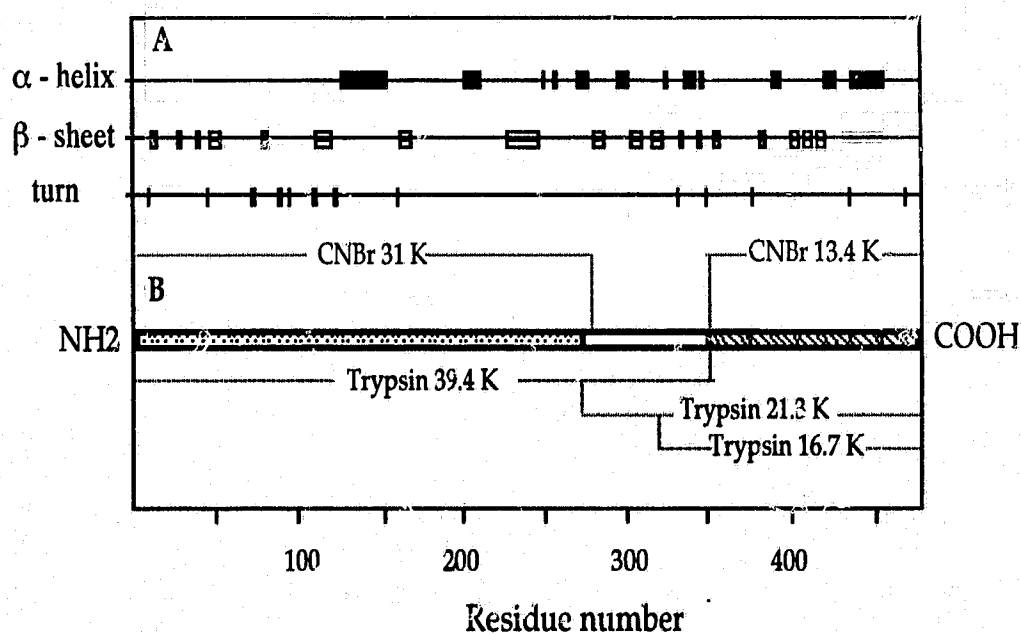


Fig. 9. A: Predicted secondary structure of *A. salmonicida* A450 A-protein based on combined Chou-Fasman and Robson-Garnier methods. B: Map of A-protein showing location of major peptides produced by CNBr hydrolysis and TPCK-trypsin digestion. The trypsin resistant N-terminal 274 residue region of A-protein is dotted, and the C-terminal CNBr peptide is cross-hatched.

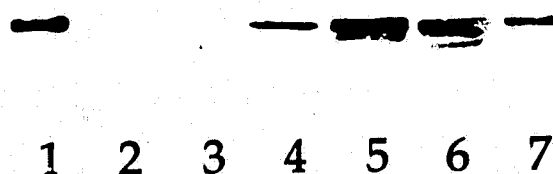
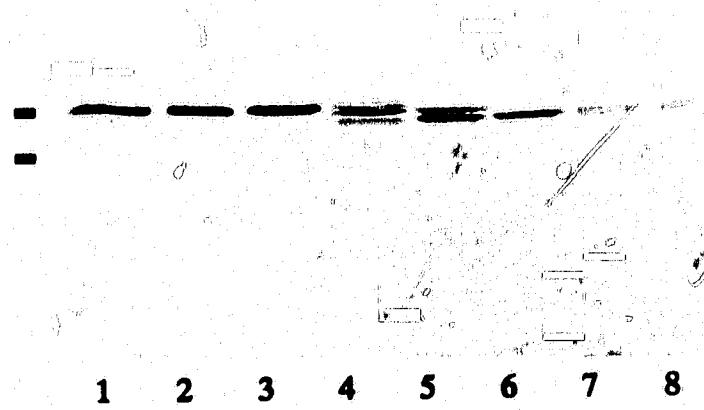
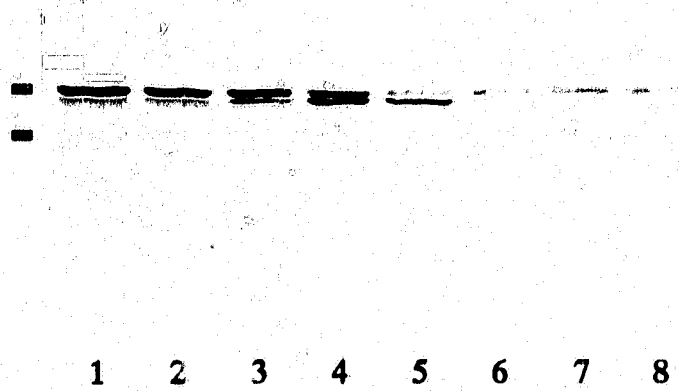


Fig. 10. Western immunoblot analysis of 7.5% SDS-PAGE showing the cellular localization of A-protein coded by pSC150 in *E. coli* DH5 α . Polyclonal affinity purified rabbit antibodies to *A. salmonicida* A450 A-protein were reacted at a dilution of 1:2,000. Lane 1, purified A-protein from A450; lane 2, culture supernatant; lane 3, outer membrane fraction; lane 4, periplasmic fraction; lane 5, cytoplasmic membrane fraction; lane 6, cytosol; lane 7, whole cell lysate.

The finding that A-protein accumulated in the *E. coli* periplasm raised the possibility that the process of A-protein translocation from the periplasm to outer membrane was different between *E. coli* and *A. salmonicida*, and that the conformation of A-protein may play a role in this process. To verify this hypothesis, trypsin accessibility of the periplasmic A-protein from both *A. salmonicida* and *E. coli* was examined by TPCK-trypsin partial digestion and Western blot. The partial digestions were carried out with the TPCK-trypsin in a series of 1:2 dilutions at room temperature for 1 h and stopped by excess amount of soybean trypsin inhibitor. While the expected 39,400 Kd trypsin resistant fragment was produced from A450 periplasmic preparation, it was absent in *E. coli* periplasmic digestion. In contrast to the A-protein accumulated in *E. coli* periplasm, A-protein accumulated in *A. salmonicida* TM1 periplasm, a Tn5 insertion mutant of A449 which accumulates A-protein in the periplasm, showed the same 39,400 Kd trypsin fragment as that in A450 (Fig. 11). This suggested that the trypsin accessibility of periplasmic A-proteins from these strains was different.

A.**B.**

C.

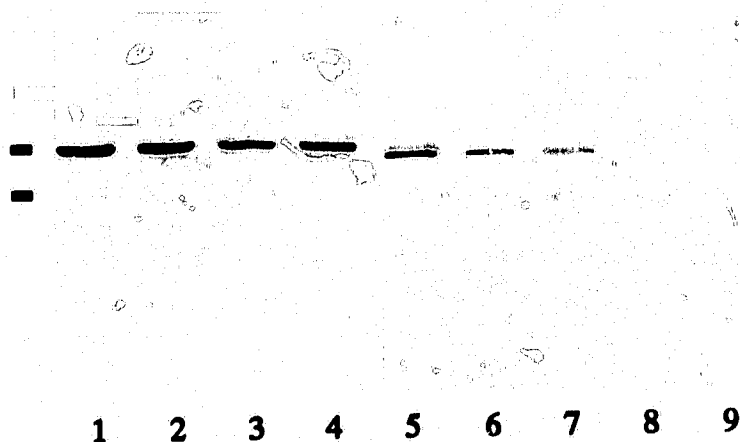


Fig. 11. Western blot analysis of 12% SDS-PAGE of TPCK-trypsin partially digested periplasmic A-protein from *A. salmonicida* and *E. coli* strains. In each panel, TPCK-trypsin concentration was 1:2 diluted for each digestion, with the highest final concentration of 0.25 mg/ml (lane 8 in panel A and B, lane 9 in panel C). Rabbit antiserum against A450 A-protein was used at a dilution of 1:2,000. The upper and lower markers on the left indicate 50 Kd and 39 Kd respectively. A: pSC150/*E. coli* DH5 α . B: A450. C: TM1.

II. Characterization of the *vapA* gene promoter area

Subcloning and sequencing of the flanking region in front of the *vapA* gene

As described before, pSC150 expressed A-protein at a low level in *E. coli* and carried a short *Aeromonas* DNA sequence (62 bp) in front of the *vapA* structural gene. Since the *vapA* expression in wild type *A. salmonicida* is significantly higher than the expression from pSC150, we suspected that a wild type promoter(s) or other *Aeromonas* DNA sequences necessary for normal *vapA* expression were missing in pSC150. This led to a search for the possible wild type promoters of *vapA* in other clones.

DNA sequencing of the insert in pRJ3 and pRJ5 (Fig. 3) revealed a 374 bp DNA sequence immediately in front of the *vapA* gene and 324 base pairs in the gene (Fig. 12). Although the *vapA* structural gene in pRJ5 was truncated by a 816-base pair deletion, the integrity of the sequence upstream from this deletion was confirmed by a series of Southern analyses. In these Southern analyses, the chromosomal DNA and the plasmid DNA were digested with various restriction enzymes and probed with oligonucleotide TJ41 (from residue 123 to 139 in the *vapA* gene). Restriction mapping based on the Southern blot data showed that the area before the 816-base pair deletion in pRJ5 and the related region on the chromosome were identical.

In agreement with this finding, the sequence upstream of -62 in the A450 clone pSC150 was shown to be vector sequence. A data base search (GenBank and EMBL) showed that the sequence in pSC150 was homologous to the neomycin phosphotransferase gene from transposon Tn5 (18). Southern hybridization of *Bam*HI digested A449 and A450 chromosomal DNAs using oligonucleotides and the 0.5 kb *Kpn*I-*Pst*I insert in

TCGAAAAACGGAGTTGGTACTGTTAATCACCCCTATGTGATCACTTCAAAGAAGAGG-421
RAA2

CCGAATTTTATACTGATCAATTCCGTACCTTGAGTGGCTGGATCCGATGCCCAAACGTT-361
BamHI

CTCAATCTGACGCAGGACTAGATCGATGACCATCTTCGGTCATCAGTGCTGTGACTTGAC-301
TaqI -11.6

ACAAAAATCGTCGATCGGCGGTGTTTTTTGTGCGCTCAGTTCAGGAAAACGATGAAAGG-241
TaqI

CGATCTTTGTACAGATTAGTAATTGACATGCCTGAGTCTGATGGCTAAGAATATGCAAG-181
-35 -10

CGCACTTCGGAGCGCGGTGAAACTGTTGTATCTCACAGGACATGAGCATCAGTAGTTTCC-121
A450-2▼
M S I S S F

CATTTTTTTCGGGAAAACAGCGGTGTTTGTGTTTCGGTGCTCACAGTGCACCGAAGGTTGA -61
-9.6 -15.2
P F F A G K Q R C L C S V L T V H R R L

TCAACGGATAGGTTCAACCCTATTTGTATATAATCAATAAACTTCCTTAAGGAAAAAGT -1
-35 -10 CEG4▼ palindromic
I N G ****
SD

ATGTTTAAGAAGACTTTGATTGCAGCTGCCATTGTGGTTCGGTTCGCTGCACCTGCGTTT 60
vapA... TJ52 PviII

GCTGATGTCGTGATTAGCCCGAACGACAACACTTTCGTTACTACCTCCCTCGCATCTGTA 120

ACTAAGCAGCCGGTACTGGACTTCTCCACTGCTCAACAAAACCTGACCCTTAACCTCAGT 180
TJ41

Fig. 12. DNA sequence of the 5' region of the *vapA* gene of A450, the immediate upstream flanking DNA, and the predicted amino acid sequence. Nucleotide residues are labeled on the right relative to *vapA*. The transcriptional start site is double underlined and the putative SD sequence labeled with (*). Three stem loop structures are underlined and their free energy values marked on the top. A palindromic sequence, restriction sites, conserved promoter sequences and oligonucleotide probes used are also shown. Amino acid sequence of a predicted short peptide is shown under the DNA sequence. The ISA1 insertion in A450-2, and the ISA2 insertion in CEG4 are indicated by arrows.

pSC650 (mapped to the central region of the *vapA* gene, Fig. 3) showed an identical 2.5 kb fragment, which was exactly the size between the *Bam*HI site in pRJ5 and the *Bam*HI site in the pSC150 insert (Fig. 3). Furthermore, A449 is sensitive to kanamycin and the vector pLA2917 contains the neomycin phosphotransferase gene.

Analysis of the 374 bp of DNA sequence upstream from the A449 *vapA* gene revealed a number of interesting features. The GC content of this region is 44.5%, fairly close to *vapA* gene 48% but lower than the 55 +/- 1.8% reported for the *A. salmonicida* genome (22). In front of the gene, there are three potential stem loop structures located at -336 to -317 ($\Delta G = -11.6$ kCal), -125 to -105 ($\Delta G = -9.6$ kCal) and -89 to -67 ($\Delta G = -15.2$ kCal), respectively (Fig. 12). These are the three lowest free energy values among all predicted stem loop structures in the 3.4 kb of *vapA* containing *Aeromonas* DNA sequenced in this laboratory to date. None of other predicted stem loop structures have energy values lower than -7.2 kCal. No long ORFs were observed in front of these stem loop structures, however, a small 86-base pair ORF was observed from -137 to -24, coding for a putative 3,266 Dalton peptide of 29 amino acid residues (Fig. 12). This ORF spans the second and the third potential hair pin structures (Fig. 12). At -245 - -225, there is a AA-GGCGA--T---TCA-AGA sequence which shows 66% conservation with the consensus cAMP-CRP binding site (49). A 12-base pair palindromic sequence is present 6 base pairs upstream from the *vapA* structural gene (Fig. 12). This may indicate a site for DNA binding proteins (229). The previously identified ribosome binding site, AGGA, resides in the 3' half of the palindromic sequence, from -11 to -8, and, as previously mentioned, is optimally positioned relative to *vapA*.

As was the case for the *vapA* structural gene, when the upstream region of six A-layer-producing *A. salmonicida* strains (A185, A450 and A591, A400,

A460 and A480) was analyzed by PCR fragment digestion using the primers RAA2 (-438 to -462, referring to *vapA* gene), TJ41 and restriction enzyme *TaqI*, the region was found to be conserved. Strains A185, A400 and A450 were also sequenced in the promoter region (from oligonucleotide RAA2) and the sequences were identical to that of A449.

Primer extension analysis

To determine the natural transcriptional start site for *vapA* in *A. salmonicida*, primer extension analysis was employed. Because pRJ5 contained 374 bp of *A. salmonicida* sequence immediately 5' to *vapA* gene as well as 220 bp of the 5' sequence of *vapA*, the pRJ5 insert was subcloned into M13 to prepare the DNA sequencing ladder. RNA templates were prepared from *A. salmonicida* strains A449 and A450, and the extension and DNA sequencing ladders were started from the 17-mer oligonucleotide TJ-52 (*vapA* bp 3-19, Fig. 12).

The results in Fig. 13 show a single identical start site for *vapA* transcription in *A. salmonicida* strains A449 and A450. Comparison with the DNA sequence ladder showed that the transcription started at G -181 relative to the structural gene (Fig. 12). No other band was observed, even in the area following the predicted P2 promoter. Perfectly positioned relative to this start of the *vapA* message was a -10 TAagAAT box at -13 to -7, and 17 bases further upstream was a -35 TTGACA box at -36 to -31. These sequences were designated the P1 promoter of *vapA* and the previously predicted proximal promoter was designated the P2 promoter. Primer extension experiments were also performed with *A. salmonicida* 30°C mutants A450-3 and A450-CEG6

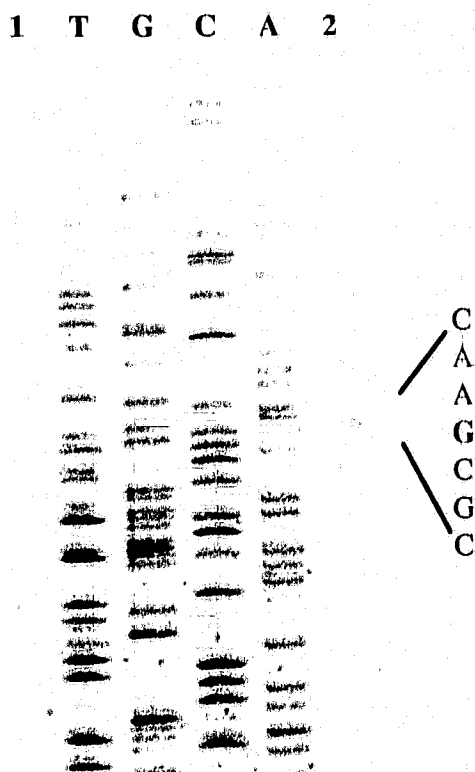


Fig. 13. Primer extension of the *vapA* gene. Lane 1 and lane 2 represent RNAs from A449 and A450 respectively. Lanes labeled with A, C, G and T are DNA sequencing lanes. The transcriptional start residue G (bold) and surrounding bases are labeled on the right.

which produced reduced quantities of A-protein and were unable to produce A-layer, and with *E. coli* DH5 α carrying pSC150 DNA which contained an intact *vapA* gene, as well as 62 bp of immediate upstream flanking DNA including the ribosomal binding site and the predicted P2 promoter sequence, but lacked the predominant P1 promoter sequence. The insert in this plasmid produced low levels of A-protein in *E. coli* DH5 α . However, in none of these cases was an RNA band detected in primer extension experiments.

***vapA* transcript size**

Northern hybridization with the 17-mer oligonucleotide probe TJ41 to the 5' end of *vapA* was used to determine the size of the *vapA* transcript and to evaluate whether *vapA* mRNA was monocistronic. Fig. 14 shows that a single mRNA band of 1.65 kb was observed in both the wild-type S-layer producing strains examined. This size was consistent with the predicted 1.7 kb size for a monocistronic mRNA based on DNA sequence and primer extension analysis. Northern analysis was also used to examine the relative amount of *vapA* transcript produced in reduced A-protein producing mutants A450-3 and A450-CEG6. No *vapA* mRNA was detected in either case, or in Northern blots of mRNA isolated from either TM4 which had Tn5 inserted in the *vapA* gene, or strain A438 in which *vapA* is deleted (Fig. 14).

Growth phase dependence of *vapA* transcription

Northern analysis of total cell RNA (5 μ g/lane) with 32 P-radiolabeled *vapA* specific oligonucleotide TJ41 allowed the quantitation of *vapA* transcript during cell growth. Fig. 15 shows that at 15°C the quantity of *vapA* mRNA increased rapidly early in growth, peaking in early log phase at 11 h. This was followed by a relatively sharp decline in *vapA* mRNA as the cells reached late log phase. A similar profile of *vapA* transcript production was observed in

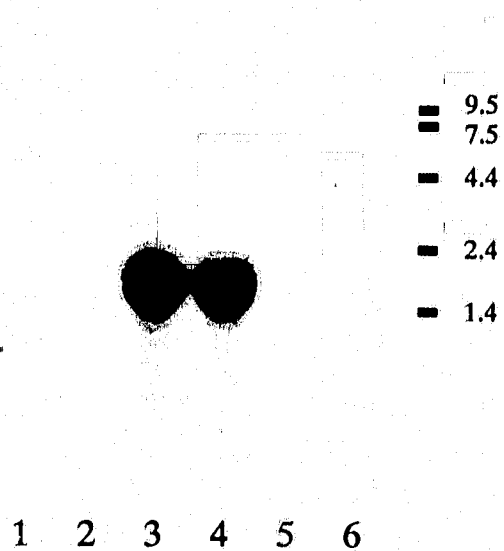


Fig. 14. Northern hybridization of wild type and mutant *Aeromonas* strains. Size markers are on the right side. Lane 1 through lane 6 represent RNAs from TM4, A438, A450, A449, A450-3 and CEG6 respectively.

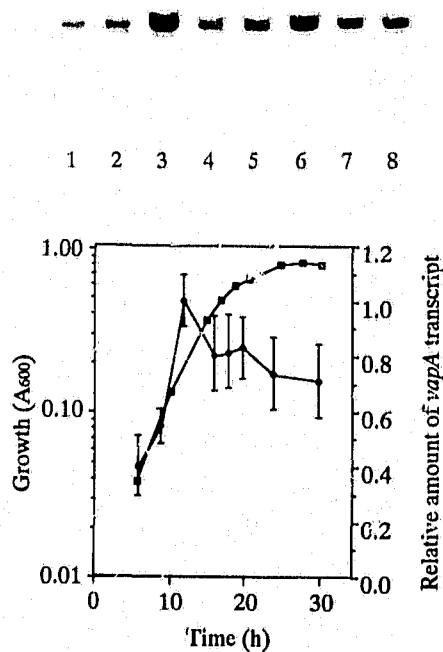


Fig. 15. *vapA* transcript dynamics during cell growth at 15°C. Upper panel: Northern hybridization of A449 RNA probed with radiolabeled oligonucleotide TJ41. Lanes 1 through 8 are A449 RNAs isolated after 6, 9, 12, 16, 18, 20, 24 and 30 h growth respectively. Lower panel: solid squares represent relative *vapA* transcript level obtained from densitometer analysis; hollow squares indicate absorbance of the cell culture at 600 nm during growth. The plots represent the average of three independent experiments.

cultures grown at 20°C, although the decline after peak *vapA* transcript production was more pronounced (data not shown).

Effects of growth temperature on *vapA* transcription

Growth at different temperatures also affected the quantity of *vapA* transcript produced by *A. salmonicida* cells. When cells of strain A449 were grown in L-broth at 10, 15, 20, 25 and 30°C to early log phase and the relative quantity of *vapA* transcript determined, the highest quantity of *vapA* mRNA was observed in cells grown at 15°C (Fig. 16). Cells grown at the other temperatures had reproducibly lower quantities of *vapA* mRNA. Indeed cells grown at 30°C had approximately only 12% of the *vapA* transcript compared to cells grown at 15°C.

***vapA* transcript stability**

Growth temperature also affected the stability of *vapA* mRNA. This was shown by adding rifampicin (0.2 mg/ml) to log phase cultures of *A. salmonicida* A449 growing at 15°C and 20°C to inhibit transcription. At 5 min intervals thereafter cell samples were removed from the cultures and placed on ice. RNAs isolated from the cell samples were then analyzed by Northern hybridization. Fig. 17 shows the time-dependent decay of the 1.65 Kb *vapA* mRNA band after rifampicin addition, with the transcript being noticeably more stable in cells growing at 15°C. Densitometric analysis of the autoradiograms from two independent experiments showed that the average half life of the *vapA* mRNA in cells growing at 15°C was 22 min compared to 11 min for cells growing at 20°C. No difference in transcript stability was seen between early log phase (10 h) and stationary phase (18 h) cells (data not shown).

A.



10 15 20 25 30

B.

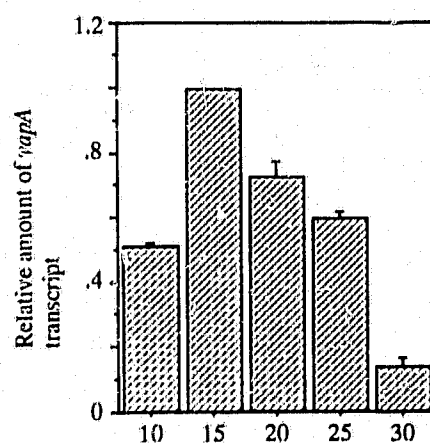


Fig. 16. *vapA* transcript level at various temperatures measured by Northern hybridization. Panel A: Northern hybridization of A449 RNA probed with radiolabeled TJ41. Panel B: Plot of densitometer analysis of the Northern hybridization gels from three independent experiments. The numbers at the bottom of each panel indicate temperatures (°C) at which these cells were grown.

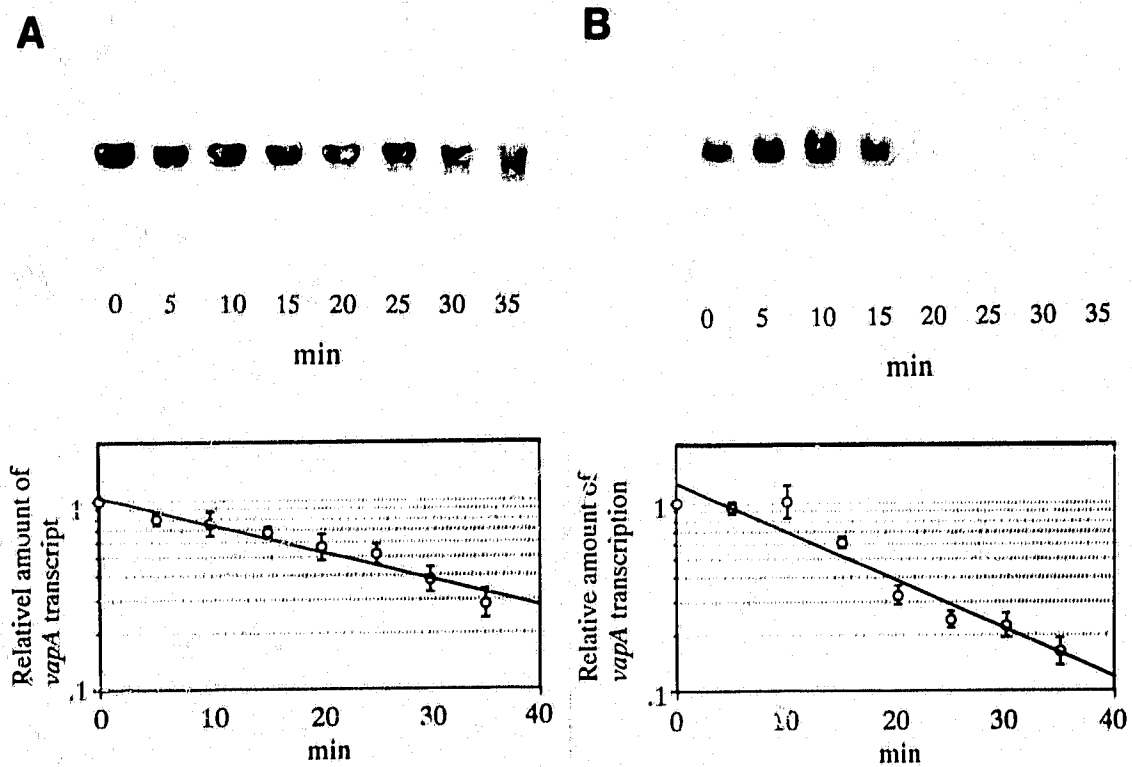


Fig. 17. *vapA* transcript stability assay. Panel A: 15°C culture. Panel B: 20°C culture. Pictures show Northern hybridization of A449 probed with radiolabeled TJ41. Samples were collected at 5 min intervals after addition of rifampicin. Semilog graphs show plots of densitometric analysis results from three independent experiments and were used for half life measurements.

Variation of *vapA* transcript levels in other growth conditions

Selected other growth conditions were tested for their effect on *vapA* transcription, including growth atmosphere, growth pH (pH 6 - pH 9), and ion addition and depletion. No statistically significant changes were observed in the amount of *vapA* transcript as a result of changes in growth pH (Table 4), from Ca^{2+} or Mg^{2+} addition, or from Fe^{3+} and Ca^{2+} depletion (Tables 5, 6 and 7). However when log phase cells were incubated anaerobically for 4 h, a 39% reduction was seen in the quantity of *vapA* transcript (Table 8).

Effects of nalidixic acid and novobiocin on *vapA* transcription

The effect of the DNA gyrase inhibitors, nalidixic acid and novobiocin, on *vapA* transcription was also examined by Northern analysis. In the case of nalidixic acid, while 0.313 $\mu\text{g/ml}$ had no effect on the quantity of *vapA* transcript, 0.625 $\mu\text{g/ml}$ reduced *vapA* mRNA to 50% of that of cells grown in the absence of the DNA gyrase inhibitor (Table 9). Similarly, while novobiocin at 10 $\mu\text{g/ml}$ appeared not to affect *vapA* transcription, 20 $\mu\text{g/ml}$ novobiocin reduced *vapA* transcript production by 50% (Table 10). Growth of *A. salmonicida* was not detectably inhibited by the concentrations of nalidixic acid and novobiocin used.

Table 4. *vapA* transcript level affected by pH

pH	Relative amount of <i>vapA</i> transcript $\pm$ SD	% $\pm$ SD
6	724 $\pm$ 67	79 $\pm$ 9
7	916 $\pm$ 37	100 $\pm$ 4
8	946 $\pm$ 112	103 $\pm$ 12
9	1020 $\pm$ 69	111 $\pm$ 8

Table 5. *vapA* transcript level affected by Ca⁺⁺ addition and depletion

EGTA (mM)	CaCl ₂ (mM)	Relative amount of <i>vapA</i> transcript $\pm$ SD	% $\pm$ SD
0	0	1291 $\pm$ 89	100 $\pm$ 7
0	1	1280 $\pm$ 79	99 $\pm$ 6
0	10	1332 $\pm$ 115	103 $\pm$ 9
0	30	1165 $\pm$ 134	90 $\pm$ 10
10	0	1089 $\pm$ 159	84 $\pm$ 12
10	1	1185 $\pm$ 80	92 $\pm$ 8
10	10	1181 $\pm$ 181	92 $\pm$ 15
10	30	1271 $\pm$ 62	99 $\pm$ 5

Table 6. *vapA* transcript level affected by Fe⁺⁺⁺ depletion

2, 2'-dipyridyl (mM)	FeCl ₃ (mM)	Relative amount of <i>vapA</i> transcript $\pm$ SD	% $\pm$ SD
0.00	0.00	956 $\pm$ 48	100 $\pm$ 5
0.01	0.00	994 $\pm$ 67	104 $\pm$ 7
0.01	0.01	927 $\pm$ 86	97 $\pm$ 9
0.10	0.00	908 $\pm$ 67	95 $\pm$ 7
0.10	0.01	879 $\pm$ 115	92 $\pm$ 12
0.10	0.10	975 $\pm$ 105	102 $\pm$ 11

Table 7. *vapA* transcript level affected by Mg⁺⁺ addition

MgCl ₂ (mM)	Relative amount of <i>vapA</i> transcript $\pm$ SD	% $\pm$ SD
0	1664 $\pm$ 48	100 $\pm$ 3
1	1580 $\pm$ 133	95 $\pm$ 8
10	1647 $\pm$ 150	99 $\pm$ 9
30	1847 $\pm$ 166	111 $\pm$ 10

Table 8. *vapA* transcript level affected by oxygen

Oxygen content	Relative amount of <i>vapA</i> transcript $\pm$ SD	% $\pm$ SD
Normal	3264 $\pm$ 245	100 $\pm$ 8
Microaerophilic	2483 $\pm$ 369	76 $\pm$ 11
Anaerobic	1991 $\pm$ 620	61 $\pm$ 19

Table 9. *vapA* transcript level affected by nalidixic acid

Nalidixic acid ($\mu\text{g/ml}$)	Relative amount of <i>vapA</i> transcript $\pm$ SD	% $\pm$ SD
0.000	1591 $\pm$ 213	100 $\pm$ 13
0.313	1501 $\pm$ 78	94 $\pm$ 5
0.625	820 $\pm$ 231	51 $\pm$ 15
1.250	561 $\pm$ 68	35 $\pm$ 4

Table 10. *vapA* transcript level affected by novobiocin

Novobiocin ($\mu\text{g/ml}$)	Relative amount of <i>vapA</i> transcript $\pm$ SD	% $\pm$ SD
0.0	1447 $\pm$ 293	100 $\pm$ 20
2.5	1250 $\pm$ 323	86 $\pm$ 6
5.0	1542 $\pm$ 254	107 $\pm$ 17
10.0	1310 $\pm$ 85	91 $\pm$ 6
20.0	722 $\pm$ 14	50 $\pm$ 1

III. The *vapA* gene in some *A. salmonicida* 30°C mutants is interrupted by insertion elements

DNA rearrangements in 30°C mutants

It was previously reported that when *A. salmonicida* cells were grown at 30°C, while most of them would die, surviving cells experienced irreversible loss of virulence and A-layer (105). The loss of A-layer was shown by SDS-PAGE and thin section electromicroscopy, as well as by functional evidence such as the loss of self-aggregation, increased sensitivity to bacterial phages and increased yeast cell binding affinity. We also examined a 30°C mutant A450-3 by immunogold electromicroscopy with polyclonal antiserum against A-protein, and found significantly less gold particles on the cell surface compared to the wild type strain A450 (Fig. 18).

On the DNA level, Belland and Trust have previously observed that in an area near the beginning of the *vapA* structural gene in A450-3, an uncharacterized DNA rearrangement had occurred (21). To provide more information on this DNA rearrangement, a series of Southern hybridizations were carried out on A450 and A450-3 using oligonucleotide TJ44 from near the 5' end of the gene. The chromosomal DNAs were digested with *AccI*, *BamHI*, *HindIII* and *PstI*, and each of these digestions showed a uniform size difference, i.e., the A450-3 fragments were about 1.2 kb bigger than the A450 fragments (Fig. 19). These results led to an A450-3 chromosome map which, when compared with A450, revealed a 1.2 kb expansion between the upstream *BamHI* site and the *PstI* site in the *vapA* structural gene (Fig. 20).

To further understand the molecular mechanism(s) involved in the phenotypic change, we made more 30°C mutants of A449 and A450 and

A.



B.



Fig. 18. Electron micrographs of immunogold labeling of A450 (A) and A450-3 (B) cells.

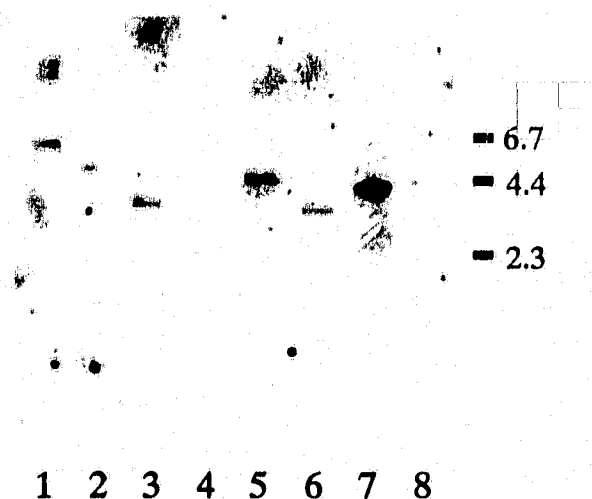


Fig. 19. Southern hybridization of A450 and A450-3 chromosomal DNAs probed with oligonucleotide TJ44. Odd numbered lanes are A450-3 DNAs and even numbered lanes are A450 DNAs. Lanes 1 and 2 are *Hind*III digestions. Lanes 3 and 4 are *Bam*HI digestions. Lanes 5 and 6 are *Acc*I digestions. Lanes 7 and 8 are *Pst*I digestions. Size markers are indicated on the right (kb).

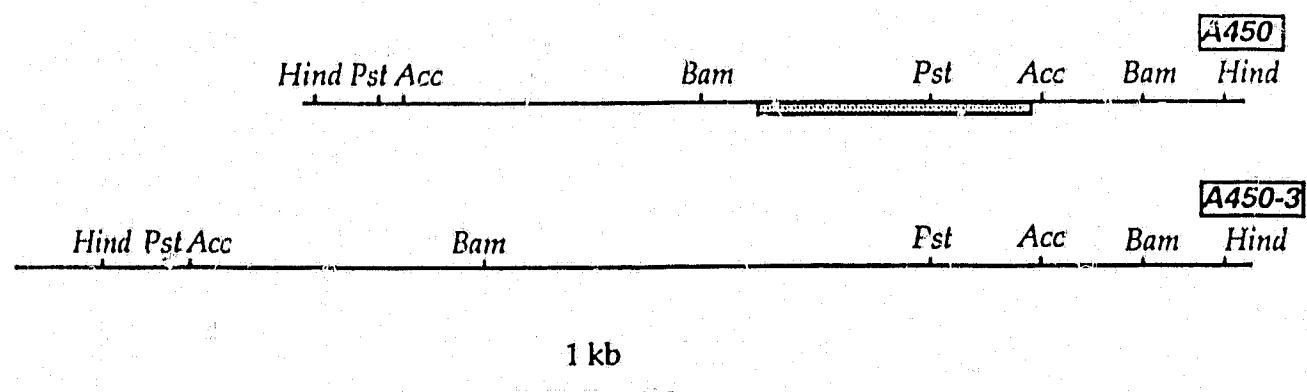


Fig. 20. Comparison of physical maps of A450 and A450-3 chromosomes. The shaded box in the A450 map indicates the *vapA* gene. Restriction sites are indicated.

found that the A-protein production in the mutants varied but all produced lower amounts of A-protein than the wild type strain A450 (Fig. 21). Southern hybridization with *Bam*HI digested chromosomal DNAs probed with TJ44 showed that the 30°C mutants had bands of various sizes. These variations suggested that the DNA rearrangements were heterogeneous, and resulted in different reductions in A-protein production.

Identification of two insertions in the *vapA* area in *A. salmonicida* 30°C mutants

Using oligonucleotides derived from the *vapA* area in the *Aeromonas* chromosome, PCR analysis was performed to localize the rearrangements in A450-3 and other 30°C mutants. Among the strains tested, the sites of the rearrangement were found scattered in the *vapA* area including the structural gene and the promoter area in front of the gene (Gustafson, unpublished data). Interestingly, these PCR products containing the rearranged area all showed a 1.2 kb size increase similar to that seen in A450-3. This suggested that these expanded DNA sequences, although localized in different sites, might be of the same origin. To demonstrate this hypothesis, the expanded DNA sequence in CEG6 was cloned (pCG106), excised, radiolabeled, and used as a probe in Southern analysis of other 30°C mutants (Gustafson, unpublished data). Under stringent conditions, PCR products containing the expanded region in A450-2, A450-3, CEG6, CEG22 showed positive hybridizations (Table 11), suggesting a similar composition or the same origin. From among the mutant strains which showed negative reactions with pCG106, CEG4 was chosen to prepare a second probe for the DNA rearrangements. The expanded DNA sequence in CEG4 was amplified by PCR and radiolabeled. In the Southern hybridization using this CEG4 probe,

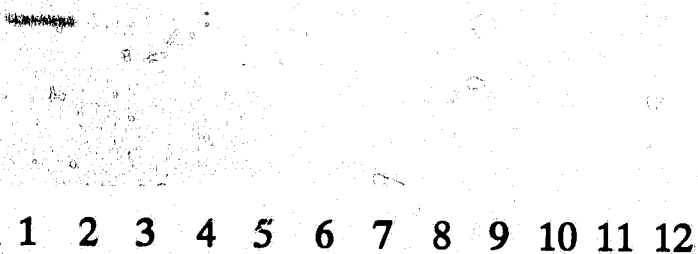


Fig. 21. Western blot of whole cell lysate of *A. salmonicida* 30°C mutants with 1:1,000 diluted rabbit anti-A-protein antiserum. From lane 1 to lane 12: A450-2, CEG4, A450-3, CEG7, CEG24, CEG27, CEG28, CEG29, CEG6, CEG22, TM4, and A450. Position of wild type A-protein is marked on the right.

strains displaying negative reactions with pCG106 all showed positive results (CEG7, CEG24, CEG27, CEG28 and CEG29), while those which hybridized with pCG106 were all negative with CEG4 (Table 11). This suggested that the DNA rearrangements in these *A. salmonicida* 30°C mutant strains involved two classes of insertion element. Since these insertion elements are the first ones identified in *Aeromonas* cells, the element identified from CEG6 and like mutants has been designated ISA1 and the element identified in CEG4 and like mutants has been designated ISA2.

Cloning and sequencing of ISA2

As DNA sequencing of ISA1 displayed a typical insertion element structure (Gustafson, unpublished data), ISA2 was likely to be an insertion element as well. The 1.9 kb PCR fragment synthesized by RAA2 and TJ44 on CEG27 containing the insertion DNA sequence (ISA2) was cloned into pGEM plasmid (Promega) yielding pSC103, and the entire ISA2 DNA sequence of 1,163 bp was determined (Fig. 22). From the DNA sequencing data, the ISA2 sequence was found between a pair of duplicated 3-bp targeting sequences from base C₁₃₆ to G₁₃₈ in the *vapA* gene. A pair of perfect terminal inverted repeats of 13 bp or a pair of imperfect inverted repeats of 29 bp with 6 mismatches were identified at both ends of the sequence. In addition, a palindromic sequence of 21 bp with an unmatched T in the middle was observed near the center of the sequence. An ORF of 1,017 bp was predicted spanning almost the entire region of the ISA2, starting from an ATG codon at base 34 and coding for a putative protein of 339 residues with a molecular weight of 38,810. The G+C content of this ORF is 60%. There was no obvious rbs and conserved promoter sequences observed in front of the ORF. Like the predicted protein sequence in ISA1, the hydropathy of this protein showed

Table 11. Results of DNA hybridization of *A. salmonicida* 30°C mutants

Strains	Probes	
	CEG6	CEG4
A450-2	+	-
A450-3	+	-
CEG6	+	-
CEG22	+	-
CEG4	-	+
CEG7	-	+
CEG24	-	+
CEG27	-	+
CEG28	-	+
CEG29	-	+

CTG CCGGAATTCAGATAATAAGTGCGACACTCATGG

ATGAATGGCGAGAGGTGGCCAACTGCTGATTGGTGGTACCCCTTCTGCTCGCCAAAGTAAA 60
 M N G E R W P T A D W W Y P S A R Q S K
 AGCAGTATCACCATGAGCTATCAGCAGTTGACCGAGGGGCAACGATACCAGTTATCGGTG 120
 S S I T M S Y Q Q L T E G Q R Y Q L S V
 CTTCGCGCACAGGGGATGTCCATCCTTGCCACCGCACGCGCCATTGGGGTTCATCGCTCC 180
 L R A Q G M S I L A T A R A I G V H R S
 ACCCTCTACCGGGGAGCTGCGTCGTAACGCCGGGCGCAGGGCTACCAGCCAGACAATGCG 240
 T L Y R E L R R N A G P Q G Y Q P D N A
 CATCAGCACGCCACCCACCGACGGGCCAGTGCGGCCAAATCTCGGCTATCTGCCGATGTC 300
 H Q H A T H R R A S A A K S R L S A D V
 ATCCAGTTTCTCGAGCTGACACTCGCCTGGTGGTGGAGCCCGGAGCAGATAAGCGCGGTG 360
 I Q F L E L T L A W W W S P E Q I S A V
 GGCAAGCAGATAGGCCCTCATGGTGAGTCACGAGTGGATATACCGCCATGTGGCGCGCGAC 420
 G K Q I G L M V S H E W I Y R H V A A D
 AAAGCCCCGGGTGGCCAGTTGTACCGCCATCTGCGCCAGGGCCACAAGCGCTACCGCAAG 480
 K A R G G Q L Y R H L R Q G H K R Y R K
 GGGCGAGCAGCCTGCGTTCTCCCATCAAGGAAGCCCGCTCTATTGATGAACGGCCCGCT 540
 G A S S L R S P I K E A R S I D E R P A
 ATCGTCTGATAGCCGGGAGCGGCTCGGTGATTGGGAGGCGGACACGGTGCTGGGCAAGCAA 600
 I V D S R E R L G D W E A D T V L G K Q
 GGCACAGGTGCACTGGTCAACCTGGTGGAGCGCAAGAGCCGGCTCTATCTGGTCAAGCGG^{SC8} 660
 G T G A L V T L V E R K S R L Y L V K R
 GTCGCGAACAACAGGGCTGGGGTGGTGGCGGATGCGATAATCGAGATGTTAACGCCCTAC 720
 V A N K Q A G V V R D A I I E M L T P Y
 ATCGAGCAGGTGCATACCATCACCTTTGACAACGGCGGCGAGTTTGCCGAACACAAGGCG 780
 I E Q V H T I T F D N G G E F A E H K A
 ATAGAGGAAGCGCTGGGTGCCGAGACTTACTTTGCGCACCCCTTACTCTTCGTGGGAGCGC 840
 I E E A L G A E T Y F A H P Y S S W E R
 GGTCTGAACGAGACAGCAATGGTCTGTTGAGGCAGTTTATCCCAAAGGGAACAGACCTG 900
 G L N E N S N G L L R Q F I P K G T D L
 AGAGAGGTACCGACGAGGATGTCAGGAGAGCGGAGCAGTGGCTCAACCTACGACCTCGG 960
 R E V T D E D V R R A E Q W L N L R P R
 AAATGTCTCGGATTCAAGCAGCCTGTCAAGGTATTGCAAGAGTACCGTCAGGCGGCA 1017
 K C L G F R Q P V K V F E E Y R Q A A
 TAGGCGAGTGTCCCACTTCGAAGTTGAATTCGCGCTG

Fig. 22. *A. salmonicida* ISA2 sequence. Duplicated targeting sequence is indicated at both ends as outlined letters. Inverted repeats near the ends of the sequence and the palindromic sequence near the middle are underlined. Nucleotide numbers for the putative transposase gene are marked on the right and the deduced amino acid sequence is shown. Oligonucleotide SC8 is indicated.

essentially a hydrophilic composition, as predicted by the method of Kyte and Doolittle (124). Comparison of the ISA1 and ISA2 protein sequences did not shown any significant homology, neither did their DNA sequences. However, a homology search of the ISA2 DNA and protein sequences in NCBI databases using the FASTA protocol (163) revealed high similarities with other bacterial insertion elements at both the DNA and protein levels. The proteins encoded by these insertion elements with high similarities to ISA2 included a probable transposase in the *E. coli* insertion element IS30 (40), a predicted protein from the *Alcaligenes eutrophus* insertion element IS1086 (42), and a protein encoded by the *Bacteroides fragilis* transposon Tn4551 direct repeat sequence-R (196) and insertion element IS4351 (173). Amino acid sequence alignment showed that the homology was widespread over most of the protein with some regions near the C-terminus displayed especially significant homology (Fig. 23).

ISA2 MNG-----ER WPTADWW--- --YPS----- ARQSKSSITM --SYQQLTEG 33
 IS1086 MTR-----TK YQQLQ----- --PE----- ERMRIEIWKA --EDVSLRAM 30
 IS30 MRRITITAEK ASVFELWKNG TGFSEITNIL GSKPGTIFTM LRDTGGIKPH 50
 IS4351 MSK-----H ITEEQR----- --YAI----- -SMMLQI-PM --SKKAIAEA 29

ISA2 QRYQ----LS V-LRA---QG MSILATARAI GV--HR--ST LYRELRRNAG 71
 IS1086 AR-R-----LG ---RA---PS -TLMRELRRN AT--AR--GG -YGAM----S 59
 IS30 ERKRAVAHLT LSEREERIRAG LSAKMSIRAI ATALNRSPST ISREVQRNRG 100
 IS4351 IG-----VD ---KS----- -TVYREIKRN CD--AR--SG SY-SM----E 55

ISA2 PQGYQPDNAH QHATHRRASA AKSRLSAD-- VIQFLELTLA WWWSPECISA 119
 IS1086 AQACRTQRLK ASRPVAKL-A PDGVLWGV-- VRHFLDQK-- --WSPQEISG 102
 IS30 RRYKKAVDAN NRANRMAKRP KPCLLDQNL P LRKLVLEKLE MKWSPECISG 150
 IS4351 LAQRKADRRK QQKHKRKEVLT PA--MRKR-- IIKLLKKG-- --FSPECIVG 97

ISA2 VGK-----QI GLMVSHETIY RHVAA-DKAR -GGQLYRHLR QGH-KRY-RK 160
 IS1086 TLKRAFPDQP DLNVSHETIY NAIYAYPRGE LRRQLIACLR QARTKRL-PR 151
 IS30 WLRRTKPRQK TLRISSETIY KTLYFRSREA LHHLNIOHLE RSHSLRHGRR 200
 IS4351 RSR----LEG IAMVSHETIY RWIWE-DKRR -GGKLHKYLA R-QGRYAKR 140

ISA2 G-ASSIRSHI K--EARSTDE RPAIVDSRER IGQWEADTVL GKQGTGALVT 207
 IS1086 SRGTDHRCOI P--DMVSIHV RPPEVNDRLM FGHWEGDLIK GAGNQSAVGV 199
 IS30 HTRKGERGTI NIVNGTPIHE RSRNIDNRRS IGHWEGDLVS GTKN-SHIAT 249
 IS4351 GSKNAGRGFI P--GRVDIDE RPEIVELKER FGDLEIDTII GKNHKGAILT 188

ISA2 LVERKSRLYL VKRVANKQAG VVRDAIIEML TPYIEQV-HT ITFDNGGEFA 256
 IS1086 LVERMSRAVL LVKMPDATAA SALAGFTGKL QSLVAPLRQT ITMDQGREMA 249
 IS30 LVDKRSRYTI ILRLRGKDSV SVNQALTDKF LSLPSELRSK ITWDRGMELA 299
 IS4351 INDRATSRVW IRKLSGKEAI PV-AKIAVWA LRKVKNLIHT ITADNGKEFA 237

ISA2 EHKAIEEALG AETVFAHPSY SWERGLNENS NGLIRQYIPK GTDLREVTDE 306
 IS1086 RPAELSAATG VRVYFCDPHS FWRGTCENT NGLIRQYIPK GTDLSVYSQE 299
 IS30 RHLEFTVSTG VKVYFCDPOS FWRGTNENT NGLIRQYIPK KTCLAQYTOH 349
 IS4351 KHEEIAQKLE IKFYFCKPYH SWERGANENT NGLIRQYIPK GKDFSEVTNK 287

ISA2 DVRRAEQWLN LRPRKILGFR QPVKFEEY- -----R-QAA 339
 IS1086 ELDAIADSLN GRPRKILNWH SPLQVLAQVL ANPTDRLPVQ 339
 IS30 ELVLVAAQLN NRPRKILKFK TPKEIIERGV A-----LTD 383
 IS4351 QIKWIENKLN NRPRKILGYL TPNEK/KQII N-QNSVAFAS 326

Fig. 23. Amino acid sequence alignment of the predicted ISA2 encoded protein and other proteins coded by prokaryotic insertion elements. Names of the insertion elements which code for the proteins are labeled on the left and the residue numbers are labeled on the right. All the sequence residues are shown and completely conserved residues are boxed. $\blacktriangleright$ indicates completely conserved Arg residues. $\blacktriangleright$ indicates Arg residues conserved in three of the four sequences. $\rightarrow$ indicates Arg residues conserved in two of the four sequences.

Effect of insertion elements in the *vapA* promoter region on A-protein production

A. salmonicida mutants A450-2 and CEG4 have been shown by PCR to have ISA1 and ISA2 inserted in their *vapA* promoter regions (Gustafson unpublished data). In immunoblot analysis, these two mutants expressed A-protein at a level at least ten fold less than their parental strains. This suggested that the promoter region had been disrupted by these insertion elements so that the *vapA* transcription was affected. If this was the case, these insertion mutants could provide opportunities to examine the transcriptional activities of the P1 and P2 promoters separately. Therefore, DNA sequencing was performed and the insertion sites in these two strains were localized. The DNA sequencing templates were prepared by PCR synthesis using primers SC8 (in ISA2, Fig. 22) and CG3 (in *vapA*, Fig. 5) for CEG4, and CG5 (in ISA1) and CG3 for A450-2. The oligonucleotide CG3 was used as the primer for DNA sequencing which started from *vapA* and sequenced upstream. In the case of A450-2, the ISA1 sequence was found 153 bp in front of the *vapA* gene between the *vapA* transcriptional start site and the first stem loop structure in the *vapA* mRNA leader sequence, while the ISA2 sequence was detected 19 bp in front of *vapA* in CEG4, between the P2 promoter and the predicted SD sequence (Fig. 12).

IV. Characterization of a gene downstream from *vapA*

A-protein expression in *E. coli* affected by a downstream *Aeromonas* DNA sequence

As mentioned previously, when a series of subcloning experiments was performed by removing DNA fragments from the 3' end of pSC150 insert (Fig. 24) and A-protein production from the subclones was compared with pSC150, it was found that removal of a 0.5 kb DNA segment from the 3' end (clone pSC151) resulted in decreased production of A-protein in *E. coli* (Fig. 25). A-protein could only be seen in Western blots of whole cell lysates at heavy loadings, and comparison with dilutions of lysates from cells carrying pSC150 showed that the decrease was approximately 16 fold (Fig. 25). Further deletion to the *SphI* site (pSC152), which was less than 300 bp downstream from *vapA*, resulted in the same decreased level of expression as pSC151, while deletions into the *vapA* structural gene (pSC153) resulted in apparent total loss of production of A-protein, or immunoreactive truncated A-protein products.

***vapA* expression in *E. coli* can be complemented by the downstream sequence**

To initially confirm that the downstream DNA influenced *vapA* expression in *E. coli*, complementation assays were performed. A 1.4 kb *BglIII*-*HindIII* fragment from pSC150 was subcloned into a P15A replicon vector pSU2718 (pSC163) (135). This plasmid was then introduced into the DH5 α cells containing either pSC151 or pSC152. Western blot analysis showed that DH5 α cells carrying either of the two pairs of plasmids, *i.e.*, pSC151 and pSC163 or pSC152 and pSC163, produced A-protein in amounts

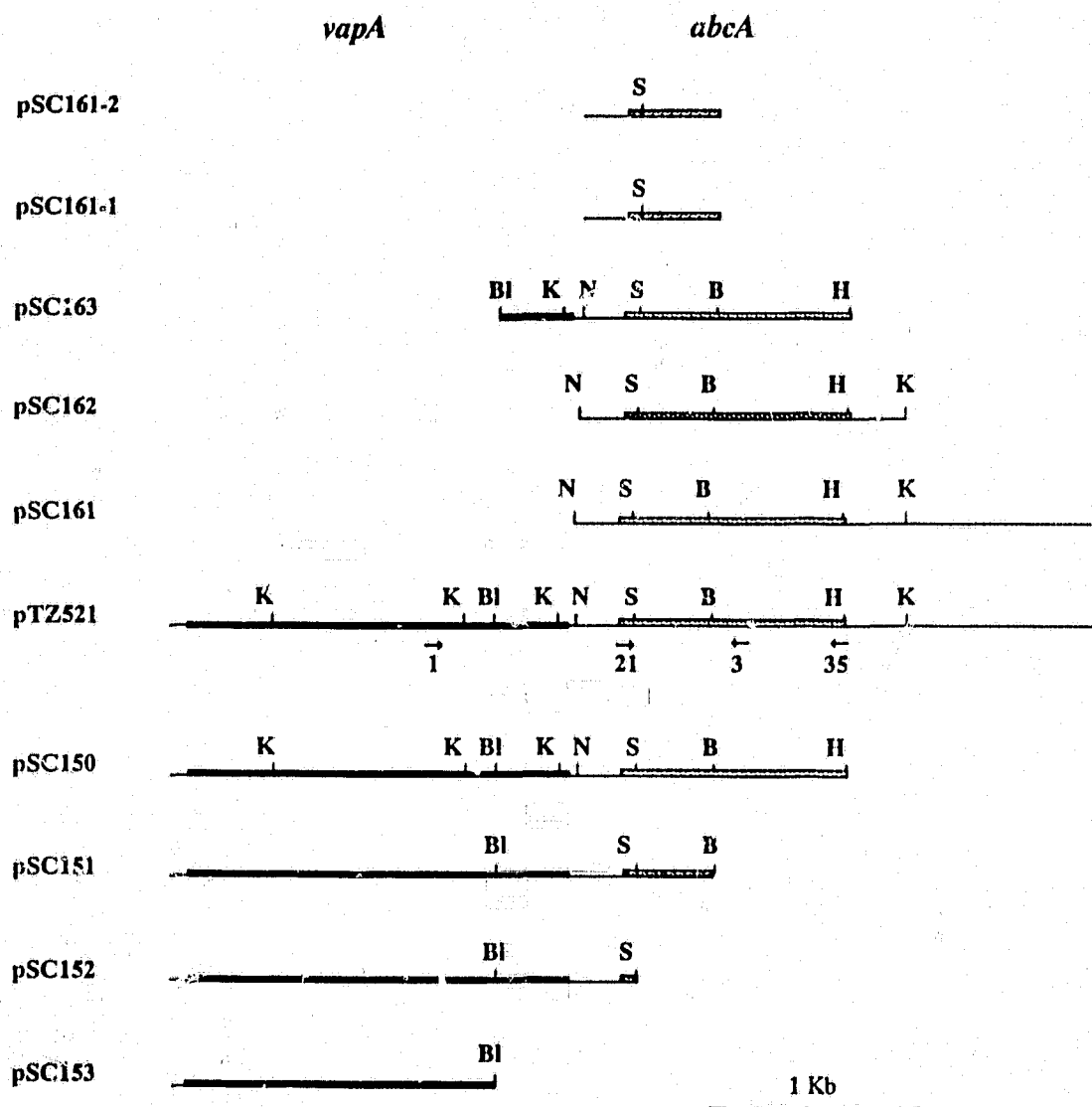


Fig. 24. Restriction maps of inserts in each subclone used for *abcA* subcloning and complementation. Names of the subclones are marked on the left. All subclones are vertically aligned to each other. Arrows indicate position of oligonucleotide primers used for PCR analysis of gene distribution. Numbers 1, 21, 3 and 35 beneath the arrows indicate primers AP-1, TJ21, RAA3 and TJ35 respectively. Thicker lines indicate *vapA* coding regions. Dotted boxes represent *abcA* gene. B: *Bam*HI; B1: *Bgl*II; H: *Hind*III; K: *Kpn*I; N: *Nar*I; S: *Sph*I.

indistinguishable from pSC150/DH5 α (Fig. 25), *i.e.*, production of A-protein was significantly increased by re-introduction of the 1.4 kb *Bgl*III-*Hind*III downstream DNA fragment.

The effect of the downstream DNA sequence on *vapA* expression was also confirmed with an *in vitro* expression system (Prokaryotic DNA-directed Translation Kit, Amersham, Arlington Heights, IL). Plasmids pSC150 and pSC152 were used as DNA templates and the DNA concentrations were calibrated by UV absorbance and agarose gel so that equal amounts of template DNA (5 μ g) were added into each reaction. Consistent with the *in vivo* results, pSC150 produced a strong band of the same molecular weight as A-protein, while there was no visible band in the reaction with pSC152 (Fig. 26).

DNA sequence analysis of the downstream region

Sequencing of the DNA downstream from *vapA* revealed an ORF (Fig. 27) on the same strand as *vapA* which started 205 bp after the *vapA* termination codon. A number of additional stop codons were present in all three reading frames in the intervening sequence between the two genes. This downstream ORF was the only one in the area which codes for a peptide of more than 300 amino acids. Other predicted ORF's coding for more than 150 amino acids were all from methionines included in this larger ORF. The complete ORF, *i.e.*, gene *abcA* (for ATP-binding-cassette protein), contained 924 bp and was terminated by a TGA stop codon. In pSC151 and pSC152, although the *vapA* gene was still intact, *abcA* was truncated at the 3' end by 541 bases and 846 bases respectively.

The intact *abcA* has a GC content of 47%, which is similar to the 48% G + C content of *vapA*, but lower than the reported 55% G + C content for the *A*.

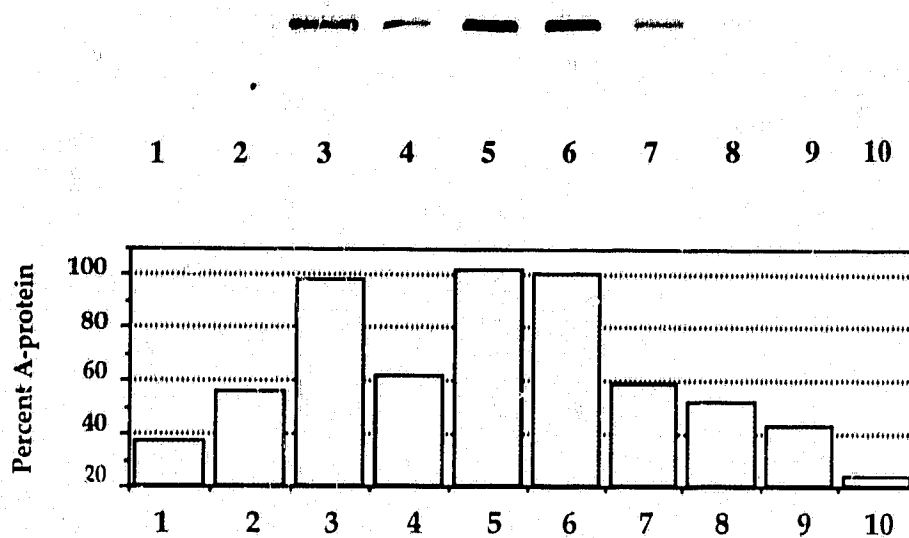


Fig. 25. Western blot detection of A protein (upper panel) using 1:1,000 diluted affinity purified anti-A-protein antiserum, and densitometric analysis of the blot (lower panel). All samples are whole cell lysates of *E. coli* carrying the following plasmids. Lane 1: pSC151/pSU2718; lane 2: pSC151; lane 3: pSC151/pSC163; lane 4: pSC152; lane 5: pSC152/pSC163; lane 6: pSC150; lanes 7 to 10: pSC150, lysates diluted 1/16, 1/32, 1/64 and 1/128 respectively.

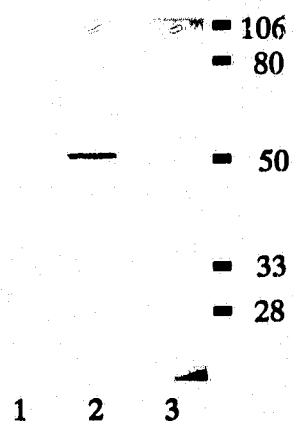


Fig. 26. A-protein production from *E. coli* strains with and without *abcA* gene detected by in vitro transcription-translation. Molecular weight markers are on the right. Lane 1 through lane 3 represent pSC152, pSC150 and no-DNA control respectively.

vapA stop
 CCCGCGGCACAGCACCGCTGGTAGATTCTACTCTGTAAGAGCGAATCTAGTCGATAAAGG -181
 ..ArgGlyThrAlaProLeuValAspPheThrLeu
 GCGCGGAGGCGCCCTTTTAATTAATGTAAACTAAGCAGTTTCATTGTTCCTCACTC -121
 ATCAATCTCTTCATTAGCGAGGTAGACAAGCTCTGCCGTTGCTCGTGACCAAGCCCTT -61
 -35 -10 TJ30
 ATTTTCAGTTGTCATTATTGTATATCTCTTACCTCGTTAGCTACCTTGCGATTATCT -1
abcA
 ATGTCGAGCGTGTACTTGCCGTTAGCGGTGTCAATAAATCAITTCCTATCTATCGTTCC 60
 MetSerGluProValLeuAlaValSerGlyValAsnLysSerPheProIleTyrArgSer 20
 CCTTGGCAAGCGCTATGGCATGCACTCAATCCCAAGGCTGATGTCAAGGTTTTTCAAGCA 120
 ProTrpGlnAlaLeuTrpHisAlaLeuAsnProLysAlaAspValLysValPheGlnAla 40
 TTACGTGATATTGAAGTACGGTATATCGCGGGAGACAATAGGCAITGTGGGCCACAAT 180
 LeuArgAspIleGluLeuThrValTyrArgGlyGluThrIleGlyIleValGlyHisAsn 60
 GlyXxxXxx
 GGTGCGGGCAAGTCTACCTTGCTACAGCTTATCACAGGAGTCATGCAACCTGATTGTGGC 240
 GlyAlaGlyLysSerThrLeuLeuGlnLeuIleThrGlyValMetGlnProAspCysGly 80
 GlyKxxGlyLysSerThr
 CAGATTACCGTACAGGGCGTGTGTGGGCTTGTAGAGCTTGGTTCTGGTTTTAACCT 300
 GlnIleThrArgThrGlyArgValValGlyLeuLeuGluLeuGlySerGlyPheAsnPro 100
 GAATTTACTGGACGTGAAAACATTTTTTTAATGGCGCTATCTTGGGAATGTCACAGCGA 360
 GluPheThrGlyArgGluAsnIlePhePheAsnGlyAlaIleLeuGlyMetSerGlnArg 120
 GAGATGGATGATCGTTTGAACAGATCCTTTCTTTCGCTGCCATAGGTGATTTTATTGAT 420
 GluMetAspAspArgLeuGluArgIleLeuSerPheAlaAlaIleGlyAspPheIleAsp 140
 CAGCCTGTTAAAAACACTCCTCTGGGATGATGGTACGCTTGCTTTTTCAGTCATCATC 480
 GlnProValLysAsnTyrSerSerGlyMetMetValArgLeuAlaPheSerValIleIle 160
 AATACTGATCCTGACGTATTGATCATTGATGAGGCATTGGCAGTGGGAGATGATGCATTT 540
 AsnThrAspProAspValLeuIleIleAspGluAlaLeuAlaValGlyAspAspAlaPhe 180
 CAACCTAAATGCTATGCCAGGCTAAAGCAGTTCAGTCAAGGCGTAACGATTCTGCTG 600
 GlnArgLysCysTyrAlaArgLeuLysGlnLeuGlnSerGlnGlyValThrIleLeuLeu 200
 GTATCCCATGCGAGCGGCGCGTATGAGTTATGTGACCGTGCAGTATTACTGGATCGA 660
 ValSerHisAlaAlaGlySerValIleGluLeuCysAspArgAlaValLeuLeuAspArg 220
 GGTGAGGTGCTGCTTCAAGGGGAGCCCAAAGCAGTGGTACACAACTATCACAAGCTGCTC 720
 GlyGluValLeuLeuGlnGlyGluProLysAlaValValHisAsnTyrHisLysLeuLeu 240
 CATATGGAAGGCGATGAGCGTGCCCGCTTTCGTTATCACTTGCGCCAGACTGGCCGTGGA 780
 HisMetGluGlyAspGluArgAlaArgPheArgTyrHisLeuArgGlnThrGlyArgGly 260
 GACAGTTATATTAGTGACGAATCTACCTCGGAGCCCAAAATTAAGTCTGCCCCGGTATT 840
 AspSerTyrIleSerAspGluSerThrSerGluProLysIleLysSerAlaProGlyIle 280
 TTATCAGTAGACCTTCAGCCTCAGTCTACAGTTTGGTATGAGAGTAAAGGTGCGCTTCTT 900
 LeuSerValAspLeuGlnProGlnSerThrValTrpTyrGluSerLysGlyAlaValLeu 300
 CG4 stop
 AGCGATGTACACATTGAAAGCTTTTGAAGTCGGCTTGGTATGATGATTAAACTCGTTC 960
 SerAspValHisIleGluSerPhe 308
 CGGCGTTGATCTTGGCGGACGAACCTCAACGCGTAACCACGAACATCGCTTGGCTTATGT 1020

Fig. 27. Nucleotide sequence of the *abcA* gene and flanking DNA from *A. salmonicida* strain A450, and translated amino acid sequence. The end of the *vapA* gene is indicated, as are the C-terminal residues of the A-protein. Numbers on the right indicate nucleotide positions and amino acid residues (underlined) in *abcA*. The predicted translational start of the *abcA* gene is shown in bold, as are the termination codons of both the *abcA* and *vapA* genes. A possible ribosomal binding site, and a -10 and -35 RNA polymerase binding sequence in front of the gene are underlined, residues composing the heptad Leu-Val repeat are double underlined, the predicted nucleotide binding sequence is underlined and the consensus sequence is shown underneath. An inverted repeat forming the stem of a possible transcriptional terminator is underlined with dots. Oligonucleotides TJ30 and CG4 are underlined.

salmonicida genome (22). The codon adaptation index (CAI) (189) for *abcA*, according to *E. coli*, was 0.242, much lower than the *vapA* CAI of 0.516. Codon usage was quite different from *vapA* (Table 12). This was most obvious for the codons for Phe, Pro, Ala, Tyr, and Gly. For example, while UUU is used for 11 of the 12 Phe's in *abcA* it only receives 36% usage in *vapA*. Also, in the case of Pro, CCA and CCG are not used in *abcA* but receive 70% usage in *vapA*. The only other codon not used in the case of *abcA* was the Arg codon AGA. Possible -35 and -10 promoter regions of *abcA* are indicated in Fig. 27. The predicted -10 region promoter hexamer sequence TATACT is 33 base pairs upstream from the first codon ATG, and a poorly conserved putative -35 sequence TTATTT ends 17 nucleotides further upstream. Attempts at mapping the transcriptional start site were unsuccessful as were Northern blots, suggesting low transcriptional rates. There is also a possible ribosomal binding site seven base pairs from the start of the gene. A predicted stem-loop structure was located 12 bases downstream from the stop codon with a free energy of -6.2 ΔG . It includes a 7 bp stem and a 16-base loop which could serve as a transcriptional terminator.

Two approaches were used to determine the presence of *abcA* coding sequence in the chromosomal DNA's from a range of *A. salmonicida* strains including the three subspecies isolated from diverse species of fish, diverse geographical regions, and from diseases with different pathogenesis. Southern blot analysis of eight representative strains with plasmid pSC162 (Fig. 24) which contains a 1.4 kb *NarI-KpnI* insert including the entire 924 bp *abcA* gene showed that a 1.6 kb *KpnI-KpnI* DNA fragment was conserved among the *A. salmonicida* strains A449, A450, A362, A500, A400, A477, A419, A480, and A461. No hybridization was seen with chromosomal DNA's of the four tetragonal S-layer producing strains of *A. hydrophila* (TF7 and A274) and

Table 12. Codon usage for the *A. salmonicida* A450 *abcA* gene and *vapA* gene

Amino acid	Codon	Codon usage	% codon usage		Amino acid	Codon	Codon usage	% codon usage	
			<i>abcA</i>	<i>vapA</i> ^a				<i>abcA</i>	<i>vapA</i>
Phe	UUU	11	92	36	Ala	GCA ^b	9	43	16
	UUC ^b	1	8	64		GCG ^b	2	10	6
Leu	UUA ^c	5	16	2	Tyr	UAU	7	88	40
	UUG ^c	8	25	17		UAC ^b	1	12	60
	CUU ^c	7	22	11	His	CAU	3	38	50
	CUC ^c	3	9	9		CAC	5	62	50
	CUA ^c	3	9	0	Gln	CAA	6	40	58
	CUG ^b	6	19	61		CAG ^b	9	60	42
Ile	AUU	12	57	42	Asn	AAU	5	56	36
	AUC ^b	7	33	58		AAC ^b	4	44	64
	AUA ^c	2	10	0	Lys	AAA ^b	6	50	30
Met	AUG	7	100	100		AAG	6	50	70
Val	GUU ^b	6	23	57	Asp	GAU	14	74	63
	GUC	4	15	7		GAC	5	26	37
	GUA ^b	10	39	24	Glu	GAA ^b	7	39	79
	GUG ^b	6	23	12		GAG	42	17	6
Ser	UCU	7	29	41	Cys	UGU	2	67	0
	UCC	4	17	30		UGC	1	33	0
	UCA ^c	5	21	50	Trp	UGG	3	100	100
	UCG	1	4	0					
	AGU	3	12	7	Arg	CGU ^b	11	58	72
	AGC	4	17	22		CGC ^b	4	21	14
Pro	CCU ^c	7	58	31		CGA	2	11	0
	CCC ^c	5	42	0		CGG	1	5	7
	CCA ^b	0	0	8		AGA ^c	0	0	0
	CCG ^b	0	0	62		AGG ^c	1	5	7
Thr	ACU ^b	3	25	48	Gly	GGU ^b	8	30	80
	ACC ^b	2	17	40		GGC ^b	10	37	16
	ACA ^c	5	41	12		GGA ^c	5	18	2
	ACG	2	17	0		GGG ^c	4	15	2
Ala	GCU ^b	4	19	66					
	GCC	6	28	13					

^a As listed in Table 3, **^b** codons corresponding to the most abundant tRNA species in *E. coli* (101), **^c** rare codons whose corresponding tRNA species in *E. coli* occur with an abundance of 0.3 or less on a scale of 0 to 1.0 (102) and whose percentage use is approximately 10% or less.

A. veronii biotype *sobria* (A700 and A702) tested. In addition, PCR analysis with primers allowing amplification of an internal sequence of the *abcA* gene (TJ21 / TJ35), or internal and 5' sequence (AP1 / RAA3, RAA2 / RAA3 and AP1 / TJ35) (Fig. 24) (Primer RAA2 is 416 bp before *vapA* and is shown in Fig. 12) resulted in the appropriately sized sequences from eleven 'typical' strains (A202, A203, A251, A362, A395, A440, A447, A449, A450, A451, A488, A500, A505 and A506) and five 'atypical' strains (A206, A400, A401, A419 and A430).

Predicted AbcA protein structure

The *abcA* gene of *A. salmonicida* A450 encodes a predicted protein of Mr 34,015 containing 308 amino acid residues (Fig. 27). The predicted protein contains 44.5% hydrophobic and nonpolar residues (Val, Met, Ile, Leu, Ala, Phe, Trp and Pro) and the Kyte and Doolittle (124) hydropathic index using an interval of 9 amino acids gives an average hydrophobicity score of -0.8 (the average hydropathy for soluble proteins is -0.4). Using the method of Klein *et al.* (115) the predicted protein was classified as an integral membrane protein with a putative membrane associated segment between Val₁₉₆ and Ile₂₀₉ (Peripheral:Integral odds of 0.23) (Fig. 28). Other algorithms showed no membrane spanning segments. In addition, the predicted AbcA protein contains 31.8% polar, 12.0% acidic, and 12.7% basic residues and a predicted pI of 5.7.

The predicted secondary structure of this protein includes 42.8% α -helix, 39.2% β -sheet, 9.4% β -turns and 8.4% coiled structure (73). The distribution of this secondary structure is shown in Fig. 28. The longest predicted α -helix containing region is between Ser₂₀₂ and Arg₂₄₉, with all but four residues predicted to fall within the helix. In this region, a heptad repeat of hydrophobic residues was present starting at residue 211,

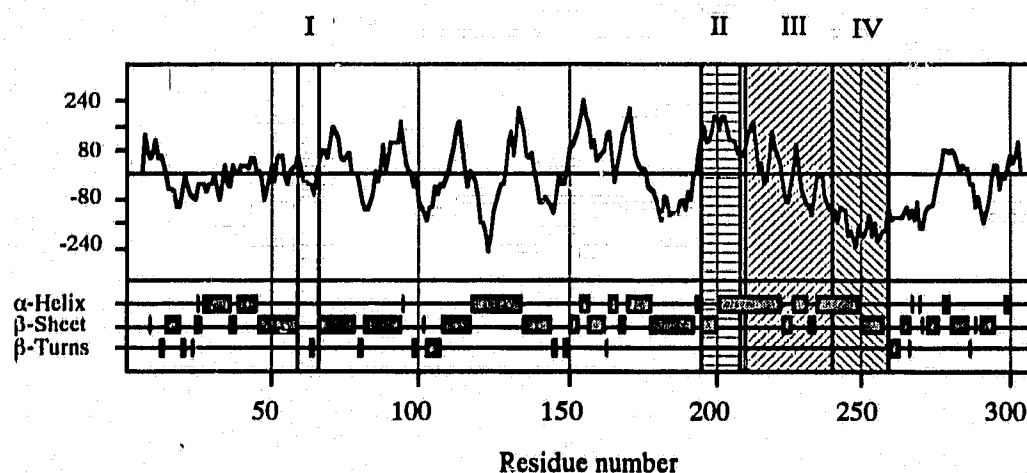


Fig. 28. Schematic map of deduced sequence of AbcA. Upper panel shows a hydrophobicity plot using Kyte-Doolittle's method (124). Hydrophobic regions are represented by positive values. Lower panel shows secondary structure prediction by MacVector using Garnier's method (73). I indicates P-loop, II indicates putative membrane associated sequence, III indicates leucine zipper region, and IV indicates the basic region.

with leucine in four of the five heptad repeats, and valine in the remaining heptad repeat (Fig. 27). These Leu and Val residues lined up on one side of the hypothetical helix (Fig. 29) which may facilitate dimerization with another protein with the same or similar structure (127). This potential leucine zipper sequence overlaps a highly positively charged sequence of 22 residues (Tyr237 - Arg259) which has a predicted isoelectric point of 11.02, and is within the longest hydrophilic region of the deduced protein (Fig. 28).

Sequence similarity searches showed that the protein contained regions of sequence with high similarities to a number of proteins belonging to a superfamily of ATP-driven membrane transporters (91). The NodI protein of *Bradyrhizobium japonicum* (Blast score = 160) (79), the CtrD protein of *Neisseria meningitidis* (Blast score = 144) (62), the polysialic acid transport protein KpsT of *E. coli* (Blast score = 141) (162), and the BexA protein of *Hemophilus influenzae* (Blast score = 130) (121) were examples among the proteins giving the highest homology scores (Fig. 30). Homologies with the *Pseudomonas* ATP-binding protein NosF (240) and the *Bacillus* glutamate permease transporter GlnQ (233) are also shown in Fig. 30. The sequence containing the highest similarity is the previously reported ATP or GTP binding sequence known as a P-loop (phosphate binding loop), GXXGXGKST (184, 227).

Identification and localization of AbcA protein

Plasmid pSC161 (Fig.24) and the T7 polymerase expression system were used to demonstrate the product of the *abcA* gene, and to determine the cellular location of the gene product. This plasmid contains a 2.5 kb *NarI-PstI*

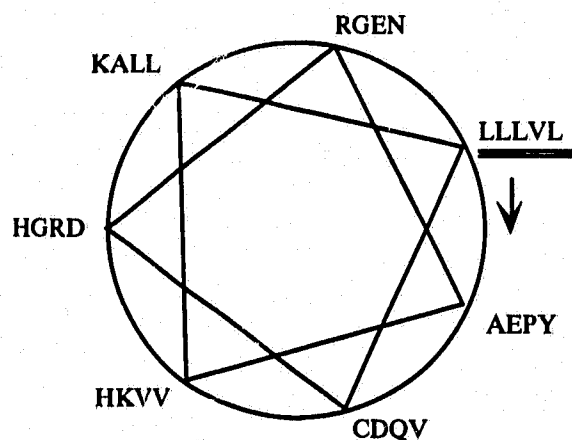


Fig. 29. α -helix structure of the putative leucine zipper region between L211 to L239 viewing from one end of the helix. The sequence starts from the first (inside) leucine residue (211) in the double underlined residues. Subsequent residues are along the straight lines in the direction indicated by the arrow. Residues are arranged from inside to outside in each group around the circle.

	60	
MSEPVLAVSGVNKSFPIYRSPWQALWHALNPKADVQVFQALRDIELTVYRGETIGIVGHN	GXX	AbcA
L++I+ + +GE IGI+G+N		BexA
L DI+ ++ +GE +GI+G+N		CtrD
+G I++G+N		KpsT
D+ +V RGE G++G N		NodI
L+D++L + GE +G+ GHN		NosF
F+ L+CI+LT+ +GE + I+G +		GlnQ
	120	
GXGKST		
GAGKSTLLQLITGVMPDCGQITRTGRVVGLLELGS GFNPEFTGRENIFFGAILGMSQR		AbcA
GAGKSTL++L++GV P G I R+ + L +++F TG +N+ F		BexA
GAGKSTL++LI+GV P G+I RT + L +++F TG +N+ F		CtrD
GAGKSTLL++I G+ +PD G I ++ + L++GF TGREN+ F		KpsT
GAGKST+ +++ G++ PD G IT VG++ +PEFT REN+ G GMS R		NodI
GAGK+T + LI G++ P GQ+ GR +G L F P+ +GRE + + + G +		NosF
G+GKSTL++		GlnQ
	180	
EMDDRLEIRILSFAAIGDFIDQPVKNYSSGMMVRLAFSVIINTDPDVLIIDEALAVGDDAF		AbcA
F+ +GD++ +PVK+YSSGM RLAF++ ++ + D +IDE +AVGD F		BexA
F+ +G+++ +PVK YSSGM RLAF++ + + D +IDE +AVGD F		CtrD
E+++R++ + F+ +G + D P+K YSSGM RLAF++ + D +IDE AVGD F		KpsT
++ + +L FA + D V S+GM RL+ + + DP +L++DE+ + D		NodI
++D+ LE++ D+ VK YS GM RL+ + + +P +L++DE+ D		NosF
S+G R+A + + P +++ DE+ + D		GlnQ
	240	
QRKCYARLKQLQSQGV TILLVSHAAGSVIELCDRAVLLDRGEVLLQGEPKAYVHNYHKL		AbcA
KC +I+LVSH+++++ CD AV+L+ G		BexA
KC +I+LVSH+ +++ + CD A++L++G		CtrD
+KC +++VSH+ ++ E CD A+ L++		KpsT
++ RL+ L ++G TILL +H LCDR +L+ G + +G P A++ +		NodI
+ Y + +L+ +G +I+L SH +V +RA +L+ G		NosF
+KQL +G+T+++V+H G E+ DR V +D+G +L ++ P		GlnQ
	300	
HMEGDERARFRYHLRQTGRGDSYISDESTSEPKIKSAPGILSVDLPQSTVWYESKGAVL		AbcA
	308	
SDVHIISF		AbcA

Fig. 30. Alignment of the AbcA protein (308 residue protein) of *A. salmonicida* with ABC-transporter proteins BexA (217 residue protein), CtrD (216 residue protein), KpsT (224 residue protein), NodI (306 residue protein), NosF (308 residue protein), and GlnQ (224 residue protein). Residues representing conservative substitutions are indicated by +. AbcA residue number in sequence is indicated, the consensus P-loop sequence is shown above the AbcA sequence on the top line, and residues of the putative leucine zipper are underlined.

insert with the *abcA* gene located about 170 bases downstream from the vector's T7 promoter. To induce the T7 promoter, pSC161 was introduced into *E. coli* strain KX100, and a second plasmid, pGP1-2, was also introduced. pGP1-2 contains the T7 polymerase gene which can be induced by raising the temperature to 42°C (179). After induction of the T7 polymerase gene and ³⁵S methionine labeling, the cells were fractionated and fractions examined by SDS-PAGE. The negative control employed vector plasmid pTZ18R in place of pSC161. A ³⁵S radiolabeled protein of apparent Mr 43,000 was detected in the envelope fraction in pSC161 assays (Fig. 31). This protein was not detected in the cytosol and periplasmic fractions, or in the negative controls. After treatment of the envelope fraction with sodium lauryl sarcosinate, and centrifugation to pellet the outer membrane fraction, the Mr 43,000 protein remained in the sarcosyl-soluble cytoplasmic membrane fraction (Fig. 31 inset). Two dimensional electrophoretic analysis showed that the protein displayed a pI of 6.3 (data not shown). Another *NarI-PstI* insert coded protein of apparent Mr 30,000 was also detected in the envelope fraction and was later identified as a protein encoded by a gene 324 bp after *abcA* and localized in the *E. coli* outer membrane fraction.

To confirm that the Mr 43,000 protein was the gene product of *abcA*, a prokaryotic DNA-directed translation kit was used with a 1.7 kb *NarI-KpnI* fragment cut from pSC162 (Fig. 24) as template. This DNA contained the entire *abcA* gene, but no other ORFs capable of coding for a peptide longer than 200 amino acid residues. With this 1.7 kb *NarI-KpnI* fragment as the template, the only protein observed in the test lane which was not present in the control lane had an apparent Mr of 43,000 (data not shown).

Analysis of *abcA* expression

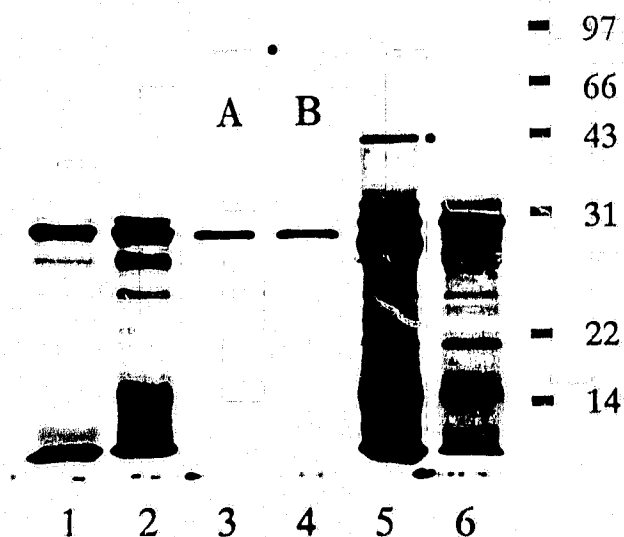


Fig. 31. In vivo plasmid expression and localization of AbcA protein (●) from pSC161 using the T7 polymerase expression system. Lane 1 and 2 are cytosol fractions, lane 3 and 4 are periplasmic fraction, and lanes 5 and 6 are the cell envelope fractions of pSC161/DH5 α and pTZ18R/DH5 α respectively. Molecular weight is indicated on the right. Inset shows the presence of the apparent Mr 43,000 protein in the sarcosyl soluble (A) fraction, and absence of the protein in the sarcosyl insoluble (B) fraction obtained from the cell envelope fraction.

Expression of the *abcA* gene in *E. coli* was further examined by construction of a *lacZ* fusion. The vector chosen for this experiment was pMC1871 which contains a promoter-less *lacZ* gene (188). There is also a *Sma*I site at the beginning of the *lacZ* gene facilitating construction of LacZ fusion proteins. *Aeromonas* DNA in pSC162 was digested by *Bam*HI which cut at the vector polycloning site (168 bases in front of the *abcA* gene) and at the site 387 bases into the gene (Fig. 24). The *Bam*HI ends were made blunt by filling in with Klenow fragment. pMC1871 was then digested with *Sma*I and ligated to the blunt ended *Bam*HI fragment. Blue colonies on X-gal plates were selected and whole cell lysates were screened for the presence of a AbcA- β -galactosidase fusion protein by Western blot analysis with a monoclonal anti- β -galactosidase antibody. The plasmid containing the fused gene, pSC161-1, produced a fusion protein of apparent Mr 130,000 which was confined to the cell cytosol (Fig. 32 A). The construction of the fusion was validated by restriction digestions, i.e., the insertion was confirmed to be 0.5 kb, and the *Sph*I site in the insertion was shown to be in the correct location. Based on DNA sequence, this fusion protein should contain 129 residues coded by the *abcA* gene, a number consistent with the observed molecular weight of the fusion protein.

To confirm that the *abcA* gene could also be expressed in *A. salmonicida*, and to obtain a measure of promoter activity, the *abcA-lacZ* fusion gene was introduced into *A. salmonicida* A450 using the broad host range plasmid pMMB67 (pSC161-2). In this clone, a 3 kb *Pst*I-*Pst*I fragment from pSC161-1, containing the whole fusion gene and 168 bp in front of the gene, was inserted into the *Pst*I site in pMMB67. Since only a very short *Aeromonas* DNA fragment was included in front of the fusion gene in pSC161-2, it would be possible that the gene was transcribed from an outside promoter.

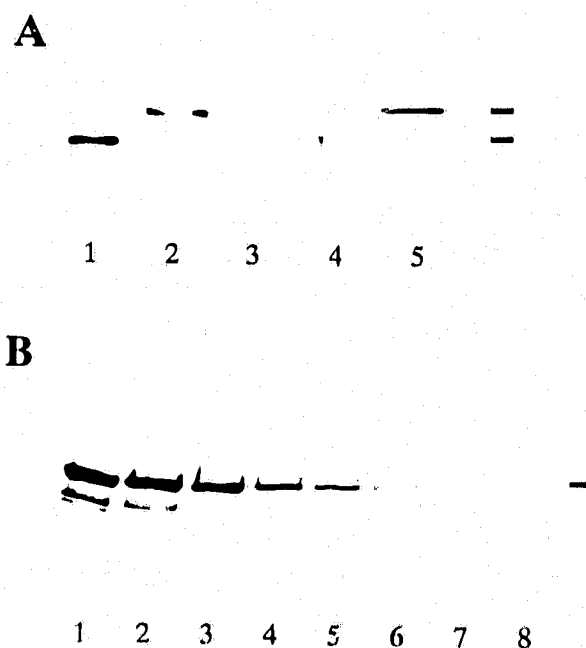


Fig. 32. A. Expression and localization of AbcA- β -galactosidase fusion protein as determined by Western blot analysis using monoclonal antibody against *E. coli* β -galactosidase. Lane 1: cytosol fraction of pTZ18R/DH5 α (β -galactosidase +); Lane 2: cytosol fraction of pSC161-1/DH5 α (AbcA- β -galactosidase fusion protein +); Lane 3: membrane fraction of pSC161-1/DH5 α (AbcA- β -galactosidase fusion protein -); Lane 4: whole cell lysate of pMC1871/DH5 α (AbcA- β -galactosidase fusion protein -). Lane 5 is Coomassie Blue stained SDS-PAGE gel showing purified AbcA- β -galactosidase fusion protein. The upper marker indicates the 116.5 Kd β -galactosidase, and the lower marker represents the position of the 106 Kd marker. **B.** Western blot analysis (anti- β -galactosidase monoclonal antibody) of fractions eluted from the ProtoSorb *lacZ* agarose beads column during affinity purification of AbcA- β -galactosidase fusion protein. Marker indicates 130,000 molecular weight.

Therefore the fusion gene was cloned into pMMB67 in both orientations, and the both activities measured. The β -galactosidase activity in both directions was identical at 1,000 Miller Units. This was almost the same level compared to the 900 Miller units of β -galactosidase activity of pSC161-1 in *E. coli* DH5 α . Control assays with pMC1871 in *E. coli* were negative and pMMB67 in A450 was nearly negative (less than 5 units).

Purification of the AbcA- β -galactosidase fusion protein and identification of ATP binding activity.

Because a sizable portion of the AbcA sequence was contained in the AbcA- β -galactosidase fusion protein, including a predicted ATP binding site, the ATP-binding ability of the purified fusion protein was tested. This required that the fusion protein be purified from a pSC161-1/DH5 α culture. Western blot analysis with monoclonal anti- β -galactosidase antibody showed that the fusion protein was precipitated in the 10% - 40% (NH₄)₂SO₄ fraction of the cell cytosol. Further purification was achieved by immunoaffinity column chromatography using ProtoSorb *lacZ* agarose beads bound with anti- β -galactosidase monoclonal antibody. Bound protein was eluted with pH 10.8 buffer. The majority of the fusion protein was found in first two bed volumes of eluent (Fig. 32 B). The first six bed volumes of eluent were pooled and concentrated, and the purity of the preparation confirmed by SDS-PAGE (Fig. 32 A). The ATP-binding ability of the purified fusion protein was then tested using an ATP affinity column. Western blot analysis showed that the 130 Kd fusion protein bound to the ATP affinity column, and required 0.125 mM ATP for elution (Fig. 33 A), confirming the nucleotide binding activity of the AbcA protein. In control assays, β -galactosidase did not bind to the ATP affinity column, eluting in the void volume (Fig. 33 B).

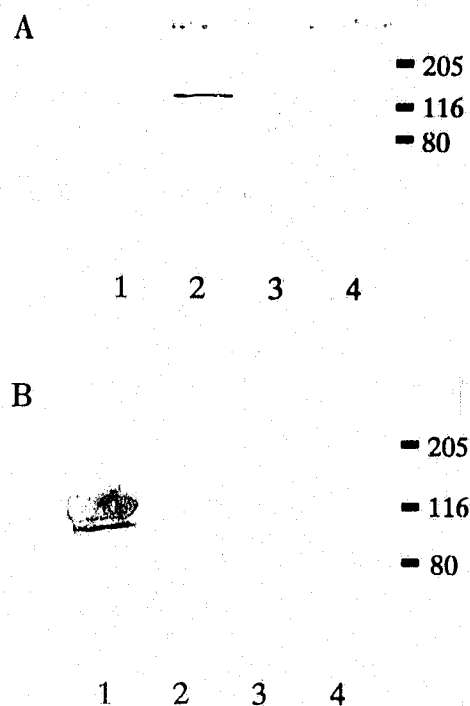


Fig. 33. Nucleotide binding ability of (A) the purified AbcA-β-galactosidase fusion protein and (B) *E. coli* β-galactosidase was tested using an ATP affinity column. The figures show Western blots of fractions eluted from the ATP affinity column. Lane 1, void volume; lane 2, 0.125 mM ATP; lane 3, 0.25 mM ATP; lane 4, 0.5 mM ATP. Molecular weight is indicated on the right.

***abcA* gene complements LPS O-chain expression in TM4**

The Tn5 insertion mutant TM4, which was constructed by R. Belland, was previously reported to be A⁻ and LPS O-polysaccharide chain negative and it was hypothesized that the reduction of A-protein and O-chains was somehow co-regulated (20). However, when TM4 was reexamined more closely by silver-stained SDS-PAGE, trace amount of O-polysaccharide chain was detected, and the level was estimated as about 1% of that of A449 based on the same cell culture density and the same amount of fast-migrating lipid A-core components (Fig. 34). The Tn5 insert in TM4 has been localized to the *vapA* structural gene, between bases 417 and 418 by DNA sequencing (S. Cavaignac, unpublished data). The *abcA* gene in TM4, when examined by PCR using primers TJ30 and CG4 (Fig. 27), was found to have a size increase of 1.2 kb compared to the wild type gene (C. Gustafson, unpublished data). This raised a possibility that the reduced O-chain production of TM4 could be caused by the mutation in the *abcA* gene or its promoter region, especially as a number of other bacterial ABC proteins have been shown to function in the transport of sugars. To examine this hypothesis, a complementation study was carried out by introducing the *abcA* gene into TM4.

As described before, pSC162 is the smallest clone containing the entire *abcA* gene (Fig. 24). The 1.8 kb *KpnI*-*KpnI* insert was subcloned into the broad host range plasmid pMMB67, yielding pSC162-1, which was then introduced into TM4 by plate conjugation selecting for kanamycin and ampicillin resistance. For a negative control, pMMB67 vector DNA was also conjugated into TM4 by the same method. Whole cell lysates from A449, TM4, pMMB67/TM4 and pSC162-1/TM4 were compared by both silver stained SDS-

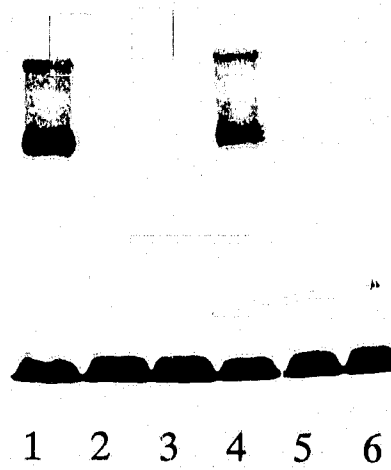


Fig. 34. Silver stained SDS-PAGE gel of LPS samples. Lane 1 through lane 6: A449, TM4, pMMB67/TM4, pSC162-1/TM4, pSCGV58-1/TM4, and pSCGV61-1/TM4.

PAGE gel analysis (Fig. 34) and Western blot using 1:1,000 diluted anti-*A. salmonicida* SJ-15 LPS antiserum. Both showed O-chain complementation by pSC162-1 but not pMMB67. Liquid cell cultures were also examined for self-aggregation. Compared with A449, which showed obvious self-aggregation in 4 h, TM4 and pMMB67/TM4 showed the similar amounts of aggregation at the bottom of the tubes, while pSC162-1/TM4 showed little or no aggregation in 4 h.

Site-directed mutagenesis in the P-loop of *AbcA*

To confirm that *AbcA* was responsible for the LPS O-chain complementation in TM4, and that the highly conserved ATP binding sequence was required for this activity, site-directed mutagenesis was carried out in plasmid pSC162. Two mutants were isolated. In mutant pSCGV58, G173 in *abcA* has been changed to T173, resulting in an amino acid sequence change from Gly58 (codon GGC) to Val58 (codon GTC); in mutant pSCGV61, G182 in *abcA* was replaced with T182, yielding a change of Gly61 (codon GGT) to Val61 (codon GTT) (Fig. 35). Both of the mutants were examined by DNA sequencing and shown to have correct base replacements.

These two mutants were then used for LPS O-chain complementation in TM4. The inserts of pSCGV58 and pSCGV61 were excised by *EcoRV* and *PstI* and subcloned into *SmaI* and *PstI* digested pMMB67 plasmid, yielding pSCGV58-1 and pSCGV61-1 respectively. These two plasmids were subsequently conjugated into TM4 and the conjugants were selected by ampicillin and kanamycin resistance at 20°C. To examine the ability of these mutant *abcA* genes to complement O-chain production in TM4, LPS preparations of these conjugants were subjected to SDS-PAGE and silver

	180 190
Wild type sequence	GGCCACAATGGTGCGGGCAAGTCTACC
	GlyHisAsnGlyAlaGlyLysSerThr
	58 61
pSCGV58 sequence	GT CCACAATGGTGCGGGCAAGTCTACC
	Val HisAsnGlyAlaGlyLysSerThr
pSCGV61 sequence	GGCCACAAT GT TGCGGGCAAGTCTACC
	GlyHisAsn Val AlaGlyLysSerThr

Fig. 35 Sequences indicating mutation sites in the *abcA* gene P-loop constructed by site-directed mutagenesis. Base pair numbers are indicated above the wild type DNA sequence and amino acid residue numbers are indicated below the wild type protein sequence. Mutated base pairs and amino acid residues are printed in bold.

staining. Fig. 34 shows that O-chain production was not detected in either pSCGV58-1 or pSCGV61-1.

DISCUSSION

As an extension to previous data on the *A. salmonicida* A-layer subunit A-protein obtained from electron microscopy, biochemical characterization and functional studies, this study has analyzed the A-protein gene *vapA*, the gene mapping immediately downstream from *vapA*, and insertion elements which affect the expression of *vapA*. In so doing, the study has provided important information on the structure of *vapA*, A-protein and A-layer, as well as an explanation of why the *vapA* gene was unstable when expressed at high level in a foreign host such as *E. coli*. This study has also examined transcription of the *vapA* gene, described two *vapA* promoters, and suggested possible reasons why *vapA* is efficiently expressed in *A. salmonicida* and poorly expressed in *E. coli* clones. The previously unexplained phenotype of the 30°C mutants of *A. salmonicida* was also explored further and transposition involving endogeneous insertion elements was demonstrated as one of the mechanisms involved in this mutational event. In addition, the *abcA* gene, which is immediately downstream of *vapA*, was shown to affect *vapA* expression in *E. coli* and LPS O-chain formation in *A. salmonicida*, the latter observation clarifying an unexplained LPS O-chain negative phenotype of a Tn5 mutant in the *vapA* gene of *A. salmonicida*-TM4. The gene product of *abcA* appeared to be an ATP binding transport protein which may also have regulatory functions. Taken together, these data have revealed a hitherto unrecognized complexity of S-layer gene expression and in particular have provided important information on the S-layer system of *A. salmonicida*.

I. Structure of *vapA* and analysis of its promoters

Previous studies had shown that the A-protein gene was particularly difficult to clone, and problems with gene stability were experienced. This is

often the case with S-layer protein genes especially when the entire promoter region is involved such as in the case of *B. brevis* 47 *cwp* operon (234). The instability encountered when the *vapA* gene was cloned in λ gt11 appeared to be related to the presence of two direct repeats in the sequence, and the deletion of almost half of the gene can be explained as a cross-over event involving these repeats. Finally, one cloning attempt which selected for low expression provided a full copy of the gene which was stable and produced a wild-type-sized A-protein because the major promoter was not present in the clone.

The *vapA* gene appears to be conserved in *A. salmonicida* as shown by its presence in a diverse selection of strains of *A. salmonicida* representing its three subspecies, as well as strains isolated from different geographic sources, from different species of both marine and freshwater fish, and from diseases with markedly different pathogenesis. The *vapA* gene was not found in other S-layer-bearing *Aeromonas* species such as *A. hydrophila*, although the morphology and subunit molecular weight of these S-layers and subunit proteins were quite similar (44, 151). This is in agreement with earlier N-terminal sequence comparisons of A-protein and the S-layer proteins of mesophilic aeromonads (112). No significant homologies were demonstrable in the data bank search for the *vapA* gene and the deduced VapA amino acid sequence. This has commonly been seen in other S-layer proteins in which little homology in primary structure has been reported.

The composition of the mature A-protein was similar to other S-layer proteins in terms of polar, acidic, and basic amino acid content. The hydrophobic content of A-protein (45.1%) is also consistent with that of other bacterial surface layer proteins (40 -50%). In A-layer, it seems probable that much of the hydrophobicity is located in surface exposed regions of the

subunit and may play a role in the initial interaction of *A. salmonicida* with cells such as macrophages, and in the other binding activities of the A-layer. Analysis of the deduced composition of A-protein using the algorithm of Barrantes (16) gave a ratio 3 value of 1.06, consistent with a peripheral protein, and analysis of the deduced amino acid sequence by the method of Klein et al. (115) also classified A-protein as a peripheral rather than a integral membrane protein.

Conformational information about A-protein was obtained from trypsin digestions. In addition to the cleavage at the two central sites to produce a C-terminal Mr 21,335 peptide beginning at residue 275 and its subsequent cleavage to a Mr 16,608 peptide beginning at residue 324, trypsin also sequentially cleaves the purified protein at a series of sites beginning from the C-terminal end of A-protein to produce a stable N-terminal peptide of approximate Mr 39,400, probably by cleavage after Arg₃₇₀. The resulting overlapping N- and C-terminal compact peptides both appear to adopt compact tertiary structures refractile to trypsin. The C-terminal peptide displayed intermediate resistance to trypsin even though it contains 14 potential trypsin cleavage sites, while the N-terminal Mr 39,400 compact peptide unit with 29 potential trypsin sites appeared to be totally refractile to trypsin activity. Indeed, the 23 potential trypsin sites carried in the N-terminal 274 residue sequence of A-protein appeared to be inaccessible until the protein was denatured. The total refractility of the protein to trypsin digestion when assembled as A-layer on the cell surface suggests that none of the Arg or Lys residues in the protein are located or accessible on the surface of the layer.

The presence of the two major morphological domains appears to be a motif shared by all S-layers (186). Three-dimensional image analysis of A-

protein showed that the subunits contain a heavy mass domain with a linker arm to a domain of lesser mass (44, 198). The present findings with trypsin digestions support these morphological findings by providing the first biochemical evidence for a two-domain structure for A-protein, i.e., a C-terminal domain of lesser mass represented by the 16.7 Kd trypsin-resistant fragment and the 13.4 Kd CNBr cleavage fragment and an N-terminal major domain demonstrated by the 39.4 Kd trypsin-resistant product and the CNBr cleavage product of 31 Kd. In the case of *A. hydrophila*, two morphological units with different mass in S-layer subunit were observed by computer-generated enhancements of the negatively stained micrographs (151), and a Mr 38,000 peptide at the N-terminal of the protein was shown to be refractile to proteases and was suggested to fold into a compact conformation (119). These comparisons suggest that despite their different primary structures, there are considerable similarities in *Aeromonas* S-layer protein tertiary structures.

Secondary structure predictions (37, 73) indicate that the sequence which forms the larger mass trypsin resistant compact unit can also be subdivided into two structurally defined regions: a β -sheet (28%)- β -turn (18%) N-terminal 125-residue region followed by a region of 227 residues with alternating α -helix (37%) and β -sheet (26%), largely devoid of turns (3%). The remaining C-terminal portion of the molecule can also be separated into two structurally defined regions: the first 72 residues containing predominantly β -sheet (43%) (9% α -helix, 3% β -turns), followed by the C-terminal 57 residues which are devoid of β -sheet but rich in α -helix (49%).

Another bacterial supramolecular structure which is resistant to trypsin cleavage is the flagella filament. Like A-protein, *Salmonella* flagellin has a major internal sequence of approximate Mr 40,000 which is relatively

stable to trypsin digestion despite the presence of numerous potential cleavage sites for the enzyme (225). This 40 Kd sequence appears to comprise two compact structural units, one of which is an internal 27 Kd peptide which under non-denaturing conditions is totally refractile to trypsin, while the other unit comprising two terminal regions of the flagellin is disordered and mobile in solution and are susceptible to trypsin (226). These terminal regions of flagellin are predicted to be α -helical and are thought to interact with the corresponding regions of adjacent monomers upon polymerization into the flagella filament, and in so doing lose their susceptibility to trypsin (5). Since the C-terminal region of A-protein is analogous with the terminal regions of the flagellin which are moderately resistant to trypsin and contains more α -helices, it is tempting to speculate that this region may be involved in interactions with adjacent monomers upon polymerization into the tetragonal layer. A similar observation has been reported in a recent mutagenesis study in *A. hydrophila*, in which, a transposon mutant strain produced a truncated S-layer subunit of Mr 38,650 containing the N-terminal portion of the protein (202). Although exported, this truncated subunit was incapable of forming a tetragonal array and did not anchor to the cell surface.

While the A-protein was not normally transported in *E. coli*, this species was capable of A-protein synthesis at 37°C, a temperature which is lethal for *A. salmonicida*. Indeed in the case of the natural host of the gene, growth at temperatures above 25°C results in the selection of mutants which are permanently impaired in their ability to synthesize A-protein. Cell fractionation studies provided one likely explanation of the problem *E. coli* has when called upon to produce A-protein. Although a proportion of the A-protein produced in *E. coli* clearly reaches the periplasm in this foreign host, transfer across the cytoplasmic membrane appears to be inefficient with some

A-protein being retained in the cytoplasm, as well as in the inner membrane. Such an accumulation of A-protein may be toxic for *E. coli*. Certainly the small amount of A-protein that was produced did delay growth. The inability of *E. coli* to export A-protein across the outer membrane is not surprising since *E. coli* is not regarded as a protein exporter. In contrast, *Aeromonas* is regarded as a protein exporting species (78, 97, 203). In addition, the trypsin digestion pattern of the periplasmic A-protein from *E. coli* was shown to be different from that from *A. salmonicida*, suggesting that either the conformation of the periplasmic A-protein in the two hosts were different or different chaperones were bound to the periplasmic A-proteins, which may affect its interaction with outer membranes or other proteins. Indeed in its native background, A-protein is likely to have a unique export pathway, since transposon insertion mutants which are unable to translocate A-protein across the outer membrane and accumulate A-protein in the periplasm (20) still export other proteins with proteolytic and hemolytic activity, and the Tn5 insertion has been localized in a gene about 6 kb downstream from the *vapA* gene (155). This suggests that even in *A. salmonicida* at least one additional gene product is required for translocation of A-protein across the outer membrane. Furthermore, it has been demonstrated that an *exeE* mutant *A. sobria* strain which was incompetent for extracellular export of hemolysin, still normally assembled its surface array (96).

Despite the wild type size of the A-protein produced from the cloned *vapA* gene, the quantity of production was significantly lower than that in the wild type *A. salmonicida*. The codon adaptation index of the gene, which is a measure of codon usage bias (189), was 0.516 suggesting that the gene could be relatively well expressed in *E. coli*. From the transcriptional studies of the *vapA* gene, the major promoter P1 was found to be missing in this clone.

Therefore, the poor expression is likely to result from the loss of this wild type distal promoter and possibly other additional control sequences further upstream.

The predicted P2 promoter was sited within 62 bp of the start of *vapA* in pSC150, which was subcloned from pBP512 in the tetracycline and kanamycin resistant vector pLA2917, and carried 680 bp of pLA2917 sequence in front of the *A. salmonicida* DNA insert. A search of this 680 bp sequence for *tetR*, its promoter (228), or other potential promoter sequences was negative. In addition, this piece of sequence was found to be homologous to the kanamycin resistance gene from transposon Tn5 (18). Together these results indicate that A-protein production from pSC150 does not result from transcriptional read-through from the *tetR* promoter as previously suspected (167), and suggest that the predicted P2 sequence can serve as a promoter, albeit a poor promoter. Indeed the amount of A-protein expressed in *E. coli* from the P2 promoter was sufficiently low that immunological enhancement was required for its detection, and not surprisingly we have been unable to detect *vapA* mRNA from pSC150.

Although a few other genes have been sequenced in *Aeromonas* species, i.e., *A. hydrophila* (78, 83, 93, 97, 109, 136, 203), *A. proteolytica* (15, 74, 84), *A. sobria* (99), and *A. salmonicida* (221), promoters and transcriptional start sites have only been determined in *A. sobria* aerolysin gene *aerA* and its regulatory gene *aerC* (99). The P1 promoter is therefore the first *A. salmonicida* promoter to be structurally and functionally characterized. The predominant transcriptional activity of P1 in the wild type *Aeromonas* is also supported by the fact that the ISA1 insertion site in A450-2 has been localized between P1 and P2. This might block the P1 transcription and certainly appears to lead to a ten fold reduction in A-protein production over A450.

The P1 promoter appears to be a σ^{70} homologue, having an identical -35 consensus sequence, a related -10 motif, and a 17 bp spacer between the two RNA polymerase binding sites (176). The promoter provides for a transcription start site 181 bp in front of the S-layer protein gene, and is contained within a conserved 441 bp region immediately 5' to *vapA*. This expression control region is complex in structure, carrying a potential cAMP-CRP binding site which could play a role in the regulation of the P1 promoter (38), a 12-base pair palindromic sequence overlapping the presumptive Shine-Dalgarno ribosomal binding site which could serve as a site for DNA binding proteins (229), as well as the P2 promoter. In addition to these two promoter sequences, a potential σ^{54} promoter sequence spans the -10 box of the σ^{70} P1 promoter. This TGGC (N)₈ TGC consensus is located at -3 relative to the transcriptional start site determined in this study, rather than at the typical -12 spacing (90) and would appear not to be functional under the growth conditions tested. Several other S-layer systems have multiple promoters, however in these cases primer extension experiments have shown that these promoters are active during growth in vitro. For example several transcriptional start sites have been shown in a 360 bp sequence in front of the structural genes for the S-layers of *Bacillus brevis* 47, the P2 and P3 promoters being most active (2), while the *Lactobacillus brevis* S-layer protein gene possesses two promoters which appear to be equally active during exponential growth phase (222).

In keeping with other S-layer subunit proteins (59, 128, 218, 222), the *vapA* transcript expressed from the P1 promoter was found to be monocistronic. This is also supported by the fact that a transcriptional terminator-like sequence is located 20 bp downstream from the end of *vapA* and the downstream *abcA* gene is expressed off its own promoter. Indeed

only the *Bacillus* S-layers have been shown to be polycistronic (2). However these organisms produce a double layered array. Each layer contains a different S-protein species, i.e., the outer and middle wall proteins (OWP and MWP's), and the *Bacillus owp* and *mwp* genes are in tandem and are transcribed by the single cell wall protein (*cwp*) mRNA. The monocistronic character of S-layer protein gene transcription resembles that of another supramolecular assembled surface protein, the flagella filament. Even in the case of an organism such as *Campylobacter* which produces a complex flagella filament encoded by tandem *flaA* and *flaB* genes, both genes are transcribed separately (132). Unlike the *fla* transcripts however, the monocistronic *vapA* transcript contains a relatively long untranslated leader. Indeed the presence of a long mRNA leader appears to be a characteristic of certain S-layer subunit protein mRNAs. For example the constitutive P2 promoter of the *B. brevis* 47 *cwp* operon provides for a 247-bp transcriptional leader (215), and 111 - 127 bp transcriptional leaders have been reported for other S-layer genes (56, 128, 218). Long mRNA leaders are also seen in attenuated operons such as the amino acid biosynthetic operons (117, 126). In these operons peptides of 14 to 30 residues are coded from the mRNA leader. The translation of these short peptides appears to function in the conformation of the mRNA leader sequences and the attenuation of transcription. Interestingly the 181 bp leader in the *vapA* transcript also contains a coding region for a possible 29 residue peptide. In case of the *cwp* operon of *B. brevis* 47, the gene contains a short coding region preceding another translational start site, and Yamagata et al. (234) have speculated that any product arising from this short coding region may play a role in efficient translation of the downstream gene.

Structural predictions suggest that the 181 bp leader segment of the *vapA* transcript will contain two stem-loop structures with ΔG 's = - 9.5 kCal

and - 15.2 kCal respectively. Similarly, in the case of *B. brevis* 47, a palindromic sequence CGAAAAAACTTTAAATTTTTTTCG has been found in the *cwp* operon between the first two transcriptional start sites (234). Indeed, this palindromic sequence could also be a hair pin structure if the imperfectly paired middle sequence CTTTAAA formed a loop. Stem loop structures in the 5' RNA can function as attenuators as is the case in the amino acid biosynthetic operons in the presence of short peptides coded by mRNA leader sequences, and can increase the stability of transcripts as is the case in the *ompA* transcript of *E. coli* (19, 52). In this regard the *A. salmonicida vapA* transcript can be considered to exhibit pronounced stability with a half-life of 22 min in cells growing at 15°C. Indeed, since the average half-life typically found for most prokaryotic mRNAs is approximately 3 min (152, 178), the 11 min half-life of the *vapA* mRNA at 20°C can also be considered to represent high stability. This is the first determination of S-layer protein transcript stability, and the pronounced stability we observed is probably a major contributing reason for the high-level production of the S-layer protein subunit by the *Aeromonas* cell. A similar situation is seen with the Pap fimbriae of *E. coli* where the major fimbrial subunit protein PapA is expressed in high copy number, and where the mRNA half life is approximately 27 min (13). In the case of another highly expressed protein, the OmpA of *E. coli*, the transcript has a half life of approximately 17 min (153), again consistent with that of *vapA*. Production of the highly stable monocistronic *vapA* transcript peaks in the early log-phase of growth, and again there is a similarity with the expression of certain flagellin genes. For example production of *B. subtilis* (*hag*) transcripts peak during exponential growth and decreases significantly when the culture enters stationary phase (148). *Campylobacter flaB* gene expression also peaks at mid-log phase (8). However transcription of the *A.*

salmonicida S-layer protein promoter clearly differs from *B. brevis* 47 where transcription from P2, one of the major promoters of the cell wall protein gene operon, is markedly enhanced at the early stationary phase of growth (4).

It is tempting to speculate that the long and complex mRNA leader may also have a role in the regulation of *vapA* expression. σ^{70} promoters can also be subject to both positive and negative regulation (38), and certainly *vapA* transcription does appear to be subject to some regulation. For example, in this study we found that both environmental temperature and anaerobiosis appeared to regulate *vapA* transcription. In the case of growth temperature the transcript was more stable at 15°C than 20°C. This contrasts with the case of Pap pili in *E. coli* where reduction of the growth temperature from 37°C to 25°C turns down transcription of the pilus operon (80). However the finding is entirely consistent with the fact that in nature, typical strains of *A. salmonicida* will normally be found in salmonid fish living at temperatures <20°C.

The divalent cations we tested did not appear to affect *vapA* transcription. Again this contrasts to the situation in *B. brevis* where transcription from the P2 promoter was inhibited by magnesium and calcium ions (4). However *vapA* transcription did appear to be regulated by DNA supercoiling, since sub inhibitory concentrations of the DNA gyrase inhibitors novobiocin and nalidixic acid which reduce the amount of DNA supercoiling both inhibited *vapA* transcription. Because these inhibitors may have pleiotropic effects (159), these results need to be viewed with caution, however the expression of a number of virulence genes in *Salmonella typhimurium*, *Shigella flexneri*, and *Vibrio cholerae* have also been shown to be affected by gyrase inhibitors (46, 67, 159). Indeed in the case of *S. typhimurium*, the genes involved are required for invasion (67), and in this regard it should

be noted that the S-layer of *A. salmonicida* facilitates invasion of macrophages and other cell types (69, 71).

II. *Aeromonas* insertion elements

The fact that 30°C incubation induces the loss of A-layer production in *A. salmonicida* has been observed previously (105). From the Southern hybridization data on A450 and A450-3, it became obvious that a DNA rearrangement had occurred and this rearrangement could be described as a 1.2 kb expansion of the DNA localized between the *Bam*HI and *Pst*I sites, which included the promoter area and the 5' half of the structural gene. If the DNA rearrangement had occurred in the structural gene, the gene expression could be totally abolished; on the other hand, if the sequence in the promoter area was rearranged, various levels of gene expression could be expected. Both of these scenarios seemed to be evident in a set of *A. salmonicida* 30°C mutants isolated, since some of them produced low levels of A-protein and others did not produce any detectable A-protein. In addition, the polymorphism in their Southern hybridization pattern also indicated the heterogeneous nature of the rearrangements. However, the similar size increase detected by PCR in these mutants hinted that there was a certain relationship among them, and this eventually led to the identification of two different and unrelated insertion elements ISA1 and ISA2. The identification of ISA1 and ISA2 as IS elements was based on the presence of a number of characteristics similar to other known bacterial insertion elements such as inverted repeats flanking a putative transposase gene, duplication of target sequences at the sites of insertion such that it appeared on both sides of the ISAs, no homology between the inverted repeats and the recipient DNA, the length of their DNA sequences and their predicted protein products, and the

homology between the predicted ISA2 protein and other bacterial transposases.

Also, apparently similar to other bacterial insertion elements, as has been observed in *Alcaligenes eutrophus* (123), the transposition of the ISA elements seemed to be induced by elevated temperature. However, growth of other strains such as A451 at high temperature also results in loss of A-layer and reduced A-protein production even though this strain does not carry these ISA elements. This suggests that *A. salmonicida* might possess other molecular mechanisms for the loss of the A-layer. This could include other insertion elements. On the other hand, it is also likely that the loss of A-layer facilitates survival of the bacteria at high temperatures, and the particular mechanism by which A-layer is lost is irrelevant. In this latter case, the increased rate of ISA isolation in the *vapA* area should be considered as a result of selection of A-layer negative phenotype rather than induced transposition.

Survival of *A. salmonicida* at 30°C seemed to require loss of A-layer, but not necessarily loss of A-protein production as shown in mutants 450-2 and CEG4 which expressed low levels of A-protein. These mutants had insertions located in the *vapA* promoter area which apparently disrupted optimal promoter activity resulting in the decreased levels of A-protein production. The ISA1 in A450-2 is likely to have blocked the P1 promoter as mentioned previously, while the low level *vapA* expression in CEG4 is probably a result of transcriptional read-through from the putative transposase gene in ISA2. Although it has been reported in other cases that bacterial insertion elements may carry portable promoters or form hybrid promoters resulting in low level transcription of their downstream genes (68), no such evidence was found in

the case of CEG4. A complete loss of A-protein occurred only when either ISA1 or ISA2 was inserted into the *vapA* structural gene.

Insertion sites of ISs are usually homology independent. However, some transposable elements exhibit a degree of preference for particular "hotspots" in target DNA. For example, *E. coli* K12 IS30, which duplicates 2 bp of its transposition target, has been found between the same two base pairs in three independent insertion mutants, suggesting a strong targeting specificity (40). Similarly, ISA2 appeared to exhibit more preference for its targeting sites, especially in the cases of 30°C mutants CEG27, 28, and 29 where ISA2 insertions have been localized to a 160 bp area by PCR. The IS30-like insertion element also showed similarity in their short targeting sequences. Whereas duplicated target DNA sequences of insertion elements range from 2 to 13 bp (68), IS30-like insertion elements showed mostly short target sequences, e.g., 2 bp for IS30 (40), 3 bp for IS4351 and Tn4551 direct repeat sequence element (173, 196), 5-7 bp for ISAE1 (123) and 3-bp for ISA2.

The data base search for the ISA2 and its predicted protein product showed similarity to several other bacterial insertion elements found in *E. coli*, *Alcaligenes eutrophus*, and *Bacteroides fragilis* (40, 42, 196). The distribution of the same insertion elements among different bacterial species has been observed before such as IS200, which is a *S. typhimurium* resident element and has been found in 20% of *E. coli* strains (23). The horizontal transmission of an insertion element has been suggested to be related to plasmids or bacterial phages carrying the insertion element (6). The similarity between ISA2 and other insertion elements may also suggest evolutionary relationships as has been reported for the IS2, 3 family (68) or the IS150 family (187) comprising IS2, IS3, IS150, IS600 and IS51 from *E. coli*, *Shigella dysenteriae* and *Pseudomonas savastanoi*. It has been noted when IS30 was sequenced that the protein

contained 23% basic amino acid residues (40). This feature might be important for either structure or function of IS30-like transposases as basic residues seem to be well conserved among them, i.e., more than a quarter of the R residues (10/36) in the ISA2 protein are completely conserved and some others are partially conserved. The preliminary phylogenetic tree shown in Fig. 36 may be indicative of a new IS30 family.

III. The *abcA* gene

In addition to the P2 promoter, another determinant which appears to control the *vapA* expression from pSC150 in *E. coli* is carried in the sequence downstream of *vapA*. Cleavage of this downstream sequence significantly lowered level of *vapA* expression in *E. coli* and reintroduction of this sequence into the *E. coli* cell restored the original expression level. This effect on *vapA* expression was also confirmed by an in vitro transcription-translation experiment where plasmids containing *vapA* with and without the downstream sequence were added as DNA templates. These results indicated that the downstream DNA sequence was required for better *vapA* expression in *E. coli*. This downstream DNA segment contains a gene which begins 205 bp after *vapA*, and codes for a 308 residue protein AbcA. The *abcA* gene appears to have its own functional promoter.

The AbcA protein has been shown to belong to the ATP-binding-cassette (ABC)-transport family of proteins, or so-called 'traffic ATPases' (10, 175). Proteins possessing this conserved ATP-binding sequence are associated with a variety of cellular functions including cell division and DNA repair (91), as well as a wide variety of transmembrane transport processes, including the transport of ions, heavy metals, sugars, amino acids, oligopeptides, and proteins (54, 100, 122). Bacterial ABC-proteins include the

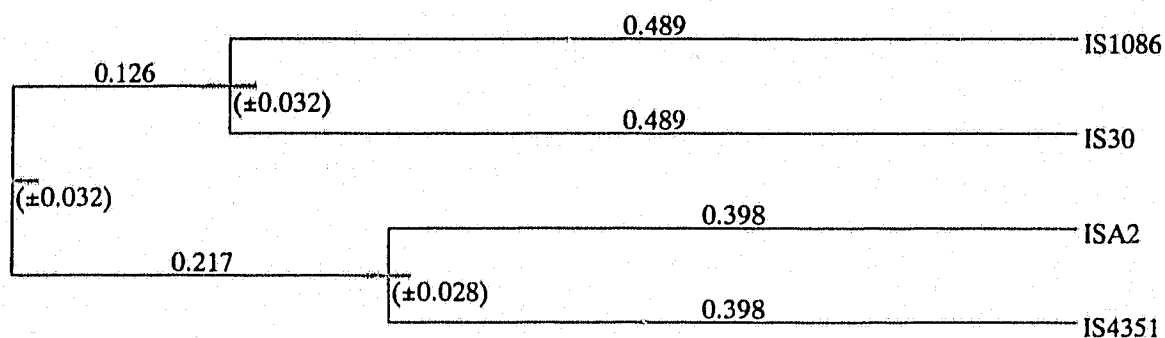


Fig. 36. Phylogenetic tree of putative transposases from ISA2 and other related bacterial insertion elements by GeneWorks (IntelliGenetics). The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. The figures in brackets represent the standard errors of the branch points.

well-characterized periplasmic permeases, capsule polysaccharide transporters (62, 121, 162), as well as protein secretion systems including the transporters for a *Bradyrhizobium* and *Rhizobium* nodulation protein (NodI) (53, 79), *E. coli* hemolysin A (HlyB) (57), *Pasteurella haemolytica* leukotoxin A (LktB) (200), *Bordetella pertussis* cytolysin (CyaB) (77), and *Pseudomonas aeruginosa* alkaline protease (PrtPA) (85). Eukaryotic members of the family include putative peptide transporters encoded at the locus for the class II major histocompatibility complex (208), the cystic fibrosis gene product cystic fibrosis transmembrane conductance regulator (CFTR) (12), P-glycoprotein responsible for multidrug resistance in tumor cells (MDR) (1, 81), and the yeast Ste6 transporter which mediates export of a peptide hormone that lacks a classical hydrophobic signal sequence (122).

This superfamily of transporter proteins shares about 30% sequence identity over an ATP-binding cassette of about 200 amino acids (100). In the *A. salmonicida* protein, the conserved ABC-transporter homologous sequence stretches between Phe38 and Asn236 and occupies approximately 65% of the length of the total deduced protein. Within this segment the most readily recognized conserved motif is the Walker motif A or P-loop (phosphate-binding loop) GXXGXGKST₆₆ nucleotide-binding sequence which is located near the N-terminal end of the protein (147, 184, 227). AbcA shares with other members of the family another loop structure, OOOODEAXXXD₁₇₇ (where O is any hydrophobic amino acid residue) with highly conserved spacing to the P-loop (131), and several other segments of sequence.

In the case of prokaryotic ABC transport systems, the basic organization normally involves four distinct subunit components, either as domains of one or more proteins, or as distinct individual proteins (10, 100, 122). The various components interact to form a membrane-associated "transport

complex". The integral membrane proteins or protein domains which form part of this complex are believed to mediate the actual transport of the substrate across the lipid bilayer. These bacterial transport systems also often require a soluble receptor (substrate-binding) in the periplasm which, when bound to substrate, directly interacts with the membrane bound complex. Two of the subunits in the transport complex are usually ABC proteins, normally encoded by a single gene, which couple ATP hydrolysis to the transport. The ABC cassette containing components are associated with the cytoplasmic membrane, and in some cases, they have been shown to be exposed to the periplasmic surface (14). Upon binding of nucleotides, substantial conformational changes have been observed (118) which could signal the substrate transport. Appropriately, the *abcA* gene product of *A. salmonicida* co-fractionated with the cell envelope fraction when it was expressed in *E. coli*, and indeed sub-fractionated into the sarcosyl-soluble, putative inner membrane fraction. The presence of the AbcA-LacZ fusion protein in the cytosol fraction is also consistent with the predicted membrane associated region which is absent in the fusion protein. While the nucleotide binding sequence present in this fusion protein has been shown to have ATP binding activity.

In contrast to the *sec* encoded protein secretion system which recognizes many different signal peptides (17), ABC transporters each appear to be relatively specific for a single substrate, or group of related substrates (100, 122). The genes encoding the ABC transporter complex normally map contiguously (91). For example the *E. coli hlyB* gene maps immediately downstream of the substrate of its transport system, *hlyA* (133), while the ABC transport complex proteins involved in capsular polysaccharide transport appear to belong to a single transcriptional unit which contains two to four

other genes, including genes coding for integral membrane proteins (62). The organization of these capsular transport genes is very similar in different bacterial species, with the gene for the ABC containing protein itself mapping after the membrane protein genes. Taken together, these various observations lend weight to the notion that VapA could be a substrate of a transport system involving AbcA.

Among the ABC transporter proteins with strong homology to the *A. salmonicida* AbcA, *N. meningitidis* CtrD protein (62), *E. coli* KpsT (162), and *H. influenza* BexA (121) (34 - 37% identity, 21 - 21.5% conserved replacements between residues 42 and 221) are involved in capsular polysaccharide transport, and have recently been shown to belong to a new sub-family within the family of ATP-type transporters, the ABC-2 family (175). In this regard, it is perhaps significant that the *abcA* gene appeared to be required for the formation of O-chains on the LPS of *A. salmonicida*. The inability of the LPS O-chain complementation in TM4 by the two mutants constructed by site-directed mutagenesis has provided further evidence that AbcA could be involved in the O-chain formation and specifically, the ATP binding sequence might be required. However, the precise role of AbcA in the O-chain formation is still not clear. As a transport protein, the absence of AbcA could lead to the accumulation of sugars inside the cell and subsequently affect the O-chain synthesis. Alternatively, it is also possible that AbcA functions as a regulatory protein which controls genes responsible for polysaccharide synthesis.

One feature which appears to distinguish this *A. salmonicida* protein from other ABC proteins is the presence of a possible leucine zipper. This predicted α -helical region has Leu residues in four of five heptad repeats, and a Val replacement in the fourth heptad repeat which should allow the region

to form a zipper (98, 185). The potential zipper sequence does contain a Pro residue, however, using PCGENE, neither the Garnier (73) nor Chou-Fasman (37) algorithms predict disruption of the extended α -helix by this residue. The heptad repeat region is juxtaposed to a highly positively charged 23 residue sequence which is characteristic of leucine zipper proteins in eukaryotes (223). In eukaryotic proteins, this region binds DNA and is located N-terminal to the leucine zipper, leading to the nomenclature bZIP protein (223). In the *A. salmonicida* protein the basic region is located in the carboxy terminal, providing a ZIPb-like structure. This differs from other reported prokaryotic leucine zipper proteins where no typical basic regions have been observed near the zipper region, and where the leucine zipper is usually at the N-terminus of the protein (137, 185). The highly positively charged region in AbcA probably contributes to aberrant migration during SDS-PAGE, accounting for the difference in deduced (34,015) and measured apparent Mr (43,000). Such effects have been noted before, for example the HlyB protein of *V. cholerae* reported by Alm and Manning (9).

In addition to AbcA's putative transport activity, the influence of *abcA* on *vapA* expression could involve gene regulation. With the ZIPb sequence, AbcA appears to have the potential to be a regulatory protein. The structurally complex *vapA* promoter region and especially the palindromic sequence found immediately in front of *vapA* might provide a binding site(s) for AbcA. Another prokaryotic membrane-associated regulatory protein (ToxR) has been reported in *V. cholerae*, which was shown to have a transmembrane sequence at C-terminal half and transcriptional activation and DNA binding domains at N-terminal part (146). However, the up-regulation of the *vapA* expression by *abcA* could also involve other gene products in *E. coli*. Several attempts to introduce a mutation into the *abcA*

gene in *A. salmonicida* chromosome were all unsuccessful. At this time, the only mutant *A. salmonicida* available with a disrupted chromosomal *abcA* gene also has a disrupted *vapA* gene, making it impossible to directly show the effect of *abcA* on *vapA* expression.

Taken together all the structural and functional features of the AbcA protein, i.e., ATP binding cassette, membrane associated region, ZIPb sequence, lack of homology at the C-terminal with other ATP-binding transport proteins, ATP binding activity, influence on *vapA* expression in *E. coli* and complementation of LPS O-chain production in *A. salmonicida*, this protein appears to be bifunctional and involved in both transportation and gene regulation. However, confirmation of its role with regard to *vapA* in *A. salmonicida* awaits future studies.

IV. Summary

This study has been focused on the structure, expression and regulation of the *A. salmonicida* surface layer protein gene *vapA* and its protein product, A-protein. The *vapA* DNA sequence has been determined, and this enabled detailed genetic characterization, as well as the prediction of the entire primary sequence of the protein which has provided valuable information for further structural and functional studies. The cellular localization of the A-protein in *E. coli* has been described and the difference in trypsin accessibility of the periplasmic A-protein has been demonstrated which may indicate a mechanism that contributes to the inefficient export of the protein in this foreign host. The transcription initiation site of *vapA* in *E. coli* has been shown to be different from that in *A. salmonicida*, since the native transcriptional start site is not included in the clone. A putative P2 promoter has been proposed that may function in *E. coli*, while the wild type *vapA*

promoter P1 has been predicted from the mapped transcriptional start site and its predominant transcriptional activity has been confirmed by transcriptional analysis and localization of the *A. salmonicida* insertion element that affected the *vapA* expression. The variation of the *vapA* transcription has been analyzed under a number of culture conditions. With 30°C incubation, mutant *A. salmonicida* strains have been shown to have various levels of reduced A-protein production and a 1.2 kb DNA expansion in the *vapA* area which was caused by *A. salmonicida* insertion elements. These *A. salmonicida* insertion elements have been sequenced and shown to be homologous to other bacterial insertion elements (ISA2). In addition, the AbcA protein has been shown to have ATP binding activity, a leucine zipper-basic region domain, and the ability to affect *vapA* expression in *E. coli* and complement the deficient LPS O-chain formation in *A. salmonicida* *abcA* mutant strain TM4. Site-directed mutagenesis experiments have shown that the ATP binding domain of AbcA may be required for the LPS O-chain complementation.

REFERENCES

1. Abraham, E. F., A. G. Prat, L. Gerweck, T. Seneveratne, R. J. Arceci, R. Kramer, G. Guidotti and H. F. Cantiello. 1993. The multidrug resistance (*mdr1*) gene product functions as an ATP channel. *Proceedings of the National Academy of Sciences of the United States of America* 90: 312-316
2. Adachi, T., H. Yamagata, N. Tsukagoshi and S. Udaka. 1989. Multiple and tandemly arranged promoters of the cell wall protein gene operon in *Bacillus brevis* 47. *Journal of Bacteriology* 171: 1010-1016
3. Adachi, T., H. Yamagata, N. Tsukagoshi and S. Udaka. 1990. Use of both translation initiation sites of the middle wall protein gene in *Bacillus brevis* 47. *Journal of Bacteriology* 172: 511-513
4. Adachi, T., H. Yamagata, N. Tsukagoshi and S. Udaka. 1991. Repression of the cell wall protein gene operon in *Bacillus brevis* 47 by magnesium and calcium ions. *Journal of Bacteriology* 173: 4243-4245
5. Aizawa, S.-I., F. Vonderviszt, R. Ishima and K. Aksaka. 1990. Termini of *Salmonella* flagellin are disordered and become organized upon polymerization into flagellar filament. *Journal of Molecular Biology* 211: 673-677
6. Ajioka, J. W. and D. L. Hartl. 1989. Population dynamics of transposable elements. p. 939-958. *In* D.E. Berg and M.M. Howe (ed.), *Mobile DNA*. American Society for Microbiology. Washington, D.C.
7. Allen, L. N. and R. S. Hanson. 1985. Construction of broad-host-range cosmid cloning vectors: Identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. *Journal of Bacteriology* 161: 955-962
8. Alm, R. A., P. Guerry and T. J. Trust. 1993. The *Campylobacter* σ^{54} *flaB* flagellin promoter is subject to environmental regulation. *Journal of Bacteriology* 175: 000-000
9. Alm, R. A. and P. A. Manning. 1990. Characterization of the *hlyB* gene and its role in the production of the El Tor haemolysin of *Vibrio cholerae* O1. *Molecular Microbiology* 4: 413-425
10. Ames, G. F.-L. and H. Lecar. 1992. ATP-dependent bacterial transporters and cystic fibrosis: analogy between channels and transporters. *FASEB Journal* 6: 2660-2666
11. Ames, G. F.-L., C. Prody and S. Kustu. 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *Journal of Bacteriology* 160: 1181-1183

12. **Audrezet, M. P., B. Mercier, H. Guillermit, I. Quere, C. Verlingue, G. Rault and C. Ferec.** 1993. Identification of 12 novel mutations in the CFTR gene. *Human Molecular Genetics* 2: 51-54
13. **Baga, M., M. Goransson, S. Normark and B. E. Uhlin.** 1988. Processed mRNA with differential stability in the regulation of *E. coli* pilin gene expression. *Cell* 52: 197-206
14. **Baichwal, V., D. Liu and G. F.-L. Ames.** 1993. The ATP-binding component of a prokaryotic traffic ATPase is exposed to the periplasmic (external) surface. *Proceedings of the National Academy of Sciences of the United States of America* 90: 620-624
15. **Barghouthi, S., S. M. Payne, J. E. Arceneaux and B. R. Byers.** 1991. Cloning, mutagenesis, and nucleotide sequence of a siderophore biosynthetic gene (*amoA*) from *Aeromonas hydrophila*. *Journal of Bacteriology* 173: 5121-5128
16. **Barrantes, F. J.** 1975. The nicotinic cholinergic receptor: different compositions evidenced by statistical analysis. *Biochemical and Biophysical Research Communications* 62: 407-414
17. **Bassford, P., J. Beckwith, K. Ito, C. Kumamoto, S. Mizushima, D. Oliver, L. Randall, T. Silhavy, F. C. Tai and W. Wickner.** 1991. The primary pathway of protein export in *E. coli*. *Cell* 65: 367-368
18. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* 19: 327-336
19. **Belasco, J. G., G. Nilsson, A. von Gabain and S. N. Cohen.** 1986. The stability of *E. coli* gene transcripts is dependent on determinants localized to specific mRNA segments. *Cell* 46: 245-251
20. **Belland, R. J. and T. J. Trust.** 1985. Synthesis, export, and assembly of *Aeromonas salmonicida* A-layer analyzed by transposon mutagenesis. *Journal of Bacteriology* 163: 877-881
21. **Belland, R. J. and T. J. Trust.** 1987. Cloning of the gene for the surface array protein of *Aeromonas salmonicida* and evidence linking loss of expression with genetic deletion. *Journal of Bacteriology* 169: 4086-4091
22. **Belland, R. J. and T. J. Trust.** 1988. DNA:DNA reassociation analysis of *Aeromonas salmonicida*. *Journal of General Microbiology* 134: 307-315
23. **Bisercic, M. and H. Ochman.** 1993. Natural populations of *Escherichia coli* and *Salmonella typhimurium* harbor the same classes of insertion sequences. *Genetics* 133: 449-454

24. Black, M. E. and D. E. Hruby. 1990. Identification of the ATP-binding domain of vaccinia virus thymidine kinase. *Journal of Biological Chemistry* 265: 17584-17592
25. Blaser, M. J. and E. C. Gotschlich. 1990. Surface array protein of *Campylobacter fetus* - cloning and gene structure. *Journal of Biological Chemistry* 265: 14529-14535
26. Blaser, M. J. and Z. H. Pei. 1993. Pathogenesis of campylobacter-fetus infections - critical role of High-Molecular-Weight S-Layer proteins in virulence. *Journal of Infectious Diseases* 167: 372-377
27. Blaser, M. J., P. F. Smith, J. E. Repine and K. A. Joiner. 1988. Pathogenesis of *Campylobacter fetus* infections. Failure of encapsulated *Campylobacter fetus* to bind C3b explains serum and phagocytosis resistance. *Journal of Clinical Investigation* 81: 1434-1444.
28. Bootsma, R., N. Fijan and J. Blommaert. 1977. Isolation and preliminary identification of the causative agent of carp erythrodermatitis. *Veterinary Archives* 47: 291-302
29. Bowditch, R. D., P. Baumann and A. A. Yousten. 1989. Cloning and sequencing of the gene encoding a 125-kilodalton surface-layer protein from *Bacillus sphaericus* 2362 and of a related cryptic gene. *Journal of Bacteriology* 171: 4178-4188
30. Bröckl, G., M. Behr, S. Fabry, R. Hensel, H. Kaudewitz, E. Biendl and H. König. 1991. Analysis and nucleotide sequence of the genes encoding the surface-layer glycoproteins of the hyperthermophilic methanogens *Methanothermobacter ferredoxiens* and *Methanothermobacter sociabilis*. *European Journal of Biochemistry* 199: 147
31. Bullock, G. L. and H. M. Stuckey. 1975. *Aeromonas salmonicida* detection in asymptotically infected trout. *Progressive Fish-Culturist* 37: 237-239
32. Burke, V., J. Robinson, M. Cooper, J. Beaman, K. Partridge, D. Peterson and M. Gracey. 1984. Biotyping and virulence factors in clinical and environmental isolates of *Aeromonas* species. *Applied and Environmental Microbiology* 47: 1146-1149
33. Carl, M., M. E. Dobson, W. M. Ching and G. A. Dasch. 1990. Characterization of the gene encoding the protective paracrystalline-surface-layer protein of *Rickettsia prowazekii* - presence of a truncated identical homolog in *Rickettsia typhi*. *Proceedings of the National Academy of Sciences of the United States of America* 87: 8237-8241

34. Carnahan, A. M. and S. W. Joseph. 1991. *Aeromonas* update: new species and global distribution. *Experientia* 47: 402-403
35. Chart, H., T. W. Pearson and T. J. Trust. 1984. Detection of specific fish antibody using an inhibition enzyme-linked immunosorbent assay (inhibition ELISA). *Journal of Immunological Methods* 68: 19-24
36. Chart, H., D. H. Shaw, E. E. Ishiguro and T. J. Trust. 1984. Structural and immunochemical homogeneity of *Aeromonas salmonicida* lipopolysaccharide. *Journal of Bacteriology* 158: 16-22
37. Chou, P. Y. and G. D. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Advances in Enzymology and Related Areas of Molecular Biology* 47: 45-148
38. Collado-Vides, J., B. Magasanik and J. D. Gralla. 1991. Control site location and transcriptional regulation in *Escherichia coli*. *Microbiological Review* 55: 371-394
39. Colwell, R. R., M. T. MacDonell and J. De Ley. 1986. Proposal to recognize the family *Aeromonadaceae* fam. nov. *International Journal of Systematic Bacteriology* 36: 473-477
40. Dalrymple, B., P. Caspers and W. Arber. 1984. Nucleotide sequence of the prokaryotic mobile genetic element IS30. *EMBO Journal* 3: 2145-2149
41. Doig, P., L. Emody and T. J. Trust. 1992. Binding of Laminin and Fibronectin by the Trypsin-Resistant Major Structural Domain of the Crystalline Virulence Surface Array Protein of *Aeromonas-Salmonicida*. *Journal of Biological Chemistry* 267: 43-49
42. Dong, Q., A. Sadouk, D. der Lelie, S. Taghavi, A. Ferhat, J. M. Nuyten, B. Borremans, M. Mergeay and A. Toussaint. 1992. Cloning and sequencing of IS1086, an *Alcaligenes eutrophus* insertion element related to IS30 and IS4351. *Journal of Bacteriology* 174: 8133-8138
43. Dooley, J. S. G., W. D. M. Cubbin, C. M. Kay and T. J. Trust. 1988. Isolation and biochemical characterization of the S-layer protein from a pathogenic strain of *Aeromonas hydrophila*. *Journal of Bacteriology* 170: 2631-2638
44. Dooley, J. S. G., H. Engelhardt, W. Baumeister, W. W. Kay and T. J. Trust. 1989. Three-dimensional structure of an open form of the surface layer from the fish pathogen *Aeromonas salmonicida*. *Journal of Bacteriology* 171: 190-197
45. Dooley, J. S. G., R. Lallier, D. H. Shaw and T. J. Trust. 1985. Electrophoretic and immunochemical analyses of the lipopolysaccharides

from various strains of *Aeromonas hydrophila*. *Journal of Bacteriology* 164: 263-269

46. Dorman, C. J., N. N. Bhriain and C. F. Higgins. 1990. DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. *Nature* 344: 789-792
47. Duff, D. C. B. and B. J. Stewart. 1933. Studies on furunculosis of fish in British Columbia. *Cont. Can. Biol. and Fish.* 8: 103-122
48. Ebisu, S., A. Tsuboi, H. Takagi, Y. Naruse, H. Yamagata, N. Tsukagoshi and S. Udaka. 1990. Conserved structures of cell wall protein genes among protein-producing *Bacillus brevis* strains. *Journal of Bacteriology* 172: 1312-1320
49. Ebright, R. H., P. Cossart, B. Gicquel-Sanzey and J. Beckwith. 1984. Mutations that alter the DNA sequence specificity of the catabolite gene activator protein of *E. coli*. *Nature* 311: 232-235
50. Edwards, P. and J. Smit. 1991. A transducing bacteriophage for *Caulobacter crescentus* uses the paracrystalline surface layer protein as a receptor. *Journal of Bacteriology* 173: 5568-5572
51. Emmerich, R. and E. Weibal. 1894. Über eine durch bakterien erzeugte seuche unter den forellen. *Archiv fuer Hygiene und Bakteriologie* 21: 1-21
52. Emory, S. A., P. Bouvet and J. G. Belasco. 1992. A 5'-terminal stem-loop structure can stabilize messenger RNA in *Escherichia coli*. *Genes and Development* 6: 135-148
53. Evans, I. J. and J. A. Downie. 1986. The *nodI* gene product of *Rhizobium leguminosarum* is closely related to ATP-binding bacterial transport proteins; nucleotide sequence analysis of the *nodI* and *nodJ* genes. *Gene* 43: 95-101
54. Evans, I. J., I. B. Holland, L. Gray, S. D. Buckel, A. W. Bell and M. A. Hermodson. 1986. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature* 323: 448-450
55. Evenberg, D., R. van Boxtel, B. Lugtenberg, F. Schurer, J. Blommaert and R. Bootsma. 1982. Cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*: I. Relationship between autoagglutination and the presence of a major cell envelope protein. *Biochimica et Biophysica Acta* 684: 241-248
56. Faraldo, M. M., M. A. Depedro and J. Berenguer. 1992. Sequence of the S-Layer gene of *Thermus thermophilus* HB8 and functionality of its promoter in *Escherichia coli*. *Journal of Bacteriology* 174: 7458-7462

57. **Felmlee, T., S. Pellet and R. A. Welch.** 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *Journal of Bacteriology* **163**: 94-105
58. **Filip, C., G. Fletcher, J. L. Wulff and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *Journal of Bacteriology* **115**: 717-722
59. **Fisher, J. A., J. Smit and N. Agabian.** 1988. Transcriptional analysis of the major surface array gene of *Caulobacter crescentus*. *Journal of Bacteriology* **170**: 4706-4713
60. **Fraser, L. and J. Friesen.** 1986. Mutations in a yeast intron demonstrate the importance of specific conserved nucleotides for the two stages of nuclear mRNA splicing. *Cell* **45**: 81-93
61. **Freij, B. J.** 1986. *Aeromonas*: biology of the organism and disease in children. *Pediatric Infectious Disease Journal* **3**: 164-175
62. **Frosch, M., U. Edwards, K. Bousset, B. Krauße and C. Weisgerber.** 1991. Evidence for a common molecular origin of the capsule gene loci in Gram-negative bacteria expression group II capsular polysaccharides. *Molecular Microbiology* **5**: 1251-1263
63. **Fuhrman, O.** 1909. La Furunculose. *Bull. Suisse Peche Pisci.* **12**: 193-195
64. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**: 119-131
65. **Fyfe, L., G. Coleman and A. L. S. Munro.** 1988. The combined effect of isolated *Aeromonas salmonicida* protease and haemolysin on Atlantic salmon, *Salmo salar* L., compared with that of a total extracellular products preparation. *Journal of Fish Diseases* **11**: 101-104
66. **Fyfe, L., G. Coleman and A. S. Munro.** 1987. Identification of major common extracellular proteins secreted by *Aeromonas salmonicida* strains isolated from diseased fish. *Applied and Environmental Microbiology* **53**: 722-726
67. **Galan, J. E. and R. Curtiss III.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. *Infection and Immunity* **58**: 1879-1885
68. **Galas, D. J. and M. Chandler.** 1989. Bacterial Insertion Sequences. p. 109-162. In D.E. Berg and M.M. Howe (ed.), *Mobile DNA*. American Society for Microbiology. Washington, DC

69. **Garduño, R. A. and W. W. Kay.** 1992. Interaction of the fish pathogen *Aeromonas Salmonicida* with rainbow trout macrophages. *Infection and Immunity* 60: 4612-4620
70. **Garduño, R. A. and W. W. Kay.** 1992. A single structural type in the regular surface layer of *Aeromonas salmonicida*. *Journal of Structural Biology* 108: 202-208
71. **Garduño, R. A., E. J. Y. Lee and W. W. Kay.** 1992. S-Layer-mediated association of *Aeromonas-Salmonicida* with murine macrophages. *Infection and Immunity* 60: 4373-4382
72. **Garduño, R. A., B. M. Phipps, W. Baumeister and W. W. Kay.** 1992. Novel structural patterns in divalent cation-depleted surface layers of *Aeromonas salmonicida*. *Journal of Structural Biology* 109: 184-195
73. **Garnier, J., D. J. Osguthorpe and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* 120: 97-120
74. **Giladi, H., W. X. Wang and A. B. Oppenheim.** 1992. Isolation and characterization of the hupA gene coding for HU of *Aeromonas proteolytica*. *Nucleic Acids Research* 20: 4092
75. **Gilchrist, A., J. A. Fisher and J. Smit.** 1992. Nucleotide sequence analysis of the gene encoding the *Caulobacter crescentus* paracrystalline surface layer protein. *Canadian Journal of Microbiology* 38:193-202
76. **Gilmore, R. D., N. Joste and G. A. McDonald.** 1989. Cloning, expression and sequence analysis of the gene encoding the 120 Kd surface-exposed protein of *Rickettsia rickettsii*. *Molecular Microbiology* 3: 1579-1586
77. **Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann and A. Danchin.** 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO Journal* 7: 3997-4004
78. **Gobius, K. S. and J. M. Pemberton.** 1988. Molecular cloning, characterization, and nucleotide sequence of an extracellular amylase gene from *Aeromonas hydrophila*. *Journal of Bacteriology* 170: 1325-1332
79. **Goettfert, M., S. Hitz and H. Hennecke.** 1990. Identification of *nodS* and *nodU*, two inducible genes inserted between the *Bradyrhizobium japonicum* *nodYABC* and *nodJ* genes. *Molecular Plant-Microb Interactions* 3: 3997-4004
80. **Goransson, M., B. Sonden, P. Nilsson, B. Dagberg, K. Forsman, K. Emanuelsson and B. E. Uhlin.** 1990. Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. *Nature* 344: 682-685

81. **Gottesman, M. M. and I. Pastan.** 1988. The multidrug transporter, a double-edged sword. *Journal of Biological Chemistry* **263**: 12163-12166
82. **Griffin, P. J., S. F. Snieszko and S. B. Friddle.** 1953. A more comprehensive description of *Bacterium salmonicida*. *Transactions of American Fisheries Society* **82**: 129-138
83. **Gu, X. R., S. Giroux and R. Cedergren.** 1988. The nucleotide sequence of the *argT* locus of *Aeromonas hydrophila*. *Nucleic Acids Research* **16**: 10936
84. **Guenet, C., P. Lepage and B. A. Harris.** 1992. Isolation of the leucine aminopeptidase gene from *Aeromonas proteolytica* - evidence for an enzyme precursor. *Journal of Biological Chemistry* **267**: 8390-8395
85. **Guzzo, J., F. Duong, C. Wandersman, M. Murgier and A. Lazdunski.** 1991. The secretion genes of *Pseudomonas aeruginosa* are functionally related to those of *Erwinia chrysanthemi* proteases and *Escherichia coli* α -hemolysin. *Molecular Microbiology* **5**: 447-453
86. **Hagiya, H., T. Oka, H. Tsuji and K. Takumi.** 1992. The S-layer composed of 2 different protein subunits from *Clostridium difficile* GAI-1152 - a simple purification method and characterization. *Journal of General and Applied Microbiology* **38**: 63-74
87. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**: 557-580
88. **Harlow, E. and D. Lane.** 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory. New York
89. **Hastein, T., S. J. Saltveit and R. J. Roberts.** 1978. Mass mortality among minnows *Phoxinus phoxinus* (L) in Lake Tveitevatn, Norway, due to an aberrant strain of *Aeromonas salmonicida*. *Journal of Fish Diseases* **1**: 241-249
90. **Helmann, J. D.** 1991. Alternative sigma factors and the regulation of flagellar gene expression. *Molecular Microbiology* **5**: 2875-2882
91. **Higgins, C. F., I. D. Hiles, G. P. D. Salmond, D. R. Gill, J. A. Downie, I. J. Evans, I. B. Holland, L. Gray, S. D. Buckel, A. W. Bell and M. A. Hermodson.** 1986. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature* **323**: 448-450
92. **Hitchcock, P. J. and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver stained polyacrylamide gels. *Journal of Bacteriology* **154**: 269-277
93. **Ho, A. S. Y., I. Sohel and G. K. Schoolnik.** 1992. Cloning and characterization of *fxp*, the flexible pilin gene of *Aeromonas hydrophila*. *Molecular Microbiology* **6**: 2725-2732

94. **Houwink, A. L.** 1953. A macromolecular monolayer in the cell wall of *Spirillum* spec. *Biochimica et Biophysica Acta* 10: 360-366
95. **Howard, L. and D. J. Tipper.** 1973. A polypeptide bacteriophage receptor: modified cell wall protein subunits in bacteriophage-resistant mutants of *Bacillus sphaericus* strain P-1. *Journal of Bacteriology* 113: 1491-1504
96. **Howard, S. P.** 1993. Assembly of surface layer and outer membrane proteins in *Aeromonas* spp. is independent of the extracellular export pathway. American Society for Microbiology 93rd General Meeting. Atlanta, Georgia
97. **Howard, S. P., W. J. Garland, M. J. Green and J. T. Buckley.** 1987. Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. *Journal of Bacteriology* 169: 2869-2871
98. **Hu, J. C., E. K. O'Shea, P. S. Kim and R. T. Sauer.** 1990. Sequence requirements for coiled-coils: analysis with λ repressor-GCN4 leucine zipper fusions. *Science* 250: 1400-1403
99. **Husslein, V., B. Huhle, T. Jarchau, R. Lurz, W. Goebel and T. Chakraborty.** 1988. Nucleotide sequence and transcriptional analysis of the *aerCaerA* region of *Aeromonas sobria* encoding aerolysin and its regulatory region. *Molecular Microbiology* 2: 507-517
100. **Hyde, S. C., P. Emsley, M. J. Hartshorn, M. M. Mimmack, U. Gileadi, S. R. Pearce, M. P. Gallagher, D. R. Gill, R. E. Hubbard and C. F. Higgins.** 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 346: 362-365
101. **Ikemura, T.** 1981. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes: A proposal for a synonymous codon choice that is optimal for the *E. coli* translational system. *Journal of Molecular Biology* 151: 389-409
102. **Ikemura, T. and H. Ozeki.** 1982. Codon usage and transfer RNA contents: organism-specific codon-choice patterns in reference to the isoacceptor contents. *Cold Spring Harbour Symposium on Quantitative Biology* 47: 1087-1097
103. **Ishiguro, E. E., T. Ainsworth, W. W. Kay and T. J. Trust.** 1986. Heme requirement for growth of fastidious atypical strains of *Aeromonas salmonicida*. *Applied and Environmental Microbiology* 51: 668-670
104. **Ishiguro, E. E., T. Ainsworth, T. J. Trust and W. W. Kay.** 1985. Congo red agar, a differential medium for *Aeromonas salmonicida* detects the presence of the cell surface protein array involved in virulence. *Journal of Bacteriology* 164: 1233-1237

105. Ishiguro, E. E., W. W. Kay, T. Ainsworth, J. B. Chamberlain, R. A. Austen, J. T. Buckley and T. J. Trust. 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. *Journal of Bacteriology* 148: 333-340
106. Janda, J. M. and P. S. Duffey. 1988. Mesophilic aeromonads in human disease: current taxonomy, laboratory identification, and infectious disease spectrum. *Reviews of Infectious Diseases* 10: 980-997
107. Janda, J. M. and R. P. Kokka. 1991. The pathogenicity of *Aeromonas* strains relative to genospecies and phenospecies identification. *FEMS Microbiology Letters* 90: 29-34
108. Janda, J. M., L. S. Oshiro, S. L. Abbott and P. S. Duffey. 1987. Virulence markers of mesophilic aeromonads: association of the autoagglutination phenomenon with mouse pathogenicity and the presence of a peripheral cell-associated layer. *Infection and Immunity* 55: 3070-3077
109. Jiang, B. and S. P. Howard. 1992. The *Aeromonas hydrophila* *exeE* gene, required both for protein secretion and normal outer membrane biogenesis, is a member of a general secretion pathway. *Molecular Microbiology* 6: 1351-1361
110. Johnson, C. M., M. F. Tatner and M. T. Horne. 1985. Comparison of the surface properties of seven strains of a fish pathogen, *Aeromonas salmonicida*. *Journal of Fish Biology* 27: 445-458
111. Kay, W. W., J. T. Buckley, E. E. Ishiguro, B. M. Phipps, J. P. L. Monette and T. J. Trust. 1981. Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. *Journal of Bacteriology* 147: 1077-1084
112. Kay, W. W., B. M. Phipps, E. E. Ishiguro, R. W. Olafson and T. J. Trust. 1984. Surface layer virulence A-proteins from *Aeromonas salmonicida* strains. *Canadian Journal of Biochemistry and Cell Biology* 62: 1064-1071
113. Kay, W. W., B. M. Phipps, E. E. Ishiguro and T. J. Trust. 1985. Porphyrin binding by the surface array virulence protein of *Aeromonas salmonicida*. *Journal of Bacteriology* 164: 1332-1336
114. Keen, N. T., S. Tamaki, D. Kobayashi and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* 70: 191-197
115. Klein, P., M. Kanehisa and C. DeLisi. 1985. The detection and classification of membrane-spanning proteins. *Biochimica et Biophysica Acta* 815: 468-476

116. **Kokka, R. P., N. A. Vedros and J. M. Janda.** 1990. Electrophoretic analysis of the surface components of autoagglutinating surface array protein positive and surface array protein negative *Aeromonas hydrophila* and *Aeromonas sobria*. *Journal of Clinical Microbiology* 28: 2240-2247
117. **Kolter, R. and C. Yanofsky.** 1982. Attenuation in amino acid biosynthetic operons. *Annual Review of Genetics* 16: 113-134
118. **Koronakis, V., C. Hughes and E. Koronakis.** 1993. ATPase activity and ATP/ADP-induced conformational change in the soluble domain of the bacterial protein translocator HlyB. *Molecular Microbiology* 8: 1163-1175
119. **Kostrzynska, M., J. S. G. Dooley, T. Shimojo, T. Sakata and T. J. Trust.** 1992. Antigenic diversity of the S-layer proteins from pathogenic strains of *Aeromonas hydrophila* and *Aeromonas veronii* biotype *sobria*. *Journal of Bacteriology* 174: 40-47
120. **Koval, S. F.** 1993. Predation on bacteria possessing S-layers. p. 85-92 In T.J. Beveridge and S.F. Koval (ed.), *Advances in Bacterial Paracrystalline Surface Layers*. Plenum Publishing. New York
121. **Kroll, J. S., B. Loynds and E. R. Moxon.** 1990. The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsule polysaccharide export. *Molecular Microbiology* 4: 1853-1862
122. **Kuchler, K. and J. Thorner.** 1992. Secretion of peptides and proteins lacking hydrophobic signal sequences: The role of adenosine triphosphate-driven membrane translocators. *Endocrine Reviews* 13: 499-514
123. **Kung, S.-S., J. Chen and W.-Y. Chow.** 1992. Molecular and genetic characterization of an *Alcaligenes eutrophus* insertion element. *Journal of Bacteriology* 174: 8023-8029
124. **Kyte, J. and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157: 105-132
125. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685
126. **Landick, R., J. Carey and C. Yanofsky.** 1985. Translation activates the paused transcription complex and restores transcription of the *trp* operon leader region. *Proceedings of the National Academy of Sciences of the United States of America* 82: 4663-4667
127. **Landschulz, W. H., P. F. Johnson and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240: 1759-1764

128. Lechner, J. and M. Sumper. 1987. The primary structure of a procaryotic glycoprotein: cloning and sequencing of the cell surface glycoprotein gene of *Halobacteria*. *Journal of Biological Chemistry* 262: 9724-9729
129. Lee, K.-K. and A. E. Ellis. 1989. The quantitative relationship of lethality between extracellular protease and extracellular haemolysin of *Aeromonas salmonicida* in Atlantic salmon (*Salmo salar* L.). *FEMS Microbiology Letters* 61: 127-132
130. Lee, K.-K. and A. E. Ellis. 1990. Glycerophospholipid: cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytotoxin of *Aeromonas salmonicida*: LPS stabilises and enhances toxicity of the enzyme. *Journal of Bacteriology* 172: 5382-5393
131. Linder, P., P. F. Lasko, P. Leroy, P. J. Nielsen, K. Nishi, J. Schnier and P. P. Slonimski. 1989. Birth of the D-E-A-D box. *Nature* 337: 121-122
132. Logan, S. M., T. J. Trust and P. Guerry. 1988. *Campylobacter* flagellin: evidence for post-translational modification and gene duplication. *Journal of Bacteriology* 171: 3031-3038
133. Mackman, N., J.-M. Nicaud, L. Gray and I. B. Hilland. 1985. Genetical and functional organisation of the *Escherichia coli* haemolysin determinant 2001. *Molecular and General Genetics* 201: 282-288
134. Marsh, M. C. 1902. *Bacterium truttae*, a new bacterium pathogenic to trout. *Science* 16: 706
135. Martinez, E., B. Bartolome and F. de la Cruz. 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ* a reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene* 58: 159-162
136. Massida, O., G. M. Rossolini and G. Satta. 1991. The *Aeromonas hydrophila* *cphA* gene: molecular heterogeneity among class B metallo-beta-lactamases. *Journal of Bacteriology* 173: 4611-4617
137. Maxon, M. E., J. Wigboldus, N. Brot and H. Weissbach. 1990. Structure-function studies on *Escherichia coli* MetR protein, a putative prokaryotic leucine zipper protein. *Proceedings of the National Academy of Sciences of the United States of America* 87: 7076-7079
138. McCarthy, D. H. 1978. Ph.D. thesis. A study of the taxonomic status of some bacteria currently assigned to the genus *Aeromonas*. Council of National Academic Awards, Great Britain
139. McIntosh, D. and B. Austin. 1990. Recovery of cell wall deficient forms (L-forms) of the fish pathogens *Aeromonas salmonicida* and *Yersinia ruckeri*. *Systematic and Applied Microbiology* 13: 378-381

140. Mead, D. A., E. Szczesna-Skorupa and B. Kemper. 1986. Single-stranded DNA "blue" T7 promoter plasmids: A versatile tandem promoter system for cloning and protein engineering. *Protein Engineering* 1: 67-74
141. Mescher, M. F. and J. L. Strominger. 1976. Structural (shape-maintaining) role of the cell surface glycoprotein of *Halobacterium salinarium*. *Proceedings of the National Academy of Sciences of the United States of America* 73: 2687-2691
142. Messner, P. and U. B. Sleytr. 1991. Bacterial surface layer glycoproteins. *Glycobiology* 1: 545-551
143. Messner, P. and U. B. Sleytr. 1992. Crystalline bacterial cell-surface layers. p. 213-275. In A.H. Rose and D.W. Tempest (ed.), *Advances in Microbial Physiology*. Academic Press Ltd. London
144. Mettam, A. E. 1914. Report on the outbreak of furunculosis in the river Liffey in 1913. *Scientific Investigations of the Fisheries Branch Department of Agriculture for Ireland*. 1941: 19
145. Miller, J. H. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
146. Miller, v. L., R. K. Tayler and J. J. Mekalanos. 1987. Cholera toxin transcriptional activator *ToxR* is a transmembrane DNA binding protein. *Cell* 48: 271-279
147. Mimura, C. S., S. R. Holbrook and G. F. Ames. 1991. Structural model of the nucleotide-binding conserved component of periplasmic permeases. *Proceedings of the National Academy of Sciences of the United States of America* 88: 84-88
148. Mirel, D. B. and M. J. Chamberlain. 1989. The *Bacillus subtilis* flagellin gene (*bag*) is transcribed by the sigma²⁸ form of RNA polymerase. *Journal of Bacteriology* 171: 3095-3101
149. Mittal, K. R., G. Lalonde, D. Leblanc, G. Olivier and R. Lallier. 1980. *Aeromonas hydrophila* in rainbow trout: relation between virulence and surface characteristics. *Canadian Journal of Microbiology* 26: 1501-1503
150. Munn, C. B., E. E. Ishiguro, W. W. Kay and T. J. Trust. 1982. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity* 36: 1069-1075
151. Murray, R. G. E., J. S. G. Dooley, P. W. Whippey and T. J. Trust. 1988. Structure of an S-layer on a pathogenic strain of *Aeromonas hydrophila*. *Journal of Bacteriology* 170: 2625-2630

152. Nelson, D. R. and D. R. Zusman. 1983. Evidence for long-lived mRNA during fruiting body formation in *Myxococcus xanthus*. Proceedings of the National Academy of Sciences of the United States of America 80: 12589-12595
153. Nilsson, G., J. G. Belasco, S. N. Cohen and A. von Gabian. 1984. Growth rate dependent regulation of mRNA stability in *Escherichia coli*. Nature 312: 75-77
154. Nomura, S., M. Fujino, M. Yamakawa and E. Kawahara. 1988. Purification and characterization of salmolyisin, an extracellular hemolytic toxin from *Aeromonas salmonicida*. Journal of Bacteriology 170: 3694-3702
155. Noonan, B., S. Cavaignac and T. J. Trust. 1993. Localization and cloning of genes involved in the export of the A-protein of *Aeromonas salmonicida*. p. 315-318. In T. J. Beveridge and S. F. Koval (ed.), Advances in bacterial paracrystalline surface layers. Plenum Publishing. New York
156. Olivier, G., C. A. Eaton and N. Campbell. 1986. Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). Veterinary Immunology and Immunopathology 12: 223-234
157. Parker, N. D. and C. B. Munn. 1984. Increased cell surface hydrophobicity associated with possession of an additional surface protein by *Aeromonas salmonicida*. FEMS Microbiology Letters 21: 233-237
158. Parker, N. D. and C. B. Munn. 1985. Cell surface properties of virulent and attenuated strains of *Aeromonas salmonicida*. p. 97-105. In A.E. Ellis (ed.), Fish and shellfish pathology. Academic Press Ltd. London
159. Parsot, C. and J. J. Mekalanos. 1992. Structural analysis of the *acfA* and *acfD* gene of *Vibrio cholerae*: effects of DNA topology and transcriptional activators on expression. Journal of Bacteriology 174: 5211-5218
160. Paterson, W. D., D. Douey and D. Desantels. 1980. Isolation and identification of an atypical *Aeromonas salmonicida* strain causing epizootic losses among Atlantic salmon (*Salmo salar*) reared in a Nova Scotian hatchery. Canadian Journal of Fisheries and Aquatic Sciences 12: 2236
161. Paterson, W. D. and J. L. Fryer. 1974. Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* endotoxin. Journal of the Fisheries Research Board of Canada 31: 1743-1749
162. Pavelka Jr., M. S., L. F. Wright and R. P. Silver. 1991. Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. Journal of Bacteriology 173: 4603-4610

163. Pearson, W. R. and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences of the United States of America* 85: 2444-2448
164. Peters, J. and W. Baumeister. 1986. Molecular cloning, expression, and characterization of the gene for the surface (HPI)-layer protein of *Deinococcus radiodurans* in *Escherichia coli*. *Journal of Bacteriology* 167: 1048-1054.
165. Peters, J., M. Peters, F. Lottspeich and W. Baumeister. 1989. S-Layer protein gene of *Acetogenium kivui* - cloning and expression in *Escherichia coli* and determination of the nucleotide sequence. *Journal of Bacteriology* 171: 6307-6315
166. Peters, J., M. Peters, F. Lottspeich, W. Schafer and W. Baumeister. 1987. Nucleotide sequence analysis of the gene encoding the *Deinococcus radiodurans* surface protein, derived amino acid sequence, and complementary protein chemical studies. *Journal of Bacteriology* 5216-5223
167. Phipps, B. M. 1988. Ph.D. thesis. Purification, biochemical characterization and molecular cloning of the regular surface layer A protein of *Aeromonas salmonicida*. University of Victoria
168. Phipps, B. M. and W. W. Kay. 1988. Immunoglobulin binding by the regular surface array *Aeromonas salmonicida*. *Journal of Biological Chemistry* 263: 9298-9303
169. Phipps, B. M., T. J. Trust, E. E. Ishiguro and W. W. Kay. 1983. Purification and characterization of the cell surface virulent A protein from *Aeromonas salmonicida*. *Biochemistry* 22: 2934-2939
170. Pittel, L. 1910. La furunculose observee sur la Bordeliere, la Nase et le Barbeau. *Bull. Suisse Peche Pisci.* 2: 171-173
171. Plehn, M. 1911. Die furunkulose der Salmoniden. Zentralblatt fuer Bakteriologie Parasitenkunde Infektionskrankheiten und Hygiene Abteilung I Originale 60: 609-624
172. Popoff, M. 1984. Genus III. *Aeromonas* Kluyver and Van Niel 1936, 398^{AL}. p. 545-548. In N.R. Krieg and J.G. Holt (ed.), *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins. Baltimore, MD
173. Rasmussen, J. L., D. A. Odelson and F. L. Macrina. 1987. Complete nucleotide sequence of insertion element IS4351 from *Bacteroids fragilis*. *Journal of Bacteriology* 169: 3573-3580
174. Reddy, K. J. and M. Gilman. 1988. Preparation and analysis of RNA. p. 4.0.1-4.4.7. In F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (ed.), *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience. New York

175. Reizer, J., A. Reizer and M. H. Saier. 1992. A new subfamily of bacterial ABC-Type transport systems catalyzing export of drugs and carbohydrates. *Protein Science* 1: 1326-1332
176. Reznikoff, W. S., D. A. Siegele, D. W. Cowing and C. A. Gross. 1985. The regulation of transcription initiation in bacteria. *Annual Review of Genetics* 19: 355-387
177. Riggs, M. G. and A. McLachlan. 1986. A simplified screening procedure for large numbers of plasmid mini-preparations. *BioTechniques* 4: 312-313
178. Romeo, J. M. and D. R. Zusman. 1992. Determinants of an unusually stable mRNA in the bacterium *Myxococcus xanthus*. *Molecular Microbiology* 6: 2975-2988
179. Rosenberg, A. H., B. N. Lade, D. Chui, S. Lin, J. J. Dunn and W. Studier. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56: 125-135
180. Sakai, D. K. 1984. Opsonization by fish antibody and complement in the immune phagocytosis by peritoneal exudate cells isolated from salmonid fishes. *Journal of Fish Diseases* 7: 29-38
181. Sakai, D. K. 1985. Loss of Virulence in a protease-deficient mutant of *Aeromonas salmonicida*. *Infection and Immunity* 48: 146-152
182. Sakai, D. K. 1987. Adhesion of *Aeromonas salmonicida* strains associated with net electrostatic charges of host tissue cells. *Infection and Immunity* 55: 704-710
183. Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press. New York
184. Saraste, M., P. R. Sibbald and A. Wittinghofer. 1990. The P-loop - a common motif in ATP- and GTP-binding proteins. *Trends in Biochemical Sciences* 15: 430-434
185. Sasse-Dwight, S. and J. D. Gralla. 1990. Role of eukaryotic-type functional domains found in the prokaryotic enhancer receptor factor σ^{54} . *Cell* 62: 945-954
186. Saxton, W. O. and W. Baumeister. 1986. Principles of organization in S layers. *Journal of Molecular Biology* 187: 251-253
187. Schwartz, E., M. Kroger and B. Rak. 1988. IS150: distribution, nucleotide sequence and phylogenetic relationships of a new *E. coli* insertion element. *Nucleic Acids Research* 16: 6789-6802

188. Snapira, S. K., J. Chou, F. V. Richaud and M. J. Casadaban. 1983. New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lacZ* gene sequences encoding an enzymatically active carboxy-terminal portion of β -galactosidase. *Gene* 25: 71-82
189. Sharp, P. M. and W.-H. Li. 1987. The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Research* 15: 1281-1295
190. Shotts, E. B. J., F. D. Talkington, D. G. Elliot and D. H. McCarthy. 1980. Aetiology of an ulcerative disease in goldfish, *Carassius auratus* (L): characterization of the causative agent. *Journal of Fish Diseases* 181-186
191. Sleytr, U. B. 1978. Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly, and function. *International Review of Cytology* 53: 1-64
192. Sleytr, U. B. and P. Messner. 1983. Crystalline surface layers on bacteria. *Annual Review of Microbiology* 37: 311-339
193. Sleytr, U. B. and P. Messner. 1988. Crystalline surface layers in prokaryotes. *Journal of Bacteriology* 170: 2891-2897
194. Sleytr, U. B. and P. Messner. 1988. Crystalline surface layers on bacteria. p. 160-186. In U.B. Sleytr, P. Messner, D. Pum and M. Sara (ed.), *Crystalline Bacterial Cell Surface Layers*. Springer-Verlag, Berlin
195. Smit, J. and N. Agabian. 1984. Cloning of the major protein of the *Caulobacter crescentus* periodic surface layer: detection and characterization of the cloned peptide by protein expression assays. *Journal of Bacteriology* 160: 1137-1145
196. Smith, C. J. 1987. Nucleotide sequence analysis of Tn4551: use of *ermFS* operon fusions to detect promoter activity in *Bacteroides fragilis*. *Journal of Bacteriology* 169: 4589-4596
197. Smith, I. W. 1963. The classification of "*Bacterium salmonicida*". *Journal of General Microbiology* 33: 263-274
198. Stewart, M., T. J. Beveridge and T. J. Trust. 1986. Two patterns in the *Acromonas salmonicida* A-layer may reflect a structural transformation that alters permeability. *Journal of Bacteriology* 166: 120-127
199. Stoker, N. G., N. F. Fairweather and B. G. Spratt. 1982. Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*. *Gene* 18: 335-341

200. **Strathdee, C. A. and R. Y. C. Lo.** 1989. Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of *Pasteurella haemolytica* leukotoxin. *Journal of Bacteriology* **171**: 916-928
201. **Sumper, M., E. Berg, R. Mengele and I. Strobel.** 1990. Primary structure and glycosylation of the S-layer protein of *Haloferax volcanii*. *Journal of Bacteriology* **172**: 7111-7118
202. **Thomas, S., J. W. Austin, W. D. McCubbin, C. M. Kay and T. J. Trust.** 1992. Roles of structural domains in the morphology and surface anchoring of the tetragonal paracrystalline array of *Aeromonas hydrophila* - biochemical characterization of the major structural domain. *Journal of Molecular Biology* **228**: 652-661
203. **Thornton, J., S. P. Howard and J. T. Buckley.** 1988. Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin-cholesterol acyltransferase and other lipases. *Biochimica et Biophysica Acta* **959**: 153-159
204. **Thornton, J. C., R. A. Garduno, S. G. Newman and W. W. Kay.** 1991. Surface-disorganized, attenuated mutants of *Aeromonas salmonicida* as furunculosis live vaccines. *Microbial Pathogenesis* **11**: 85-99
205. **Titball, R. W. and C. B. Munn.** 1985. Interrelationships of extracellular products from *Aeromonas salmonicida*. p. 61-68. In A.E. Ellis (ed.), *Fish and Shellfish Pathology*. Academic Press. London
206. **Titball, R. W. and C. B. Munn.** 1985. The purification and some properties of H-lysin from *Aeromonas salmonicida*. *Journal of General Microbiology* **131**: 1603-1609
207. **Towbin, H., T. Staehelin and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**: 4350-4354
208. **Trowsdale, J., I. Hanson, I. Mockridge, S. Beck, A. Townsend and A. Kelly.** 1991. Sequences encoded in the class II region of MHC related to the "ABC" superfamily of transporters. *Nature* **348**: 741-744
209. **Trust, T. J.** 1986. Pathogenesis of infectious diseases of fish. *Annual Review of Microbiology* **40**: 479-502
210. **Trust, T. J., P. S. Howard, J. B. Chamberlain, E. E. Ishiguro and J. T. Buckley.** 1980. Additional surface protein in autoaggregating strains of atypical *Aeromonas salmonicida*. *FEMS Microbiology Letters* **9**: 35-38

211. Trust, T. J., W. W. Kay and E. E. Ishiguro. 1983. Cell surface hydrophobicity and macrophage association of *Aeromonas salmonicida*. *Current Microbiology* 9: 315-318
212. Trust, T. J., M. Kostrzynska, L. Emody and T. Wadstrom. 1993. High-affinity binding of the basement membrane protein collagen type IV to the crystalline virulence surface protein array of *Aeromonas salmonicida*. *Molecular Microbiology* 7: 593-600
213. Tsai, C.-M. and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. *Analytical Biochemistry* 119: 115-119
214. Tsuboi, A., R. Uchihi, T. Adachi, T. Sasaki, S. Hayakawa, H. Yamagata, N. Tsukagoshi and S. Udaka. 1988. Characterization of the genes for the hexagonally arranged surface layer proteins in protein-producing *Bacillus brevis* 47: complete nucleotide sequence of the middle wall protein gene. *Journal of Bacteriology* 170: 935-945
215. Tsuboi, A., R. Uchihi, H. Engelhardt, H. Hattori, S. Shimizu, N. Tsukagoshi and S. Udaka. 1989. In vitro reconstitution of a hexagonal array with a surface layer protein synthesized by *Bacillus subtilis* harboring the surface layer protein gene from *Bacillus brevis* 47. *Journal of Bacteriology* 171: 6747-6752
216. Tsuboi, A., R. Uchihi, R. Tabata, Y. Takahashi, H. Hashiba, T. Sasaki, H. Yamagata, N. Tsukagoshi and S. Udaka. 1986. Characterization of the genes coding for two major cell wall proteins from protein-producing *Bacillus brevis* 47: complete nucleotide sequence of the outer wall protein gene. *Journal of Bacteriology* 168: 365-373
217. Tsukagoshi, N., R. Tabata, T. Takemura, H. Yamagata and S. Udaka. 1984. Molecular cloning of a major cell wall protein gene from protein-producing *Bacillus brevis* 47 and its expression in *Escherichia coli* and *Bacillus subtilis*. *Journal of Bacteriology* 158: 1054-1060
218. Tummuru, M. K. R. and M. J. Blaser. 1992. Characterization of the *Campylobacter fetus* *sapA* promoter: evidence that the *sapA* promoter is deleted in spontaneous mutant strains. *Journal of Bacteriology* 174: 5916-5922
219. Udey, L. and J. L. Fryer. 1978. Immunization of fish with bacterins of *Aeromonas salmonicida*. *Marine Fisheries Review* 40: 12
220. van Wezenbeek, P. M. G. F., T. J. M. Hulsebos and J. G. G. Schoenmakers. 1980. Nucleotide sequence of the filamentous bacteriophage M13 DNA genome: comparison with phage fd. *Gene* 11: 129-148
221. Vaughan, L. M., P. R. Smith and T. J. Foster. 1993. An aromatic-dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in

fish and is effective as a live vaccine against the salmonid disease furunculosis. *Infection and Immunity* 61: 2172-2181

222. Vidgrén, G., I. Palva, R. Pakkanen, K. Lounatmaa and A. Palva. 1992. S-Layer protein gene of *Lactobacillus brevis* - cloning by polymerase chain reaction and determination of the nucleotide sequence. *Journal of Bacteriology* 174: 7419-7427

223. Vinson, C. R., P. J. B. Sigler and S. L. McKnight. 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* 246: 911-916

224. von Graevenitz, A. and A. H. Mensch. 1968. The genus *Aeromonas* in human bacteriology. *New England Journal of Medicine* 278: 245-249

225. Vonderviszt, F., S. Kanto, S. Aizawa and K. Namba. 1989. Terminal regions of flagellin are disordered in solution. *Journal of Molecular Biology* 209: 127-133

226. Vonderviszt, F., H. Uedaira, S.-I. Kidokoro and K. Namba. 1990. Structural organization of flagellin. *Journal of Molecular Biology* 214: 97-104

227. Walker, J. E., M. Saraste, M. J. Runswick and N. J. Gay. 1982. Distantly related sequences in the α - and β -subunits of ATP synthase myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal* 1: 945-951

228. Waters, S. H., P. Rogowaty, J. Grinstead, J. Altenbuchner and R. Schmitt. 1983. The tetracycline resistance determinants of RP1 and Tn1721: nucleotide sequence analysis. *Nucleic Acids Research* 11: 6089-6105

229. Watson, J. D., N. H. Hopkins, J. W. Roberts, J. A. Steitz and A. M. Weiner. 1987. The Structures of DNA. p. 240-281. In J.R. Gillen (ed.), *Molecular Biology of the Gene* The Benjamin/Cummings Publishing Company, Inc. Menlo Park, California

230. Wedemeyer, G., A. J. Ross and L. Smith. 1968. Some metabolic effects of bacterial endotoxins in salmonid fishes. *Journal of the Fisheries Research Board of Canada* 26: 115-122

231. Whitley, P. W., M. Landon and G. Coleman. 1992. The cloning and nucleotide sequence of the serine protease gene (*aspA*) of *Aeromonas salmonicida* ssp. *salmonicida*. *FEMS Microbiology Letters* 99: 65-72

232. Wieland, F., J. Lechner and M. Sumper. 1982. The cell wall glycoprotein of halobacteria: structural, functional and biosynthetic aspects. *Zentralblatt fuer Bakteriologie Parasitenkunde Infektionskrankheiten und Hygiene Abteilung 1 Originale Reihe C* 3: 161-170

233. Wu, L. and N. E. Walker. 1991. Cloning and characterization of a glutamine transport operon of *Bacillus stearothermophilus* NUB-36: effect of temperature on regulation of transcription. *Journal of Bacteriology* 173: 4877-4888
234. Yamagata, H., T. Adachi, A. Tsuboi, M. Takao, T. Sasaki, N. Tsukagoshi and S. Udaka. 1987. Cloning and characterization of the 5' region of the cell wall protein gene operon in *Bacillus brevis* 47. *Journal of Bacteriology* 169: 1239-1245
235. Yamagata, H., K. Nakahama, Y. Suzuki, A. Kakinuma, N. Tsukagoshi and S. Udaka. 1989. Use of *Bacillus brevis* for efficient synthesis and secretion of human epidermal growth factor. *Proceedings of the National Academy of Sciences of the United States of America* 86: 3589-3593
236. Yamagata, N., Tsukagoshi and S. Udaka. 1986. Characterization of the genes coding for two major cell wall proteins from protein-producing *Bacillus brevis* 47: complete nucleotide sequence of the outer wall protein gene. *Journal of Bacteriology* 168: 365-373
237. Yanisch-Perron, C., J. Vieira and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33: 103-119
238. Young, R. A. and R. W. Davis. 1983. Efficient isolation of genes by using antibody probes. *Proceedings of the National Academy of Sciences of the United States of America* 80: 1194-1198
239. Zubay, G., D. A. Chambers and L. C. Cheong. 1970. Cell-free studies on the regulation of the *lac* operon. p. 375-391. In J.R. Beckwith and D. Zipser (ed.), *The Lactose Operon*. Cold Spring Harbor Laboratory. Cold Spring Harbor
240. Zumft, W. G., A. Viebrock-Sambale and C. Braun. 1990. Nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*: genes for copper-processing and properties of the deduced products, including a new member of the family of ATP/GTP-binding proteins. *European Journal of Biochemistry* 192: 591-599

APPENDIXES

I. Entire DNA sequence determined in this study

CTTACTAAGCCAAAGCAACACCAACAATGAAGAAAAAGTACCCTTTTGGGAGATATTCCA
CTTTTGGGTAATTTGTTTAAATATCAGACAAAAAAATCGAAAAACGGAGTTGGTACTG
TTAATCACCCCCTATGTGATCACTTCAAAGAAGAGGCCGAATTTTATACTGATCAATTC
CGTACCTTGAGTGGCTGGGATCCGATGCCCAAACGTTCTCAATCTGACGCAGGACTAGAT
CGATGACCATCTTCGGTCATCAGTGCTGTGACTTGACACAAAAATCGTCGATCGGCGGTC
GTTTTTTGTGCGCTCAGTTCAGGAAAACGATGAAAGGCGATCTTTGTACAGATTAGTAA
TTGACATGCCTGAGTCTGATGGCTAAGAATATGCAAGCGCACTTCGGAGCGCGGTGAAAC
TGTTGTATCTCACAGGACATGAGCATCAGTAGTTTCCCATTTTTTGCGGGAAAACAGCGG
TGTTTGTGTTCCGGTGCTCACAGTGACCGAAGGTTGATCAACGGATAGGTTCAACCCTAT
TTGTATATAATCAATAAACTTCCTTAAGGAAAAAAGTATGTTTAAGAAGACTTTGATTGC
AGCTGCCATTGTGGTCGGTTCCGCTGCACCTGCGTTTGCTGATGTCGTGATTAGCCCGAA
CGACAACACTTTTCGTTACTACCTCCCTCGCATCTGTAATAAGCAGCCGGTACTGGACTT
CTCCACTGCTCAACAAAACCTGACCCTTAACCTCAGTGAAGTTGGTGACCTTAAGAACAA
CGGTTTCATTGTGTTGGAATCCAAGGTGAAGGCCAATTCAACGACGCGGAAATCCGTCA
GTGGCTGTCCAACGGTTTCTGGCGTAGGCCGTTTACCGGTCTGCTGGTTAACCCGAATGA
TCATGGTAATTTTGCCAATAGCGGTGAAGTTAATGACGTTCGGAAGTCTTTAAGATTAT
TTCCGACGGTACCCAGCTGACCATCGTTCACACTATCGACAGCAATGGCAAGCGTCTGCG
TCTTGCTCTTGCTTCTGATGTAGAAGAGACAATCAATTTTGCTGATGCAGAGGTTGAGCT
GAAGCTGAACCTTAGCTAACCAAGCCTTTAAGCTGACCTCCGGTTCTCAAGGTACAGTAGC
TCTGACCGCAGGAGCTCTGTGGAACGCTTCTTACACTGCTGATCCGGTTGCTACCAAGCC
GCTGTTCAAACCTGGGTAAGCTGTTCCAGTTGAGTTTGACTAACGCTGGTAAAGCTACCGC
TCTGGTTTCCGAAGGTTTCTTGAACTTAATATCGGTGATGCGAATATTTCTGCTACTGA
TTTCGCGATTACCAACGTTACTACTAACCAGACCATCCAACGTGACAAGGTTAACCTGAC

CCTGACTGGTGATGTTTCTGCCTTCAAGAAAGATGCCAACGGTAACTTGGTAAACAAAGC
TGGTGCTAGCATCGGTGGAAGCTGCTGCTGATGGTCAATCTGCTACAGCTGTCTTGGG
TGCTGGCAACATGGCCGGTGGGGTTCAAAATGCTCTGGCTGCTTTTGGTACACTGTACGT
TGCTGCAGATAACACTGTTCCGGTTCCTGCTGTTAACTTCAATGTTAAGGCTGAAATCCA
AGGTGATAGCCAAGCTACCTATACTACTTCAAGGACGAGCTGGCTGATCTCTTCATCCT
CACCCGTGATGGTATGAAGTTTGACACAATTACTACTGGTACCACTTCTGCCAACCTCAT
CCACATTCGTGATGTATCTAACATCCTGCCTACTGAAGGTGGCAAGATCTTCGTAACAT
CACTGAATATGCAGATCATGCTGCCAATGGTCGTGGTGAAGGTACTGTATTGGTTACCCG
TAAAGCACTGTCTGTTACCCTGCCAAGCGGTGGTGCACTGACTCTGAAGCCTGCTGATGT
TGCTGCTGACGTTGGTGCTTCTATCACTGCTGGCCGTCAGGCTCGCTTGGTGTTTGAAGT
TGAAACCAATCAGGGTGAAGTAGCTGTTAAGAAATCCAATGCTGAAGGCGTGGATATTCA
GAATGGTACCCGCGGCACAGCACCGCTGGTAGATTTCACTCTGTAAGAGCGAATCTAGTC
GATAAAGGGCGCCGAGGCGCCCTTTTAAATTAATGTAAACTAAGCAGTTTCATTTGTTC
CCTCACTCATCAATCTCTTCATTTAGCGAGGTAGACAAGCTCTGCCGTTGCTCGTGACCA
CAGCCCTTATTTAGTTGTCATTTATTGTTATACTCTTTCACCTCGTTAGCTACCTTGCG
ATTTATCTATGTCCGAGCCTGTACTTGCCGTTAGCGGTGTCAATAAATCATTTCATCT
ATCGTTCCCTTGGCAAGCGCTATGGCATGCACTCAATCCCAAGGCTGATGTCAAGGTTT
TTCAAGCATTACGTGATATTGAACTGACGGTATATCGCGGGGAGACAATAGGCATTGTGG
GCCACAATGGTGCGGGCAAGTCTACCTTGCTACAGCTTATCACAGGAGTCATGCAACCTG
ATTGTGGCCAGATTACACGTACAGGGCGTGTGTGGGCTTGTTAGAGCTTGGTTCTGGTT
TTAACCCTGAATTTACTGGACGTGAAAACATTTTTTTTAAATGGCGCTATCTTGGAATGT
CACAGCGAGAGATGGATGATCGTTTGGAACGGATCCTTTCTTTGCTGCCATAGGTGATT
TTATTGATCAGCCTGTTAAAACTACTCCTCTGGGATGATGGTACGCCTCGCTTTTTTCAG
TCATCATCAATACTGATCCTGACGTATTGATCATTGATGAGGCATTGGCAGTGGGAGATG
ATGCATTTCAACGTAAATGCTATGCCAGGCTAAAGCAGTTGCAGTCACAAGGCGTAACGA
TTCTGCTGGTATCCCATGCAGCAGGCAGCGTGATTGAGTTATGTGACCGTGCAGTATTAC
TGGATCGAGGTGAGGTGCTGCTTCAAGGGGAGCCCAAAGCAGTGGTACACAACATATCACA

AGCTGCTCCATATGGAAGGCGATGAGCGTGCCCGCTTTCGTTATCACTTGCGCCAGACTG
GCCGTGGAGACAGTTATATTAGTGACGAATCTACCTCGGAGCCCAAATTAAGTCTGCCC
CCGGTATTTTATCAGTAGACCTTCAGCCTCAGTCTACAGTTTGGTATGAGAGTAAAGGTG
CCGTTCTTAGCGATGTACACATTGAAAGCTTTTGAAGTCGGCTTTGGTATGATGATTAAA
ACTCGTTCCGGCGTTGATCTTGGCGGAGCAACCTCAACGCGTAACCACGAACATCGCTTG
GCTTATGTTGCTGCGGGCAGCTCTCTAACAGTATCGTATGATTTTGAATGTGCATTACGA
AATGGCACTTATTTCTTGAATTGTGGTTGTTCTGGTCTTATAGATGGAGAACGCACATTT
TTGCATCGTGGTGTGATGTCGCCATGTTCAAGTCATTTGTGAAAGTGTAGATTCTACA
GGTCTGATTGATTTTTACCTCGCATTAAATGTCCACCAGAAATGAGCTTGGTTATGATGG
AAAAAATTAATCTTTTTATTATTGGTACCCAAAAAGGTGGGACTACTGCATTAGCTGATT
TTCTGGGAAAACATCCCATATTTACATAGCAGATGGTAAAGAAGCCCAC

II. List of oligonucleotides used in this study

Name	Length (bases)	Sequence
AP1	23	GGC TGA TCT CTT CAT CCT CAC CC
AP2	24	CAG AGT GAA ATC TAC CAG CGG TGC
CG2	24	ATG TTT AAG AAG ACT TTG ATT GCA
CG3	23	TGG CAG CTG CAA TCA AAG TCT TC
CG4	26	CAA TGT GTA CAT CGC TAA GAA CGG CA
CG5	24	GCC ATC ATG AGT GGT GCC GAG GGC
CG6	21	GCT GCG GTG ACG CTC AAC GGC
RAA2	24	GAT CAC TTC AAA AGA AGA GGC CGA
RAA3	24	CAA CTT TAC GTA GTA GAG GGT GAC
SC1	20	AAT CCG AGA CAT TTC CGA GG
SC2	20	AGG GAA CAA GAC CTG AGA GA
SC3	19	GTG GTC GGC AAA ATC GTC G
SC3-2	18	GCA AAA TCG TCG CCG CCG
SC5	16	CTG CTC GGG GCT CCA C
SC6	17	TGT GTC GTA ACG CCG GG
SC7	16	AAG GGG GCG AGC AGC C
SC8	19	TAT CTG GTC AAG CGG GTC G
SC9	26	CCG CCG CCG TCA AAG GTG ATG GTA TG
SC10	21	GGC AAC GAT ACC AGT TAT CGG
SC11	21	GAT AAC TGG TAT CGT TGC CCC
SC12	19	GCG GAG CAG TGG ATC AAC C
SC13	16	CAG GCT GCT CGC CCC C
SCI-V	25	CTC ACT ATA GGG ATA TCG AGC TCG G
SCG-V61	23	GGC CAC AAT GTT GCG GGC AAG TC
SCG-V58	22	GGC ATT GTG GTC CAC AAT GGT G
TJT18	17	GCT TGG CCT TCA CCT TG
TJ18	17	CCG TGA TGG TAT GAA GT
TJ19	17	GCT GAC GTT GGT GCT TC
TJ21	17	CCC CTT GGC AAG CGC TA
TJ22	17	CTT GAC ATC AGC CTT GG
TJ23	17	ATC GAC TAG ATT CGC TC

TJ24	17	CCA AGC TCT AAC AAG CC
TJ30	17	GCC GTT GCT CGT GAC CA
TJ31	17	CAA ATG AAA CTG CTT AG
TJ32	17	CTT CAC GGA ATG CGC GG
TJ33	17	CGG GTT GGG CGA GGA TC
TJ34	17	ACC ACC GCT TGG CAG GG
TJ35	17	TCC GAG GTA GAT TCG TC
TJ36	17	CGG ATA GGT TCA ACC CT
TJ37	17	GGG TGG AGA GGC TAT TC
TJ38	17	TAC GCC TTG TGA CTG CA
TJ39	17	AAC AAA ACC TGA CCC TT
TJ40	17	ACC AAG CCG CTG TTC AA
TJ41	17	CCA GTA CCG GCT GCT TA
TJ42	17	CTG ATC CTG ACG TAT TG
TJ43	17	AAC TGT CAT TAA CTT CA
TJ44	17	CGG AGG TCA GCT TAA AG
TJ45	17	TGG ATA TTC AGA ATG GT
TJ46	17	AGC AGG AAC CGG AAC AG
TJ47	17	CGG TAA CTT GGT AAA CA
TJ48	17	CGC GAA ATC AGT AGC AG
TJ49	17	AAG AGT ATA ACA ATA AA
TJ50	17	ACT GGA TCG AGG TGA GG
TJ51	17	GCA CCA CCG CTT GGC AG
TJ52	17	TCA AAG TCT TCT TAA AC
TJ53	17	GGA AAC TAC TGA TGC TC
TJ54	17	TGG TAT GAG AGT AAA GG
TJ55	17	GGC AGC TGC AAT CAA AG
TJ56	24	CTG TGA GAT ACA ACA GTT TCA CCG