

STRUCTURE AND FUNCTION OF THE SURFACE LAYER OF THE FISH  
PATHOGENIC BACTERIUM *Aeromonas salmonicida*

by

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ACCEPTED

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

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

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## ABSTRACT

The fish pathogenic bacterium *Aeromonas salmonicida* is the causative agent of furunculosis in salmonids, a systemic disease that causes important economic losses in salmon aquaculture. Since the paracrystalline S-layer of *Aeromonas salmonicida*, known as the A-layer, is essential for virulence, the virulence mechanisms associated with this structure were studied.

Structural studies demonstrated that the A-layer is flexible and plastic, being capable of acquiring different conformations and/or structural patterns, in which divalent cations play an important role.

It was rigorously demonstrated that the A-layer acts as an adhesin, promoting adherence to macrophages, and fish cell lines. Since the macrophage is a professional phagocyte involved in ingesting and destroying bacteria, the ability of *A. salmonicida* to replicate inside macrophages was examined. *A. salmonicida* replicated inside macrophages and eventually destroyed them. This characteristic, together with the fact that *A. salmonicida* also penetrated epithelial fish cells, make it a facultatively intracellular, invasive pathogen. The A-layer provided an initial protection against oxidative agents, increasing the opportunities for *A. salmonicida* cells to induce an A-layer-independent mechanism involved in high resistance to oxidative agents, and thereby increased survival inside macrophages.

Studies with *in vivo* grown *A. salmonicida* provided further insight into the pathogenic process of furunculosis, and suggested that the A-layer plays a crucial role in colonization and penetration of the host, as well as survival inside the host (early events of the infectious process). However, it was found that *in vivo* grown *A. salmonicida* is capable of expressing a slime layer that shields its entire surface and provides full protection against complement-mediated killing and phagocytosis, thus relegating the A-layer to a secondary or minor role in the later stages of infection.

The results presented have contributed significantly to our knowledge of the virulence factors of *A. salmonicida*, and could be used practically in the prevention of furunculosis in the salmon aquacultural industry.

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## LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
BS pattern	Big squares pattern
CFU	Colony forming unit
CHSE	Chinook salmon embryo
cm	Centimeter
CR	Congo Red
DMSO	Dimethyl sulfoxide
DOC	Sodium deoxycholate
EDDA	Ethylenediamine-di(o-hydroxyphenylacetic Acid)
EDTA	Ethylenediamine Tetra-acetic Acid
EGTA	Ethylene Glycol-bis( $\beta$ -aminoethyl Ether) N,N,N',N' Tetra-acetic Acid
EM	Electron microscopy
EPC	Epithelioma papulosum cyprini
FPA	Fixative containing formalin and propionic acid in ethanol
FPM	Fish peptone medium
FR units	Free ring units
X $g_{av}$	Average relative centrifugal field
GC content	Total Guanine-Cytosine content
h	Hour(s)
HBSS	Hanks balanced salt solution
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HPLC	High performance liquid chromatography
IFN	Interferon
Ig	Immunoglobulin
IP	Intraperitoneal
IPC	Intraperitoneal chamber
$K_d$	Dissociation constant
kDa	Kilodaltons

kV	Kilovolts
LB broth	Luria-Bertani broth
LD <sub>50</sub>	Lethal dose of a toxic or infectious agent that kills 50% of a tested animal population
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAbs	Monoclonal antibodies
MEM	Minimal essential medium
min.	Minute(s)
mM	Millimolar
mØ	Macrophage
M.S. 222	3-Aminobenzoic acid ethyl ester
MW or $M_r$	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate (in its reduced form)
ng	Nanogram
nm	Nanometer
nM	Nanomolar
OD	Optical density
OM	Outer membrane
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PG	Plumbagin
pI	Isoelectric point
pK <sub>a</sub>	$-\log_{10}$ (acid dissociation constant)
PMN	Polymorphonuclear
PMSF	Phenylmethylsulfonyl fluoride
ROS	Reduced oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SNG	Streptonigrin
SPA	Surface protein antigen (S-layer protein in <i>Rickettsia</i> species)

<b>SPO</b>	<b>Superoxide</b>
<b>TEM</b>	<b>Transmission electron microscopy</b>
<b>TEPA</b>	<b>Buffer containing TRIS, EDTA, PMSF and sodium azide</b>
<b>TRIS</b>	<b>Tris[hydroxymethyl]aminomethane</b>
<b>TSA</b>	<b>Trypticase soy agar</b>
<b>TSB</b>	<b>Trypticase soy broth</b>
<b>WD pattern</b>	<b>White dots pattern</b>

## ACKNOWLEDGEMENTS

I thank my supervisor Dr. W.W. Kay for his guidance and patience, as well as his generosity in providing financial support as a graduate student, and to attend scientific meetings.

The assistance of Geraldine Wong, Emit Lee, Karen Withal, Anne Archer and John Winchester, is greatly appreciated.

Financial support from the University of Victoria (through a University of Victoria Fellowship, President's Research Award and The Mrs. Annie Greskiw Award), NSERC (through a Postgraduate Scholarship), and the Science Council of British Columbia (through a GREAT Award) is acknowledged.

Finally, I want to thank J. Nichols for his advise and support during my first year as a graduate student, as well as B.M. Phipps, J.C. Thornton, J.L. Doran and S.K. Collinson for being helpful peers.

## DEDICATION

A Elizabeth, mi ayuda idónea

A mis padres por haberme engendrado como un producto de su amor

Sobre todo y sobre todos, al revelador de misterios, en especial  
el de su presencia en todo y en todos, que ha hecho y  
hace posible todas las cosas en todo lugar.

One day humanity will have a different approach to the  
learning process. I dedicate this piece of knowledge  
to the day when the present scientific method  
will be replaced by a better one.

## GENERAL INTRODUCTION

This doctoral dissertation deals with the structure and function of the paracrystalline surface layer of the fish pathogen *Aeromonas salmonicida*. Several comprehensive reviews and monographs on paracrystalline surface layers (S-layers) have appeared recently (Baumeister *et al.*, 1989; Beveridge and Graham, 1991; Engelhardt, 1988; Hovmöller *et al.*, 1988a; Koval, 1988; Koval and Murray, 1986; Messner and Sleytr, 1991 & 1992; Phipps, 1988; Pum *et al.*, 1989; Sleytr and Messner, 1988 & 1989; Sleytr *et al.*, 1988a, 1988b & 1989; Sleytr and Sára, 1986; Smit, 1986), and the proceedings of the Third International Workshop on S-layers (NATO-ARW, London, Ontario, September 27-30, 1992), are to be published shortly (Beveridge and Koval, 1993). Consequently, another extensive presentation on S-layers would be redundant. Therefore, this general introduction will describe, briefly, the general characteristics of eubacterial S-layers, and more extensively, an area that has not been covered in detail in any of the above mentioned reviews or monographs, that is the S-layers of bacterial pathogens, including the microorganism that constitutes the subject matter of this dissertation, *Aeromonas salmonicida*.

### PARACRYSTALLINE SURFACE LAYERS OF EUBACTERIA

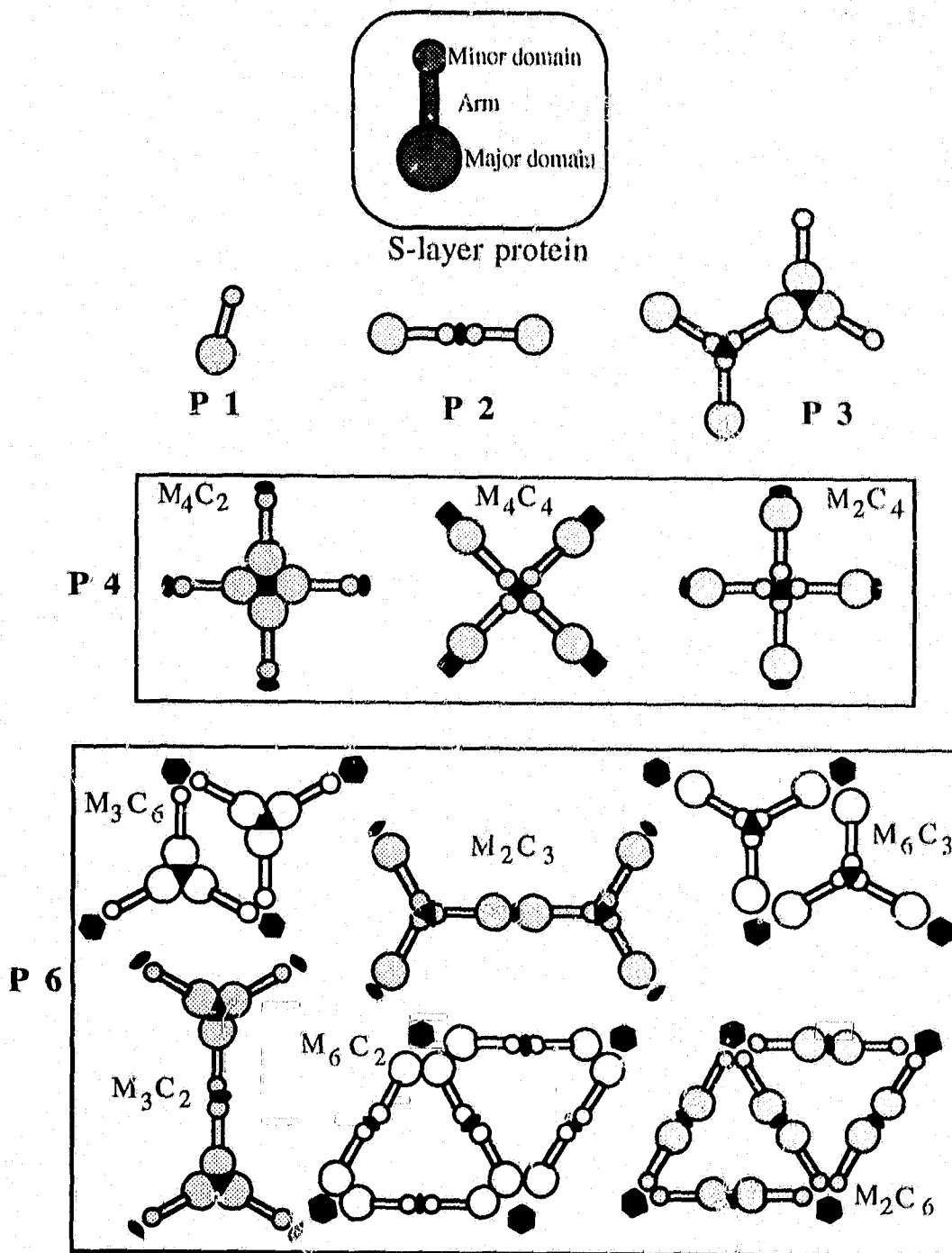
Houwink (1953) was the first to report the existence of an S-layer. This was detected by electron microscopy of cell wall fragments prepared by metal shadowing (unfortunately these layers are only detected by electron microscopy). Since then, S-layers have been reported in an increasing number of bacterial species. The most recent list of eubacteria possessing S-layers (Messner and Sleytr, 1992) includes more than 200 species and strains, comprising nearly every eubacterial taxonomic group. Although these macromolecular surface structures have received different names, at the Second International Workshop on Surface Layers, held in Vienna in 1987, it was agreed to use the term "S-layer" to connote "two-dimensional crystalline arrays of proteinaceous subunits forming surface layers on prokaryotic cells". In this context S-

layers should be distinguished from other surface layers described in prokaryotes; i.e. capsules, external slimes or glycocalices, and sheaths (Beveridge and Graham, 1991). Interestingly, many S-layer proteins constitute examples of prokaryotic glycoproteins (Messner and Sleytr, 1991), for many years thought not to exist in prokaryotes.

S-layers are two-dimensional, highly organized protein multimers disposed as single monolayers, or as naturally superimposed multilayers, that cover the entire surface of those bacteria which possess them. With few exceptions, each S-layer is composed of a single type of protein or glycoprotein subunit. Therefore, when multi-S-layers are present on the same bacterium, each has a different protein composition (Austin and Murray, 1990; Kist and Murray, 1984; Tsuboi *et al.*, 1982).

S-layer subunits are acidic proteins with a mass range of 30 to 220 kDa, high content of hydrophobic amino acids (40-50% on average), and a very low content of methionine and cysteine. Structurally, S-layer subunits in eubacteria are bilobed proteins, formed by a heavier (major) and a lighter (minor) domain connected by an arm (Fig. 1). Heavier domains convene around a primary symmetry axis to form massive cores, and the lighter domains provide connectivity by interacting at a secondary symmetry axis. Primary and secondary axes may be of the same or different symmetry, i.e. 2-fold, 3-fold, 4-fold or 6-fold symmetry, and regularly distributed within arrays of p1, p2, p3, p4 or p6 space symmetry (Saxton and Baumeister, 1986) (Fig. 1). The current understanding of the organization of S-layers in eubacteria, defines the subunit-subunit and the subunit-wall interactions as the means of S-layer integrity (Baumeister *et al.*, 1989; Koval, 1988; Sleytr and Messner, 1992; Smit, 1986). Subunit-subunit interactions determine the formation of the paracrystalline array while interactions with the cell wall provide anchorage of the assembled array to the bacterial cell.

Purified S-layer subunits have shown the ability to spontaneously self-assemble into arrays identical to those observed on cells, suggesting that array formation is an entropy-driven process, and that subunits possess all the information necessary to form lattices of defined structure (Sleytr and Messner 1989). Purified S-layer subunits or layers assembled *in vitro* also have the ability to reattach onto cell surfaces devoid of S-layers. This reconstitution process is usually species and/or strain specific, demonstrating the importance and specificity of subunit-wall interactions. Subunit-subunit and subunit-wall interactions are generally of a different nature (Austin and



**FIGURE 1.** Schematic representation of the different space symmetry groups and arrangements found in S-layers. The symmetry axes, around which the S-layer protein major (M), or minor (C) domains convene, are shown as geometric figures filled in black: ellipse = 2-fold symmetry axis, triangle = 3-fold symmetry axis, square = 4-fold symmetry axis, hexagon = 6-fold symmetry axis. P1, P2, P3, P4, and P6 designate the space symmetry groups, and the nomenclature used for the different lattice arrangements is that proposed by Saxton and Baumeister (1986). Other symmetry centres within the lattice are not shown.

Murray, 1990; Baumeister *et al.*, 1982; Doran *et al.*, 1987; Koval and Murray, 1983; Nermut and Murray, 1967; Sleytr and Glauert, 1976; Sleytr and Plohberger, 1980), and are mediated by non-covalent bonds (hydrophobic and electrostatic interactions), as inferred from the methods required to isolate intact S-layers or to solubilize S-layer proteins (König and Stetter, 1986; Koval and Murray, 1984; Messner & Sleytr, 1992). These methods usually involve the use of chaotropes, detergents, low ionic strength buffers or deionized water, extreme pH buffers, or divalent cation chelators.

S-layers are clearly important supramolecular assemblies, accounting for their ubiquity among prokaryotes. When present, they comprise up to 20% of the total cellular protein (imposing an expensive burden upon the cell economy), and it has been estimated that they represent the most abundant prokaryotic proteins in the biosphere (Messner and Sleytr, 1991 & 1992). In most cases however, their exact biological functions have not yet been assigned. Indeed, in contrast to their structural characterization, there is a severe lack of information regarding the functions of these unique structures. Owing to their strategic surface location, and to the fact that S-layers represent open networks of charged proteins with defined pores, the following potential functions have been proposed: i) protective coats, ii) molecular sieves, iii) molecular traps and/or ionic exchangers; iv) adhesins for surface recognition; v) cell shape determinants. Although some of these functions have now been confirmed in some eubacteria (Messner and Sleytr, 1992), the evidence is still fragmentary or absent in most cases.

## **S-LAYERS OF BACTERIAL PATHOGENS**

The interaction of bacterial pathogens with their host is mainly determined by macromolecular surface structures (Brown and Williams, 1985, Doyle and Sonnenfeld, 1989, Fisher, 1989, Smith, 1977, Williams, 1988). The pathogenic process of infectious diseases has been dissected in the following steps: colonization, penetration, multiplication, evasion of host defense mechanisms and damage to the host. In all these steps (with the possible exception of multiplication in the host and damage) the bacterial surface plays a pivotal role. It is the cell surface, through specialized macromolecular structures known as *adhesins*, which serves as an anchoring point for pathogenic bacteria. In turn, these adhesins may determine host and tissue specificity,

i.e. the ability of a bacterial pathogen to produce disease in some animal species in preference to others, and the ability to colonize (within a given host) some specific tissue in preference to others. It is the cell surface, through its specialized structures, which determines whether certain pathogens will be invasive, i.e. able to penetrate host cells or some physical barriers, to become established in deeper layers or tissues of the host. Surface components contribute to multiplication in the host by helping bacteria acquire essential nutrients. One of the best documented cases is their role in iron acquisition, either through specific surface receptors for siderophores or through direct binding of host iron-containing molecules. It is also the cell surface which serves as a site of immunological recognition, accounting for the importance of *surface antigens* in vaccine design. It is by modifying their cell surface (through mechanisms of *antigenic* and *phase variation*) that some bacterial pathogens evade immunological recognition. As well, it is the cell surface which serves as the target of many of the host defense mechanisms, and it is by having tough specialized surfaces that some pathogens evade these mechanisms. For instance, some surface structures (e.g. polysaccharide capsules, long O-polysaccharide chains and S-layers) inhibit complement-mediated killing, phagocytosis and/or intracellular digestion after phagocytosis. Lipopolysaccharide (LPS) from gram-negative bacteria, and lipoteichoic acids (LTA) from gram-positive bacteria, also have been implicated in modulation of the host immune response. Finally, surface components of bacterial pathogens may cause host damage evoking immunopathological reactions (e.g. immunopathology associated with surface antigens of *Treponema pallidum* or *Mycobacterium* species).

The surface location of S-layers, in many instances as the outermost structure on bacterial pathogens, means that they serve as the interface between the pathogen and its host and may significantly influence the outcome of a host-parasite relationship. S-layers must naturally come in close contact with cells, fluids, membranes and/or basement protein surfaces of the host, and therefore mediate several of the actions described above. In the following section a survey of cases of bacterial pathogens with S-layers (ordered alphabetically by genus and species) is presented. My intention is to emphasize the known virulence functions of S-layers but, unfortunately, only structural descriptions are available in the majority of cases.

### ***Aeromonas hydrophila***

According to the Bergey's Manual of Systematic Bacteriology (Popoff, 1984), the genus *Aeromonas* belongs to the family *Vibrionaceae* and includes two groups separated on the basis of optimal growth temperature and motility. One group is formed by psychrophilic, non-motile aeromonads, belonging to a single species, *A. salmonicida*, with three subspecies, *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, and *A. salmonicida* subsp. *masoucida*. The second group consists of mesophilic and motile bacteria, including three species, *A. hydrophila*, *A. caviae* and *A. sobria*. Although the taxonomy of mesophilic aeromonads has changed significantly from Popoff's scheme, the psychrophilic group has been more stable. To date, it has been proposed to move the aeromonads out from the *Vibrionaceae* to form a new family, the *Aeromonadaceae* (Colwell, *et al.*, 1986); the taxonomy of the now 11 recognized species of mesophilic aeromonads is still not resolved (Martínez-Murcia *et al.*, 1992); and a new subspecies of *A. salmonicida* has been proposed (Austin *et al.*, 1989; Beiland and Trust, 1988) to include the so-called "atypical" strains. *A. hydrophila* comprises gram-negative straight and short rods, motile by a single polar flagellum in liquid medium and sometimes peritrichous flagella on solid media. Its optimal growth temperature is 28°C. Its name means "water-loving" since this organism is commonly found in fresh water and sewage (Popoff, 1984).

In 1980, Mittal *et al.* (1980), reported a particular group of *A. hydrophila* strains that were highly virulent to salmonids. At that time, *A. hydrophila* had been regarded only as an opportunistic pathogen (Boulanger *et al.*, 1977; De Figueiredo and Plumb, 1977), residing free in the environment (Hazen *et al.*, 1978; Kaper *et al.*, 1981) or in the digestive tract of salmonids (Trust and Sparrow, 1974; Cahill, 1990b and references therein). However, the high virulence traits of this particular group indicated that some *A. hydrophila* isolates may behave as true primary pathogens. Pathogenic *A. hydrophila* cause hemorrhagic septicemic infections in fish, and gastrointestinal or systemic infections in other animal species including amphibians, reptiles, and mammals. Interestingly, certain *A. hydrophila* strains isolated from humans also display this high virulence phenotype (Dooley, *et al.*, 1985; Janda *et al.*, 1987), which significantly correlates with production of invasive infections (bacteremia and peritonitis) (Janda *et al.*, 1987). The highly virulent isolates (either from salmonids, mammals or humans) possess O-polysaccharide chains of homogeneous length (Dooley, *et al.*, 1985) that carry a thermostable serogroup-specific epitope, O:11

(Sakazaki, 1987), in turn associated with the presence of a paracrystalline surface layer (Dooley and Trust, 1988). The S-layer of the highly virulent *A. hydrophila* strains (particularly TF7) has been well characterized. In its relation with the O:11 specific O-polysaccharide chain it was shown that S-layer anchorage is O-polysaccharide-dependent and that a minimum LPS oligosaccharide size is required for anchoring the S-layer to the cell surface (Dooley and Trust, 1988; Kokka *et al.*, 1990). All the members of the high virulence group possess a similar tetragonally arrayed layer with a lattice constant of about 12 nm (Murray *et al.*, 1988). The 3-D structure of this array has been resolved (Al-Karadaghi *et al.*, 1988) showing that the S-layer subunits are bilobed proteins grouped around two different 4-fold symmetry axes with an  $M_4C_4$  arrangement (refer to Fig. 1) and a thickness of 5.6 nm. Dooley *et al.*, (1988; Dooley and Trust 1988) carried out the biochemical characterization of the S-layer subunit from strain TF7. This is a 52 kDa acidic protein, with 41% hydrophobic amino acids, a single isoelectric form (pI 4.6), and no cysteine. Very similar characteristics were also found for the S-layer protein of a strain (AH-342) involved in a case of human bacteraemia (Kokka *et al.*, 1992b). Although hydrophobic in itself, the S-layer protein does not confer hydrophobicity to *A. hydrophila* cells when present as an intact S-layer. The S-layer protein is composed by 44% beta-sheet, 19% alpha-helix, 12% beta-turn, and 25% aperiodic structure. The N-terminal amino acid sequence (first 30 residues) of the mature protein did not show any sequence similarity with other S-layer proteins (Dooley and Trust, 1988; Kokka *et al.*, 1992b).

Exploring the antigenic relatedness of the S-layers associated with the O:11 serotype, it was found that some S-layer proteins were antigenically diverse owing to differences in their primary sequence. Due to the complexity of this genus, this is not a surprising finding, and in relation to pathogenesis, it may represent an extra strategy of antigenic variation of S-layer-possessing *A. hydrophila* strains (Kostrzynska *et al.*, 1992). Establishment of the functions for the S-layer of pathogenic *A. hydrophila* has not been an easy task. The strongest direct evidence of its role in pathogenesis comes from recent experiments reported by Kokka *et al.* (1992b). S-layer negative ( $S^-$ ) strains were injected intraperitoneally, together with purified S-layer protein, in Swiss-Webster mice. Interestingly the LD<sub>50</sub> of  $S^-$  strains was reduced 30-70 fold, suggesting that the S-layer protein may play an important role in pathogenesis. The mechanism of this enhancing effect upon the ability to produce disease is not yet known. However, it was proposed that the S-layer subunits reconstituted an S-layer on the surface of  $S^-$  cells,

conferring to them some trait(s) necessary for systemic dissemination (Kokka *et al.*, 1992b). It is disappointing that, although clearly implicated in virulence and pathogenesis (Mittal *et al.*, 1980; Janda *et al.*, 1987; Ford and Thune, 1991; Kokka *et al.*, 1992b), a specific function for this well characterized S-layer of virulent *A. hydrophila*, has not as yet been unequivocally demonstrated (Kokka *et al.*, 1991).

### ***Aeromonas schubertii***

This is one of the 12 recognized species of the genus *Aeromonas* and belongs to the group of mesophilic motile aeromonads (Martínez-Murcia *et al.*, 1992 and references therein). Although different species of aeromonads possessing the O:11 serotype also possess an S-layer (e.g. *A. veronii*), *A. schubertii* is of particular interest because it causes systemic or wound infections. Significantly, although only a few strains have been isolated, none of them were isolated from the gastrointestinal tract and no animal or environmental isolates are known to exist. Kokka *et al.* (1992a) studied the 11 known strains of *A. schubertii* to determine their structural and pathogenic properties. All six strains belonging to the O:11 serogroup (but none of the strains containing LPS with O-chains of heterogeneous length) possessed a 55 kDa surface layer protein. One, out of three strains with truncated O-polysaccharide chains, secreted the S-layer protein into the medium, suggesting that, as described for *A. hydrophila*, anchoring of the S-layer to the cell surface is LPS-dependent and a minimum length of the O-polysaccharide chain is required for competent anchoring (Kokka *et al.* 1992a). Strangely, these authors did not report any ultrastructural or functional characterization of the S-layer.

### ***Bacillus anthracis***

The members of the genus *Bacillus* are distinguished from other gram-positive rods by their strict or facultative aerobic nature, and their ability to sporulate and produce catalase (Slepecky and Hemphill, 1992). *B. anthracis*, the causative agent of the fatal disease anthrax, possesses two well recognized virulence factors: a poly-D-glutamic acid capsule which inhibits phagocytosis (Green *et al.*, 1985) and a tripartite toxin (Leppa, 1988). The presence of a linear S-layer in this organism was early recognized in freeze-etched replicas (Holt and Leadbetter, 1969) where the S-layer appeared to be formed by strands of globular particles of 6-8 nm. Separation between strands and between particles was 7-10 nm. Analysis of a 2-D projection of the

negatively stained S-layer showed that it is organized in a p1 lattice (Doyle and Sonnenfeld, 1989). Although it is possible that two proteins designated as "extractable antigens" EA-I and EA-II (closely associated with the peptidoglycan layer) are the S-layer subunits (Doyle and Sonnenfeld, 1989), their identity has not been clarified, neither their function discerned.

### ***Bacillus cereus***

*B. cereus* has been recognized as an agent of food poisoning since 1955, causing true intoxications rather than infections. However, *B. cereus* has been implicated in multiple cases of a variety of infections (Farrar and Reboli, 1992), and according to Davey and Tauber (1987) this is one of the most destructive organisms to infect the eye. Since *B. cereus* produces potent exotoxins (responsible for much of the pathogenesis associated with this organism) emphasis has been placed on the study of such toxins. Thus the functions associated with its S-layer have not been characterized at all, and even structurally, very little is known about the S-layer of *B. cereus*. Holt and Leadbetter (1969) described it as a "globular layer", since in freeze-etched replicas it appeared as formed by globular units of 6-7 nm in size. Negative staining revealed more clearly a tetragonal array with a lattice spacing of approximately 10 nm, first described by Ellar and Lundgren (1967) on the ATCC strain 4342.

### ***Bacillus sphaericus* and *Bacillus thuringiensis***

In the course of sporulation, some strains of *Bacillus* synthesize a parasporal inclusion or "crystal", which may contain proteins toxic for larvae of a variety of insects. The two more prominent species of these entomopathogenic bacteria are *B. sphaericus* and *B. thuringiensis*. Commercial products (bioinsecticides), prepared from cultures of these motile *Bacillus* species, are available and they are expected to gain popularity since they do not have most of the environmentally deleterious effects of synthetic insecticides (Ignoffo and Anderson, 1979; Stahly *et al.*, 1992). Interestingly, both species have S-layers.

The S-layer of *B. thuringiensis* was described recently by Luckevich and Beveridge (1989). The three strains that they studied had structurally similar S-layers composed of a delicate oblique array. Optical diffraction of negatively stained specimens showed that the major lattice lines were spaced about 8.5 nm. Secondary lattice lines, running at 73° with respect to the major lines, were separated 7.2 nm. The

layer was 9 nm thick in thin sections. Shadowed preparations of shed S-layer fragments showed that the layer has an external smooth face and a rough face facing the cell wall. The purified S-layer protein was hydrophobic, and acidic (pI 5) with a molecular weight of 91,400, not glycosylated, and did not contain cysteine, methionine and tryptophan. Its N-terminal amino acid sequence was determined but no sequence similarity was found with any other S-layer protein. Increasing amounts of S-layer protein were found as cultures aged, and stationary phase cells shed large amounts of S-layer fragments. No functional characterization was attempted.

In contrast to the S-layer of *B. thuringiensis*, the S-layer of *B. sphaericus* was reported much earlier (Holt and Leadbetter, 1969). However, it was not until 1983 when it was discovered that the entomopathogenic strains of *B. sphaericus* had an S-layer different from that of non-pathogenic strains (Word *et al.*, 1983). The S-layer of non-pathogenic strains consists of a tetragonal array with a lattice constant of 13-13.5 nm, formed by a ~150 kDa protein. Interestingly, S-layer negative mutants have not been isolated, suggesting an indispensable role for this S-layer, also shown to constitute a bacteriophage receptor (Howard and Tipper, 1973). The S-layers from non-pathogenic *B. sphaericus* strains have been used to study the dynamic process of S-layer assembly during growth (Sleytr and Glauert, 1975; Howard *et al.*, 1982), to demonstrate the bilobal nature of the S-layer subunit and define the domains and bonds involved in array formation as well as anchorage to the cell wall (Hastie and Brinton, 1979a; Howard and Tipper, 1973; Lepault *et al.*, 1986), to study the specificity of S-layer reconstitution (Hastie and Brinton, 1979b), and the variation in fine structural detail between air-dried negatively stained and unstained frozen-hydrated specimens (Lepault and Pitt, 1984; Lepault *et al.*, 1986).

All the pathogenic strains of *B. sphaericus* possess oblique S-layers with a spacing of 5 nm and faint secondary linearity at about 27° (Lewis *et al.*, 1987). These cover the entire cell surface, and have the ability to reassemble *in vitro*. In spite of their structural homogeneity, it was found that S-layer proteins of pathogenic strains were antigenically heterogeneous. There was a perfect correlation between antibody typing and the type of peptide map obtained upon trypsin digestion, suggesting that antigenic variation was due to differences in primary amino acid sequence. However, all S-layer proteins shared many biochemical characteristics, all were glycosylated, had similar amino acid compositions, molecular masses (133-155 kDa) and isoelectric points (4.6-4.9). Interestingly, since the S-layers of pathogenic strains of *B. sphaericus* were

linear (as those of *B. thuringiensis*) whereas non-pathogenic strains possessed tetragonal arrays, a possible relationship between S-layer and pathogenicity was considered and further supported by the fact that a proteolytic degradation product of the S-layer protein of strain 2362 appeared to be toxic to insects. Preparations of the larvicidal crystal showed that it was formed by four antigenically related proteins, 125, 110, 63 and 43 kDa (Baumann *et al.*, 1985). Exciting results showed that upon completion of the exponential phase, and initiation of the sporulation process, a gradual decrease of the abundant 125 kDa protein was accompanied by a proportional increase of the 110 kDa protein. Since the latter proved to be toxic to mosquito larvae, but not as toxic as the 43 kDa protein, it was proposed that the 110 and the 43 kDa parasporal crystal components represented subsequent degradation products of the 125 kDa protoxin, each having increased toxicity against insect larvae (Broadwell and Baumann, 1986). Once the gene encoding the 125 kDa protein was cloned and sequenced, it was found that the 125 kDa was the precursor of the 122 kDa S-layer protein of strain 2362, and that the 125, 122 (S-layer protein) and 110 kDa proteins shared the same N-terminus amino acid sequence (Bowditch *et al.*, 1989). However, it was also demonstrated that no relationship existed between this group of proteins and the 43 kDa toxin, and the proposed relationship between S-layer and toxicity began to collapse. Now it is known that no relationship exists between S-layer and pathogenicity of *B. sphaericus*. The 122 and 110 kDa proteins were demonstrated not to be part of the parasporal crystal and the 110 kDa protein not to be toxic to mosquito larvae (Bowditch *et al.*, 1989; Baumann and Baumann, 1991; Baumann *et al.*, 1991). Therefore, we are left with another well characterized S-layer with no functional role yet assigned. Even the putative protective role of the S-layer against muramidases was not confirmed (Sára *et al.*, 1990).

### ***Bacteroides buccae*, *Bacteroides* spp., and other pathogens associated with periodontitis**

In the progression of the inflammatory periodontal disease, there is a clear succession of the resident microbiota from a predominantly gram-positive facultatively anaerobic one, to one that is almost exclusively gram-negative and anaerobic. A major component of this community comprises several *Bacteroides* species frequently found in association with apical periodontitis (infection of the jawbone). From these, five species have been shown to possess S-layers: *B. buccae*, *B. capillus*, *B. pentosaceus*

(Haapasalo *et al.*, 1985; Kornman and Holt, 1981), *B. heparinolyticus* (Okuda *et al.*, 1985), and *B. forsythus* (Tanner *et al.*, 1986). However, the S-layer is not usually the outermost layer on most of these bacteria since they also possess slime layers detected as an electron opaque material in specimens stained in the presence of tannic acid (Haapasalo *et al.*, 1985) or ruthenium red (Kornman and Holt, 1981). Moreover, *B. buccae* and *B. capillus* also possess pili (Haapasalo *et al.*, 1985).

Sjörger *et al.* (1985) showed that two different types of hexagonal S-layers are present on different strains of *B. buccae*, *B. capillus* and *B. pentosaceus*. The lattice constant of one S-layer is  $21.5 \pm 0.5$  nm and the other  $7.7 \pm 0.3$  nm. Although the two S-layer types can co-exist on the same supporting membrane, analysis by optical diffraction showed that they did not occur on top of each other (superimposed layers). In the same study (Sjörger *et al.*, 1985), the 3-D reconstruction of the 21.5 nm array was achieved. It consisted of a single morphological unit of 6-fold symmetry with a central pore of ~5 nm in diameter and six smaller pores (5 x 2.5 nm) surrounding it. In addition to these two S-layer types, cells of *B. buccae* have been shown to possess a hexagonally arranged periodic structure (Kerosuo *et al.*, 1987 & 1988b). This kind of array, formed by crystalline outer membrane proteins, also has been observed in other pathogenic bacteria like *Bordetella pertussis* (Kessel *et al.*, 1988) and other non-pathogenic species (reviewed in Messner and Sleytr, 1992). The relation between the 8 nm array of crystalline outer membrane proteins and the two S-layers is not known.

In the first report of Okuda *et al.* (1985) no characterization of the S-layer of *B. heparinolyticus* was attempted. Later, this was structurally characterized by Kerosuo *et al.* (1988b) as a thin electron dense layer surrounded by amorphous material and separated from the outer membrane by an electron dense, 20 nm gap. This layer has a hexagonal lattice with a spacing of about 20 nm. The structure of the S-layer from *B. forsythus* was also further characterized by Kerosuo (1988) as an oblique array of about 10 nm spacing and 10 nm height. Sometimes the array could be separated in single lines (ragged appearance), suggesting multiple subunit-subunit interactions; the interactions within lines being stronger than interactions between lines. Unfortunately, in these and other reports documenting the existence of S-layers on *Bacteroides* species no biochemical characterization was attempted and only in one case, that of *B. buccae*, was the role of the S-layer in mediating an interaction with leukocytes *in vitro* explored (Kerosuo *et al.*, 1990). Unfortunately, no S-layer deficient mutants were available and a specific role for the S-layer was not demonstrated. Moreover, some isolates studied

were apparently capsulated (K. Lounatmaa, personal communication) obscuring the role of the S-layer in adherence.

Another S-layer for which a functional role has been explored is that of *Wollinella recta*, an oral pathogen frequently implicated in mixed periodontal infections together with other flagellated, anaerobic gram-negatives: *Campylobacter concisus* and *Eikenella corrodens* (Lai *et al.*, 1981). From these organisms only *W. recta* clearly displayed an S-layer of hexagonal space symmetry and a lattice constant of about 20 nm. Dokland *et al.* (1988 & 1990), as well as Kerosuo *et al.* (1988a) further characterized the structure of the S-layer from *W. recta*. A close relationship between S-layer and outer membrane was found and a 3-D reconstruction was achieved (Dokland, 1988 & 1990), confirming the p6 symmetry and a lattice constant of 21 nm. The thickness of the layer was 15 nm (unusually thick) and it was formed by bilobed protein subunits in an M<sub>6</sub>C<sub>3</sub> arrangement (refer to Fig. 1). The six heavy domains around the 6-fold symmetry axis form a funnel-shaped massive unit that interact with adjacent units through the lighter domains at the 3-fold axis. Interestingly, a second periodic structure was present in negatively stained cell wall fragments. Its 2-D projection showed a p2 symmetry with unit cell vectors of 8.5 x 12.5 nm and a relative angle of 85°, and apparently it represented an alternate crystallization lattice of the same S-layer protein (Dokland, 1988). Comparing S-layer positive and S-layer negative strains of *W. recta* it was shown that the presence of S-layer correlated with the appearance of an acidic 142-154 kDa protein (Borinsky and Holt, 1990). The absence of S-layer also caused a 45-60% increase in bacterial adherence to a human gingival fibroblast cell line. Although clearly pointing to a potentially important function, this altered bacteria-host cell interaction in the absence of S-layer has not been further characterized. Interestingly, as previously indicated for *Aeromonas* species of the O:11 serotype, the LPS of *W. recta* possesses O-polysaccharide chains of rather homogeneous size containing 5-8 repeating units (Gillespie *et al.*, 1988). Moreover, this LPS had an unusual high content of rhamnose (88%).

Two other organisms associated with oral infections have been demonstrated to possess S-layers. These are *Campylobacter sputorum* and *Bacillus sp.* strain M3198. The former constitutes a case in which the presence of S-layer has only been documented in thin sections of the organism, since its fine structure could not be resolved by negative staining (Lai *et al.*, 1981). Interestingly (see below), the fine structure of the S-layer of another campylobacter, *Campylobacter fetus*, also has not

been resolved by negative staining. The strain M3198 is a peritrichously flagellated, facultatively anaerobic *Bacillus* species, related to *B. coagulans* and *B. circulans*. It was isolated from a root canal infection and found to have an oblique S-layer with 9.5 nm spacing between lines (Haapasalo *et al.*, 1988).

### ***Bacteroides nodosus***

This organism is the causal agent of ovine footrot disease (hoof infections of sheep and goats). It is an obligate anaerobic, gram-negative rod with an uncertain taxonomic generic position, that has been proposed to be moved out from the *Bacteroides* genus (Shah, 1992), together with many other bacteria previously classified as *Bacteroides* species (actually, most of the above mentioned oral pathogens are being reclassified into the genus *Prevotella*). The cell surface of this organism is very complex, owing to the presence of pili, capsule, unusual rod-like structures, a diffuse material associated with pili, polar rings and an S-layer (Every and Skerman, 1980). The S-layer is composed of subunits arranged in a hexagonal lattice with a spacing of 6-7 nm, clearly observed in negatively stained and freeze-etched specimens of different virulent strains. Although the role of surface structures in the virulence of *B. nodosus* was being investigated (Every and Skerman, 1980), no further findings have been reported.

### ***Campylobacter fetus***

The genus *Campylobacter* encompasses thirteen species of gram-negative, slender curved rods, motile by means of a single polar flagellum. Species of *Campylobacter* are asaccharolytic and produce their energy through respiration and metabolism of amino acids (Tenover and Fennell, 1992). *C. fetus*, the type species of the genus, is an important veterinary pathogen causing venereal genital tract infections in cattle that may lead to infertility and abortion. In humans infections are rare, but become systemic in compromised individuals. Although most cases reported in humans involve extra intestinal infections, *C. fetus* also causes acute diarrheal illness.

The first report on the existence of an S-layer on *C. fetus* was that of McCoy *et al.* (1975). The S-layer was evident in thin sections, and in contrast to other S-layers, a functional role as an antiphagocytic surface structure was readily recognized. Two types of lattice were evident in the micrographs of negatively stained specimens later reported by McCoy *et al.* (1976), but at that time this fact passed unconsidered. It took

several years to clarify the fact that indeed there are two different types of S-layer on *C. fetus* and to understand how they are formed. An oblique (possibly orthogonal) array, of about 9 nm spacing between lines, was reported by Dubreuil *et al.* (1988) and an hexagonal array with a lattice constant of 24 nm and a thickness of 10 nm was reported by Fujimoto *et al.* (1989). These observations relied on the freeze-etching technique owing to the fact that the *C. fetus* S-layer is reluctant to yield good negatively stained preparations. In a latter report (Dubreuil *et al.*, 1990) oblique (orthogonal) arrays were observed (not very clearly) by negative staining in all strains studied; both lattice vectors were spaced 5.6 nm at an angle of 75°, and the layer had a thickness of approximately 11 nm. The presence of two different S-layers on *C. fetus* was further clarified by a report showing that both lattices (linear and hexagonal) may be expressed simultaneously on the same cell and, very interestingly, that there was a strong correlation between M.W. of the S-layer protein and the type of lattice formed (Fujimoto *et al.*, 1991). High M.W. subunits formed orthogonal arrays (8 nm between lines) and low M.W. subunits, hexagonal arrays (24 nm lattice constant).

To make the structural features of the *C. fetus* S-layers even more fascinating, it was shown that there are two groups of S-layer proteins (with different amino-terminal amino acid sequences) and that each group exclusively interacts with a specific type of LPS O-chain. In reconstitution experiments, S-layer proteins belonging to the type A, were exclusively reconstituted onto cells with the type A LPS, and the same was true for type B LPS and type B S-layer proteins; type A LPS do not associate with type B S-layer subunits or vice versa. These results indicate a very specific subunit-LPS interaction and point to LPS as the anchorage molecule on the cell surface (Yang *et al.*, 1992). It is also known that divalent cations are required for proper subunit-wall interactions but not for subunit-subunit interactions (Yang *et al.*, 1992), that the LPS binding site resides in the conserved N-terminus of the S-layer protein (Yang *et al.*, 1992) and that naturally assembled S-layers cover the entire cell surface hiding the underlying LPS O-polysaccharide chains (Fogg *et al.*, 1990; McCoy *et al.*, 1975). Thus, available results (Dubreuil *et al.*, 1988 & 1990; Fujimoto *et al.*, 1989 & 1991; McCoy *et al.*, 1975 & 1976; Pei *et al.*, 1988; Winter *et al.*, 1978) acknowledge the existence of two groups of non-glycosylated bilobed S-layer proteins, each group containing proteins of different mass and antigenicities capable of forming S-layers with different lattice symmetry. The low mass S-layer subunits (94-100 kDa) form hexagonal arrays, whereas the higher mass subunits produce orthogonal arrays

irrespective of the group they belong to. Each cell of *C. fetus* may express more than one S-layer protein, simultaneously or sequentially in time, (Dubreuil *et al.*, 1990; Fujimoto *et al.*, 1991; Pei *et al.*, 1988) but these proteins would be of the type determined by the LPS type of that cell (Yang *et al.*, 1992). To date it is not known if *C. fetus* cells have the ability to express simultaneously different types of LPS, and/or S-layer proteins. In spite of this flexibility among *C. fetus* S-layer proteins, all of them are acidic proteins (group A being more acidic than group B proteins) with a similar amino acid composition typical of S-layer proteins (Dubreuil *et al.*, 1988; Messner and Sleytr, 1992; Pei *et al.*, 1988).

The gene *sapA*, encoding the S-layer protein of *C. fetus* strain 84-32, was cloned and sequenced (Blaser and Gotschlich, 1990). The first 20 amino acids encoded in the open reading frame of *sapA* match exactly the previously reported N-terminus amino acid sequence of the mature S-layer protein, indicating the absence of a leader peptide sequence. The C-terminus possesses a hydrophobic sequence (amino acids 672-689) that may function as a transmembrane spanning region and has homology with the leader sequence in a variety of pilins. Moreover, another internal hydrophobic region (amino acids 429-453) also showed some homology to signal sequences for other exported proteins (Blaser and Gotschlich, 1990). Although the recombinant protein was not exported in *E. coli*, the recovered product (upon cell lysis) was competent in reconstitution experiments (Yang *et al.*, 1992), suggesting that its folding in *E. coli* must be largely similar to that in *C. fetus*. In this respect, Dubreuil *et al.* (1988) showed that an S-layer protein from a different strain possessed 28% alpha-helix, 36% beta-sheet, 5% beta-turn and 31% aperiodic structure, and that the beta-sheet folding was quite stable.

In regard to functional aspects, it is encouraging to find out that specific virulence-related functions have been assigned to the *C. fetus* S-layer. As mentioned before, its role as an antiphagocytic "microcapsule" was early recognized (McCoy *et al.*, 1975). Later a correlation between serum resistance and the presence of surface layer proteins and type A LPS was found. Both the S-layer and type A LPS are necessary for full resistance to immune serum; some S-layer positive (S<sup>+</sup>) strains with type B LPS are serum sensitive, whereas no strains with type A LPS are serum sensitive (Blaser *et al.*, 1987; Pérez-Pérez *et al.*, 1986). These two functions (phagocytosis and serum resistance) have a common mechanism, both are the result of a failure in the complement fixation process, as determined by Blaser *et al.* (1988)

using two pairs of S<sup>+</sup>/S<sup>-</sup> strains. Although C3 consumption was similar between S<sup>+</sup> and S<sup>-</sup> strains, actual binding of C3 to S<sup>+</sup> cells was reduced and most of the bound C3 appeared in its inactive form iC3b, leading to a highly reduced C5-C9 fixation on the surface of S<sup>+</sup> strains. By using polymorphonuclear (PMN) phagocytes it was also shown that S<sup>+</sup> strains were resistant to PMN killing as a consequence of reduced phagocytosis, and this as a consequence of deficient opsonization. Opsonization with immune serum, but not with non-immune serum, immediately led to efficient internalization and killing, suggesting that internalization of *C. fetus* in PMN was mediated by Fc receptors rather than by complement receptors (Blaser *et al.*, 1988).

A series of experiments have demonstrated that the S-layer of *C. fetus* is an important virulence determinant. S<sup>-</sup> variants obtained by repeated subculture in the laboratory regained the S<sup>+</sup> phenotype after a single passage in a mouse, suggesting that the presence of an S-layer is important for growth *in vivo* (Fujimoto *et al.*, 1989). In a mouse model developed by Pei and Blaser (1990), the virulence of S<sup>+</sup> and S<sup>-</sup> strains was compared. The LD<sub>50</sub> of the S<sup>+</sup> strain was 43-fold lower than that of the S<sup>-</sup> strain. Bacteremia after an oral challenge was not detected for the S<sup>-</sup> strain, and the high-grade bacteremia detected for the S<sup>+</sup> strain was reduced 50-fold by an intraperitoneal (IP) injection of pre-immune serum raised against the purified S-layer protein. Finally, mice injected IP with a sublethal dose of S<sup>-</sup> cells or with high concentrations of purified S-layer protein, showed no mortality. In contrast, injection of S<sup>-</sup> cells mixed with S-layer protein at the same concentrations caused 30% mortality (Pei and Blaser, 1990). As discussed in the case of *A. hydrophila*, it is very likely that the S-layer protein reconstituted an S-layer on the surface of S<sup>-</sup> cells conferring them a trait necessary for pathogenesis. At the same time this last experiment showed that the S-layer protein by itself is non-toxic.

The last function of the *C. fetus* S-layer to be discussed is its antigenic variation. This characteristic makes it stand out as a unique S-layer among the ones studied to date. Dubreuil *et al.* (1990) reported three variants of the same strain with antigenically different S-layers, and showed that within each strain not all the cells reacted with the same anti-S-layer antibody. Since killing by PMN phagocytes was shown to depend upon opsonization with immune serum, S-layer antigenic variation may constitute an important function to avoid killing. Interestingly, in spite of this variation in antigenicity, that was shown to occur also *in vivo* (Schurig *et al.*, 1978), immunization with S-layer enriched extracts helped in eliminating infections from

female cattle infected with homologous and heterologous *C. fetus* strains (Schurig *et al.*, 1978).

### ***Chlamydia psittaci* and *Chlamydia trachomatis***

Members of the genus *Chlamydia* are obligate intracellular pathogens and belong to one of the three recognized species, *C. trachomatis*, *C. psittaci* and *C. pneumoniae* (the latter including the TWAR strains). These pleomorphic organisms, related to the gram-negative bacteria, have a unique biphasic developmental cycle that alternates between a spore-like, infectious, metabolically inactive form (the elementary body, EB), and a non-infectious, metabolically active, replicative form (the reticulate body, RB). Interestingly, these organisms apparently lack a peptidoglycan layer and therefore it is not clear how the cell rigidity is maintained to cope with osmotic stresses, although a series of cysteine-rich outer membrane proteins have been implicated in this function (Fields and Barnes, 1992). *C. trachomatis* causes the most frequent sexually transmitted disease in developed countries and it is also the causative agent of trachoma (the single most important cause of preventable blindness). *C. psittaci* is primarily an animal pathogen that may cause serious human respiratory infections. *C. pneumoniae* causes acute respiratory disease in humans (Fields and Barnes, 1992).

Electron microscopic observations of purified chlamydial cells and envelopes have shown very similar regular hexagonal arrays in *C. psittaci* (Matsumoto, 1973; Matsumoto and Manire, 1970) and *C. trachomatis* (Chang *et al.*, 1982) present in both the EB and the RB. For the latter species, a 3-D reconstruction was achieved showing that the array has a lattice constant of 17.5 nm, and it is formed by a single morphological unit, in turn composed of six subunits surrounding a central depression 10 nm in diameter and 8 nm deep (Chang *et al.*, 1982). This array was demonstrated to have two distinct faces, a rough inner surface and a smooth outer face; all apparent characteristics of an S-layer. Although in some recent reviews (Bavoil, 1990; Fields and Barnes, 1992) this putative S-layer has been associated with the major outer membrane protein (MOMP) its identity has not been clarified, nor its biochemistry defined. MOMP was first purified and characterized by Caldwell *et al.* (1981) who indicated that this is a very abundant ~40 kDa protein, which accounted for as much as 61% of the total envelope proteins. It has become the primary focus of vaccine-based research since it is capable of stimulating specific cell-mediated immunity, an essential response in protection against intracellular pathogens (Ishizaki *et al.*, 1992). Based on

the information currently available, it is not possible to establish any clear relationship between the MOMP (or any other chlamydial outer membrane protein) with the S-layer. Therefore, this S-layer still remains biochemically and functionally uncharacterized. It is possible that due to the lack of a typical gram-negative peptidoglycan layer, chlamydiae have developed S-layers similar to those on archaebacteria, which have been implicated in cell wall rigidity and integrity (Baumeister *et al.*, 1989). If this would hold true, then the S-layer protein may interact strongly with the outer membrane of chlamydiae or even traverse it (Baumeister *et al.*, 1989; Messner and Sleytr 1992), representing an S-layer with an important function in the lifestyle of these unique obligate intracellular pathogens.

### ***Clostridium difficile* and other clostridial pathogens**

The genus *Clostridium* represents one of the largest genera of the prokaryotes, comprising more than 100 species. It is formed by spore-forming, anaerobic, gram-positive rods, unable to reduce sulfate (Hippe *et al.*, 1992). Some members of this genus are considered pathogenic due to their ability to produce potent toxins that either necrotize tissues or interfere with nerve transmission (Smith, 1992). *C. difficile* could be considered a member of the normal human microflora that occasionally may cause pseudomembranous colitis, chronic diarrhea and necrotizing enterocolitis. The so-called CDAD (*C. difficile*-associated diarrhea) is a significant problem in many hospitals and chronic care facilities (Clabots *et al.*, 1992 and references therein). The pathogenic traits of *C. difficile* are mostly related to the production of an enterotoxin (toxin A) and a cytotoxin (toxin B).

In 1984, the presence of an S-layer was demonstrated on 10 strains of *C. difficile*. All S-layers were structurally related, consisting of a tetragonal array with a lattice constant of ~8.2 nm, but biochemically they were divided in two groups according to their composition: group-1, containing 32 and 45-47 kDa proteins and group-2, containing 38 and 42 kDa proteins (Kawata *et al.*, 1984). Takumi *et al.* (1987) characterized the 32 kDa protein from a group-1 strain (ATCC 11011). Surprisingly, the protein was readily extracted with phosphate-buffered saline washes, leaving the 45 kDa protein still associated with the cell surface. This constitutes a very interesting finding since, in a completely independent study by Dailey and Schloemer (1988), a series of recombinant *E. coli* clones were detected expressing a 32 kDa protein originally present in culture supernatants of *C. difficile*. Surprisingly, every

clone that contained the 32 kDa protein also contained a 43 kDa protein. However, a corresponding protein band for the cloned 43 kDa protein was not detected in cell-free lysates or culture supernatants of *C. difficile*. Although these two studies have not been linked experimentally, it is very tempting to speculate that the cloned DNA fragment contained the two S-layer protein genes, and that their genetic relatedness could be a reflection of a structural one. The contention that the S-layers of *C. difficile* comprise two different types of S-layer proteins has gained further credibility. Characterization of a group-2 S-layer (from strain GAI 4131) confirmed the presence of two major proteins of 38 and 42 kDa. These were not related since they produced different peptide maps upon proteolysis. The proteins were able to reassemble *in vitro* and form open cylinders possessing an array structurally similar to that observed in native S-layers. The *in vitro* reassembled cylinders were purified in a density gradient and shown to exclusively comprise the 38 and 42 kDa proteins (Masuda *et al.*, 1989). Highly purified proteins from a group-1 S-layer (from strain GAI 0714) were used to raise antibodies to the individual proteins. By using these antibodies it was shown that the two proteins were antigenically distinct and that both were evenly exposed on the cell surface (Takeoka *et al.*, 1991). Furthermore, now it is known that the two proteins (in a 1:1 ratio) are required for *in vitro* reassembly of the S-layer; none of the proteins by itself was able to form semicrystalline sheets. If the 1:1 ratio was changed to a 2:1 or a 1:2 ratio, the semicrystalline sheets were not formed; purified *in vitro* assembled sheets were shown to be exclusively composed of approximately equal amounts of the 32 and the 45 kDa proteins (Takumi *et al.*, 1991). A 3-D reconstruction would help to determine if the two types of subunits are part of the same or different lattices and what is their spatial relationship.

To date, S-layer proteins from the two groups have been purified and characterized, and the following is known: EGTA treatment disrupts the regular array and extracts the two proteins from isolated cell walls, suggesting the involvement of  $\text{Ca}^{2+}$  in the S-layer integrity (Masuda *et al.*, 1989). The two subunit types in each S-layer group are different, owing to differences in their peptide maps, immunoreactivity, N-terminal amino acid sequences, number of isoelectric forms, and susceptibility to PBS extraction. However, both proteins have similar amino acid compositions, characteristic of S-layer proteins (Hagiya *et al.*, 1992; Masuda *et al.*, 1989; Takeoka *et al.*, 1991; Takumi *et al.*, 1987 & 1991a). A direct role for the S-layer in adhesion was demonstrated by Takumi *et al.* (1991a). Fab fragments of antibodies raised against the

32 or the 45 kDa S-layer protein from strain GAI 0714 (group 1), caused 62 and 55% inhibition, respectively, in the adherence to HeLa cells. The combined use of both Fab fragments caused an 83% inhibition, indicating that both S-layer proteins play a part in mediating adherence to host cells. S-layer mediated adherence may be important in determining persistence in the intestine, but this functional role awaits further experimental demonstration.

Two other clostridia will be briefly considered here, *C. botulinum* and *C. tetani*. Both are well known pathogens, owing to the production of potent neurotoxins responsible for the pathogenesis of botulism and tetanus respectively (Smith, 1992; van Heyningen, 1992), and both are capable of growing in wound tissue. The S-layers of these two *Clostridium* species could be associated with tissue persistence, but most likely, S-layers are involved in survival of free cells in the soil environment. The S-layers have been visualized in thin sections and biochemically characterized, but no functional characterization has been attempted (Takumi *et al.*, 1983, 1991b & 1991c). Apparently, the S-layer of *C. tetani* is not associated with neurotoxicity, since toxigenic and non-toxigenic strains possessed S-layer (Takumi *et al.*, 1991c).

### ***Comamonas acidovorans* and other pathogens associated with suppurative otitis**

Only a single case report of an ear infection caused by *C. acidovorans* seems to exist in the literature (Reina *et al.*, 1991). Although this is not enough evidence to classify this organism among the bacterial pathogens, other cases of secretory otitis media, in which S-layer possessing bacteria were involved, have been reported (Lounatmaa, *et al.*, 1988). Thus, these S-layer possessing bacteria are very briefly considered here, as a potentially interesting group of pathogens. *C. acidovorans* does not possess (as could be the case with chlamydias) a *bona fide* S-layer. The regularly arrayed surface protein on this gram-negative bacterium (formerly classified as *Pseudomonas acidovorans*) is intimately associated with the outer membrane (Engelhardt *et al.*, 1991 and references therein). Three-dimensional reconstructions of the native surface array and the array obtained after *in vitro* crystallization, revealed an unusual, single morphological unit, composed of dimeric subunits and distributed around a p4 lattice with two different 4-fold axes and a spacing of 10.5 nm. No function is known for this regular surface protein, but the consequences of its tight

association with the outer membrane (in relation to the structure and function of the latter) are currently under investigation (Engelhardt *et al.*, 1991).

S-layer possessing isolates (as determined in thin sections) belonging to two species of gram-positive bacteria were obtained from a patient with secretory otitis media (Lounatmaa, *et al.*, 1988). These bacteria were identified as either *Peptostreptococcus magnus* or *Eubacterium sp.* The S-layer from two isolates of *Eubacterium sp.* (AHN 990 and ES4C) were further characterized structurally and shown to be constituted, respectively, by an hexagonal and a tetragonal array, for which 3-D reconstructions have been achieved (Hovmöller *et al.*, 1988b; Sjörgen, *et al.*, 1988). No functional characterization of these S-layers has been carried out.

### ***Corynebacterium diphtheriae***

Corynebacteria are gram-positive eubacteria with a high GC content to which the genera *Mycobacterium*, *Nocardia* and *Rhodococcus* are the closest relatives (Liebl, 1992). *C. diphtheriae* is considered a human pathogen, the causative agent of diphtheria. Pathogenicity is mainly due to the production of a potent toxin, but infections with nontoxigenic strains have been reported (von Graevenitz and Krech, 1992); suggesting the existence of virulence determinants involved in tissue persistence and host damage. Although not characterized functionally, the S-layers of this organism could constitute one of these determinants. Two types of tetragonal arrays were found on the cell envelope of *C. diphtheriae*, with lattice constants of 5.3 and 3.5 nm. Little is known about the S-layer proteins of *C. diphtheriae* except that they are not related to toxicity (Kawata and Masuda, 1972).

### ***Mycobacterium bovis***

Recently, Lounatmaa and Brander (1989) reported, for the first time, the occurrence of an S-layer with oblique lattice (5.5 nm spacing between lines) in several strains of the bacillus of Calmette and Guerin (attenuated *M. bovis*), used in the manufacturing of live vaccines against tuberculosis. The striking features of this S-layer are, first, that it has not been lost after so many years of *in vitro* subculture of these laboratory strains (implicating the existence of some selective pressure to keep it), and second, that it passed unrecognized in spite of the fact that tubercle bacilli have been studied for more than 100 years. Interestingly, Lounatmaa and Brander (1989) advanced some speculative arguments relating the S-layer with well known, highly

immunogenic surface antigens of the mycobacteria, implying the presence of S-layers in other species of the genus. It would be quite interesting to confirm that these findings and the latter hypothesis are actually true.

### ***Rickettsia* species**

The rickettsiales include several genera and their taxonomy is complex. They have a cell envelope characteristic of the gram-negative eubacteria, and with the exception of *Rochalimaea quintana* they are obligate intracellular pathogens (Dasch and Weiss, 1992). Here I deal mainly with species from two groups within the rickettsiae, the spotted fever group (represented by *Rickettsiae rickettsii*) and the typhus group (represented by *Rickettsiae typhi* and *Rickettsiae prowazekii*).

It was as a result of the early work of Palmer *et al.* (1974a & 1974b) that the rickettsiae were placed among those bacteria possessing S-layers. *R. prowazeki* and *R. akari* were shown to possess outer layers composed of tetragonally arrayed subunits with a lattice constant of about 13 nm and a depth of 7 nm. Once thought to be a capsule (Anacker *et al.*, 1967) this S-layer stains well with ruthenium red and is dissolved (losing all structure) by hypotonic shock, suggesting an acidic nature and the involvement of ionic interactions in the integrity of the layer (Dasch, 1981). Addition of divalent cations during washing prevented loss of the S-layer. Apparently, the S-layer or "microcapsule" (as it was designated then), was not the outermost structure in *R. prowazekii* or *R. rickettsii*, since these were shown to possess slime-like layers external to the S-layer (Silverman and Wisseman, 1978; Silverman *et al.*, 1978). Interestingly, it is now known that the slime-like layer is not glycosidic in nature, and it may represent polymerized host actin (G. A. Dasch, personal communication). The S-layer could be involved in actin polymerization but this remains to be experimentally determined.

The biochemical characterization (Ching *et al.*, 1990) of these S-layer proteins (or SPAs, for surface protein antigens) was not straightforward, owing to the unusual characteristics of these proteins. All the rickettsiae studied contain a ~120 kDa SPA detected by SDS-PAGE after boiling in SDS. After ion exchange HPLC of purified preparations of SPAs from different rickettsiae, a complex profile of several peaks was obtained, but surprisingly, each fraction contained SPA in high purity. Apparently this effect is due (at least in part) to formation of dimers, trimers and a diversity of higher polymers; all of them dissociated into monomeric SPA by reducing agents. These

results suggest that the polymeric SPA forms were formed by disulfide-linked subunits, an unusual property not reported for any other S-layer protein. The purified SPA is formed by 10% alpha-helix, 40-50% beta-sheet, and 40-50% aperiodic structure. The amino acid composition conforms with the general profile reported for S-layers (rich in acidic amino acids, 40-50% hydrophobic amino acids and a low content of methionine and cysteine). *R. canada* and all the spotted fever rickettsiae have a second, 130-200 kDa SPA (known as the large SPA, in contrast to the 120 kDa, or small SPA). Apparently, both SPA are part of a single S-layer, another unusual characteristic (shared by *Clostridium difficile*, see above) since most S-layers are formed by a single type of subunit (Baumeister et al, 1989; Koval, 1988; Messner and Sleytr, 1992). Both SPAs (small and large) seem to be genetically, biochemically and immunologically related, suggesting that they probably arose through duplication, deletion and/or recombination (Ching *et al.*, 1990). Both SPA are heat modifiable proteins. The low temperature form, which exists at temperatures lower than 56°C (<40°C for *R. typhi* and *R. prowazekii*), is 10-25 kDa lighter than the high temperature form.

The DNA sequence of the gene *spaP* encoding the small SPA of *R. prowazekii* has been determined. It contains an open reading frame of 4836 nucleotides, encoding a 169 kDa protein and no leader sequence (Carl *et al.*, 1990). The C-terminus is highly hydrophobic (potential transmembrane region) and contains many lysine residues (potential sites for cleavage). Since the C-terminus is evidently not present in the mature protein, this is presumably cleaved after serving as an anchoring site for SPA translocation, a similar mechanism to that proposed for the S-layer protein of *C. fetus*. No sequence homology was found with other S-layer proteins.

Immunologically, SPAs are the most intensely studied S-layer proteins so far. SPAs constitute good antigens for diagnosis as well as good protective immunogens (Dasch, 1981; Anacker *et al.*, 1986). Actually, the SPA from typhus group rickettsiae constitute the basis for immunity to typhus. Recombinant 155 kDa SPA is protective to mice (McDonald *et al.*, 1987) and guinea pigs (McDonald *et al.*, 1988a & b) and has been proposed as a vaccine. Monoclonal antibodies (MAbs) which react with the low temperature, but not high temperature, forms of both the small and large SPA have been found. These MAbs were protective. However, antibodies obtained against the heat denatured proteins, were not protective any more, suggesting that a critical heat-

modifiable site on the surface of the SPA is necessary for infectivity (Anacker *et al.*, 1987b; Li *et al.*, 1988).

Besides being highly immunogenic protective antigens, SPAs from the typhus rickettsiae specifically stimulate different classes of human lymphocytes that presumably play important roles in the protective cellular response against typhus. *R. typhi* and *R. prowazekii* induce *in vitro* production of antigen-specific antibodies by human peripheral blood mononuclear cells (PBMC). Response of PBMC to rickettsial antigens was specific, required *de novo* protein synthesis, was antigen-dependent, required pre-sensitization (e.g. natural infection *in vivo*), was T-cell dependent, and was subjected to T-cell suppression. T-cell suppressor cells were induced by pre-exposure to high concentrations of SPAs. Suppressor cell generation was inhibited by cycloheximide and suppressor cell function eliminated by radiation (Misiti and Dasch, 1985). *R. typhi* and *R. prowazekii* also induced *in vitro* formation of lymphokine-activated cytotoxic lymphocytes that efficiently killed infected target cells; non-infected cells were not killed. Supernatants from cytotoxic cells were not active, indicating a true cell-mediated process requiring contact. Preparations of PBMC from unsensitized individuals (not exposed to rickettsiae) were not induced to form killer cells (Carl and Dasch, 1986). CD-4<sup>+</sup> clones of human T cells also respond to SPA from *R. typhi* and produce interferon (IFN). These cells seem to be different from those reported by Misiti and Dasch (1985), involved in helping PBMC to produce antibodies, as inferred by the concentration of SPA used. Whereas high concentrations of SPAs induced T-cell suppressors that blocked antibody production, they still stimulated CD-4<sup>+</sup> T-cells for IFN production (Carl *et al.*, 1989). Regions of the SPA molecule, critical for T-cell recognition (T-cell epitopes) have been determined by challenging several T-cell clones with cyanogen bromide (CnBr) fragments from the SPA. Although most fragments elicited a response (indicating that T-cell epitopes are present along the entire length of the SPA), a particular fragment elicited a stronger response in all cases (Churilla *et al.*, 1990). These results are encouraging for the development of a recombinant vaccine.

Regarding the functional role of SPAs, the fact that antibodies raised against them are protective, is *per se* a strong support for their role in pathogenesis. Although the mechanism of protection has not been elucidated, it is known not to be the result of efficient opsonization, owing to the fact that antibodies against LPS efficiently opsonize the rickettsial cells but are not protective (Anacker *et al.*, 1987a). Therefore, protective antibodies must block another critical step in pathogenesis like binding to, or

penetration of non-infected host cells. There is a correlation between ultrastructural changes (loss of S-layer) and loss of virulence, as observed in inactivated rickettsiae living in starving wintering ticks. Reactivated rickettsiae (reactivated by feeding ticks or incubating them at 37°C) regained virulence and an integral S-layer (Hayes and Burgdorfer, 1982). Moreover, all pathogenic rickettsiae studied so far possess an S-layer, and the only non-pathogenic strain studied so far, lacks an S-layer (Hayes and Burgdorfer, 1982). Finally, two strains from different geographical areas which differ in virulence for guinea pigs, were found to differ in at least one epitope of the 120 kDa SPA (as detected by MAbs), while all other phenotypic and serological characteristics were very similar (Anacker *et al.*, 1987a).

### ***Treponema pallidum***

Members of the genus *Treponema* are helical, motile spirochetes (5-20 x 0.1-0.4 µm) with a gram-negative cell wall envelope. The recognized pathogenic species are *T. pallidum* (causative agent of syphilis), *T. carateum* (causative agent of pinta), *T. paraluisenculi* (causative agent of venereal spirochetosis in rabbits) and *T. hyodysenteriae* (causative agent of swine dysentery) (Miller *et al.*, 1992b). From these, a strain of *T. pallidum* has been reported to have an outer component of the cell envelope containing "polygonal" subunits (Jackson and Black, 1971). Besides this laconic evidence provided by the electron microscope, and in contrast with other treponemal S-layers (Masuda and Kawata, 1982, 1986 & 1987; Messner and Sleytr, 1992), nothing more is known about this S-layer.

As the reader may conclude after this exposition of cases, there is (with few notable exceptions) a general lack of knowledge about the biochemistry, immunology and functions of S-layers in pathogenic bacteria. Structurally, all common symmetries (p2, p4, and p6) are represented, although tetragonal and oblique arrays seem to predominate among pathogens. For those S-layer proteins with resolved amino acid composition and/or sequence, it has been confirmed that all of them are acidic proteins with no sequence homology between them, suggesting that convergent evolution rendered similar arrays formed by protein subunits of dissimilar genetic origin. Indeed, the study of S-layers from pathogenic bacteria may well be fruitful in providing an understanding of the biological significance of S-layers, as it has been demonstrated in the case of the fish pathogen *Aeromonas salmonicida*.

### ***Aeromonas salmonicida***

*A. salmonicida* is a well recognized fish pathogen, the causative agent of furunculosis and ulcerative diseases in salmonids, as well as erythrodermatitis, ulcerative diseases or septicemia in a variety of non-salmonid fish (McCarthy and Roberts, 1980; Ostland *et al.*, 1987; Trust, 1986). Udey and Fryer (1978) were the first to report the S-layer of *A. salmonicida*. They observed, in thin sections of virulent *A. salmonicida*, a distinct layer external to the outer membrane and named it the "A-layer" (for additional layer). Its invariable presence in virulent isolates, as well as the autoagglutination properties of the S-layer possessing isolates and their increased association with host cells, pointed to the now amply demonstrated importance of the A-layer as a virulence determinant. As it will be discussed in the following chapters, the A-layer is perhaps the best characterized (biochemically, genetically, structurally, and functionally) S-layer from a bacterial pathogen. The A-layer has contributed several landmarks to S-layer and bacterial pathogenesis research. The gene for the A-layer protein (the A-protein) is the first and only *A. salmonicida* gene to be sequenced to date (Chu *et al.*, 1991). The A-layer constitutes the first surface layer demonstrated to be essential for virulence, and to possess specific virulence functions (reviewed in chapter IV); the first S-layer capable of existing in alternate regular structural arrangements, and distinct functional organizational states (chapter III); the first S-layer for which a combined role of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , has been demonstrated in its structure (chapter III); the first S-layer involved in specific binding to laminin, fibronectin (Doig *et al.*, 1992) and collagen (Trust *et al.*, manuscript submitted); the first S-layer showing an ability to bind stoichiometric amounts of small host molecules like hemin (Kay *et al.*, 1985), and the first surface molecular arrangement reported in Gram negative eubacteria involved in non-immune binding of immunoglobulins (Phipps and Kay, 1988). Detailed characteristics of the A-layer will be presented, as explained in the following section, in chapters I and IV which are the introductory chapters to the two subjects in which I would like to present my experimental work: i) the structure and ii) the function, of the A-layer.

## **ORGANIZATIONAL PLAN FOR THIS DISSERTATION**

The experimental part of the present dissertation has been organized into chapters. Besides the "General Introduction", "General Discussion", and the "Literature Cited" sections, two major areas containing the chapters dealing with experimental studies have been assigned:

1. Structure of the A-layer, including an introductory chapter for this area (chapter I) and two chapters dealing with structural studies (chapters II and III).
2. Functions of the A-layer, comprising an introductory chapter for this area (chapter IV), and seven chapters dealing with functional studies (V, VI, VII, VIII, IX, X, and XI).

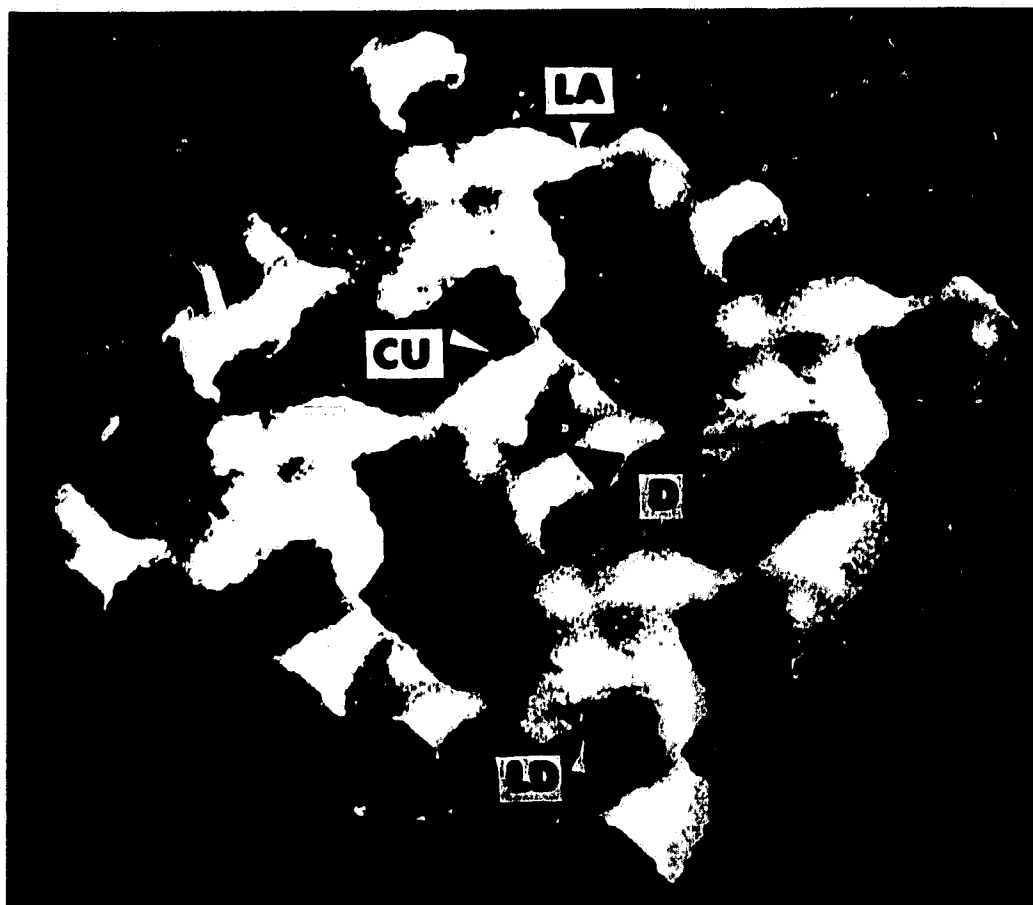
Each chapter contains its own Introduction, Results and Discussion sections. In regards to Materials and Methods, the two introductory chapters for the two major areas (i.e. chapter I for structure, and chapter IV for functions), contain, respectively, the Materials and Methods used in either the structural, or functional studies of the A-layer. Thus, those materials or experimental methods used to answer or solve more than one question or problem, are not repeated in several chapters.

## CHAPTER I

### STRUCTURE OF THE A-LAYER OF *Aeromonas salmonicida*

The A-layer is primarily composed of subunits of a ~50 000  $M_r$  single protein species, the A-protein (Kay *et al.*, 1981). The A-protein appears to be conserved among *A. salmonicida* isolates (typical or atypical) from different geographical locations (Phipps *et al.*, 1983; Kay *et al.*, 1984; Chu *et al.*, 1991). It contains a high proportion (45-47%) of hydrophobic amino acids (Chu *et al.*, 1991; Kay *et al.*, 1981; Kay *et al.*, 1984; Phipps *et al.*, 1983) that apparently concentrate at the outer surface of the arrayed layer, since it imparts high hydrophobicity to A-layer positive (A<sup>+</sup>) cells (Parker and Munn, 1984; Trust *et al.*, 1983; van Alstine *et al.*, 1986). The A-protein has a net negative charge and, on isolation, has been shown to exist in several isoelectric forms; it is not phosphorylated or glycosylated and is highly resistant to proteolytic degradation when assembled as an arrayed A-layer (Chu *et al.*, 1991; Phipps *et al.*, 1983).

As defined biochemically (Chu *et al.*, 1991) or structurally (by three-dimensional reconstruction) (Dooley *et al.*, 1989), the A-protein is bilobed, defined by a heavy major domain and a light minor domain. The bilobed A-protein subunits are organized into a p4 lattice with an M<sub>4</sub>C<sub>4</sub> arrangement (refer to Fig. 1 in the General Introduction), comprising two distinct 4-fold symmetry axes (Dooley *et al.*, 1989). Four A-protein heavy domains convene at the primary symmetry axis to form a funnel-shaped core mass unit with a prominent central depression, and four light A-protein domains convene at the secondary symmetry axis, to form the linker mass unit, to provide connectivity within the array (Fig. 2). Thus, each A-protein subunit contributes to one quarter of the respective core and linker mass units (Dooley *et al.*, 1989; Fig. 2). Upon negative staining, each of the mass units defines a morphological unit with a distinct and well characterized morphology (Stewart *et al.*, 1986; Stewart and Beveridge, 1988). It should be emphasized that the morphological units are the result of a contrast enhancing effect provided by the negative stain, and that each of these morphological units is the result of a defined pattern of stain displacement by each of the mass units of the A-layer.



**FIGURE 2.** Three-dimensional reconstruction of the A-layer. The A-layer is shown with its external face pointing to the observer. CU = Core (major) mass unit, D = Central depression in the core mass unit, LA = Linker arm of the A-layer protein subunit, LD = Mass unit formed by the linker (minor) domains. The depth of the layer shown (defined by the distance between the bottom part of the core mass unit and the highest point on the linker mass unit) is about 5.5 nm, and the lattice spacing (defined by the distance between the centers of two linker or core mass units) about 12.3 nm. Reconstruction adapted from Dooley (1988).

Genetically, the A-protein gene, *vapA*, for virulence array protein, revealed a single open reading frame of 1506 base pairs upon sequencing (Chu *et al.*, 1991). This open reading frame encodes a predicted 481-amino acid protein of 50,778  $M_r$  containing a typical 21-amino acid leader sequence for protein export. Belland and Trust (1987) previously reported that loss of A-protein expression in mutants selected for growth at high temperature (Ishiguro *et al.*, 1981; Ishiguro, 1988) was due to a genetic rearrangement within the A-protein gene that resulted in deletion of the N-terminal sequence. Sequencing of *vapA* (Chu *et al.*, 1991), revealed the existence of a 21-base pair direct repeat within the gene. This repeat sequence is separated by 795 bases and is most likely involved in the genetic rearrangement conveying a loss of A-protein expression, through looping and deletion of a 816-base pair fragment which includes one of the direct repeats. The genetically determined amino acid composition was in agreement with that determined biochemically (Evenberg and Lugtenberg, 1982; Kay *et al.*, 1981 & 1984), and followed the general composition of all S-layer proteins, i.e. high content of acidic and hydrophobic amino acids and a very low content of sulfur containing amino acids. From circular dichroism spectra the A-protein exhibited 14% alpha-helix, and 19-28% beta-sheet. This proportion changed slightly in the presence of sodium dodecyl sulfate (SDS); the alpha-structure increased to 29% at the expense of beta-structure (Phipps *et al.*, 1983). Secondary structure predictions from the primary amino acid sequence indicated 26.2% beta-structure (distributed in the first 424 amino acids), 24.5% alpha-structure (mainly confined to the last three quarters of the sequence), and 6.7% beta-turns (only present at both ends of the sequence) (Chu *et al.*, 1991). Curiously, the predicted alpha-structure (24.5%) was similar to that measured in the presence of SDS (29%). Other genetic studies (Belland and Trust, 1985) have shown that single transposon insertions may produce defects in the A-protein export process, leading to accumulation of A-protein in the periplasmic space. Now it is thought that most likely the A-protein has a unique export pathway, since these transposon insertion mutants which are unable to translocate A-protein across the outer membrane still export other proteins (T.J. Trust, personal communication). The validity of this hypothesis is currently being investigated (Noonan and Trust, 1993).

The A-layer entirely covers the *A. salmonicida* surface (Phipps *et al.*, 1983) masking underlying surface molecules. This could be one of the factors that contribute to the high hydrophobicity observed in A-layer positive ( $A^+$ ) cells of *A. salmonicida*, owing to the covering of most of the hydrophilic O-polysaccharide chains from

lipopolysaccharide (LPS) (Chart *et al.*, 1984). The layer requires a specific interaction with these *A. salmonicida* O-polysaccharide chains to remain anchored to the cell surface (Belland and Trust, 1985; Phipps and Kay, unpublished data). Interestingly, these O-chains, as those from the O:11 *A. hydrophila* LPS serotype associated with the presence of S-layer, are of an unusually homogeneous length (Chart *et al.*, 1984). O-chain deficient mutants produce assembled A-layer sheets (Dooley *et al.*, 1989) or free tetrameric units (Griffiths and Lynch, 1990) that are released into the culture medium. The relationship between A-layer and LPS O-chains appears to involve more than a mere structural interaction. Belland and Trust (1985) found that a mutant with a single Tn5 insertion lost the ability to produce both A-protein and LPS O-chains, suggesting that the synthesis of these two main surface determinants may be coordinately regulated. Also it has been observed that, upon subculturing, A-layer negative mutants easily lose their ability to produce O-polysaccharide chains, suggesting that the synthesis of high molecular weight LPS is a selective disadvantage in the absence of surface-anchored A-layer (Evenberg *et al.*, 1985).

The following two chapters will present the experimental work that led to the demonstration that the A-layer was structurally flexible and plastic. Although the A-layer usually exists in a single type of lattice structure capable of adopting different conformations (chapter II), under certain environmental conditions it may form alternate lattice patterns with functional relevance (chapter III). Also, it will be shown that the integrity and plasticity of the A-layer structure is partially maintained by Ca<sup>2+</sup> and Mg<sup>2+</sup> bridges, and that it is possible to isolate naturally occurring mutants with defects in the A-layer assembly process (chapter III).

## **MATERIALS AND METHODS USED IN STRUCTURAL STUDIES**

### **Bacterial strains**

*A. salmonicida* **A450** is a wild type, virulent, A-layer positive (A<sup>+</sup>), LPS O-polysaccharide positive (O<sup>+</sup>) strain. This strain was isolated in France by C. Michel from brown trout with the original denomination TG72/78. *A. salmonicida* **A450-1** is a spontaneous O<sup>-</sup>, A-layer secreting mutant, isolated from A450 by serial subculture. This strain produces assembled A-layers that are released into the culture supernatant as squared or rectangular double layered sheets (Dooley *et al.*, 1989) of various sizes (not

longer than 500 nm); phenotypically an A<sup>-</sup> strain. *A. salmonicida* A451-70 is also a spontaneous O<sup>-</sup>, A-layer secreting mutant, isolated from the exoprotease- and hemolysin-deficient strain A451-25. *A. salmonicida* A450-10S is a slow-growing, aminoglycoside-resistant, attenuated mutant isolated by J.C. Thornton (Thornton *et al.*, 1991). *A. salmonicida* A450-10SR is an attenuated, rapidly growing pseudorevertant from A450-10S (Thornton *et al.*, 1991).

### **Growth conditions**

Bacteria were routinely grown overnight in trypticase soy broth (TSB) (BBL), a calcium replete medium containing 173  $\mu\text{M}$  Ca<sup>2+</sup>, or for 3 days on plates of trypticase soy agar (TSA) (BBL). A450-10S was routinely grown on TSA plates with 500  $\mu\text{g}$  of streptomycin/ml until the appearance of visible colonies. Luria-Bertani (LB) broth with EDTA or EGTA at a final concentration of 5, 10, 50 or 100 mM, was also used. A minimal medium (MM) containing 0.5  $\mu\text{M}$  Ca<sup>2+</sup>, or fish peptone medium (FPM) containing 7.5  $\mu\text{M}$  Ca<sup>2+</sup> were used to grow bacteria under calcium limitation. The calcium levels of the different media were determined by atomic absorption spectroscopy. MM consisted of 0.4% glucose, and the L-amino acids glycine, alanine, valine, threonine, cysteine, methionine and histidine (each at a final concentration of 0.1 mg/ml) in basal salts solution (in g/l: NaCl, 6.0; K<sub>2</sub>HPO<sub>4</sub>, 7.15; KH<sub>2</sub>PO<sub>4</sub>, 3.0; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.78; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.008; FeSO<sub>4</sub>·2H<sub>2</sub>O, 0.005). FPM consisted of 0.4% glucose and 0.2% peptone P 0100 (desalted fish peptone from Marine Biochemicals a.s., Norway) in modified minimal Davis salts (in g/l: K<sub>2</sub>HPO<sub>4</sub>, 5.23; KH<sub>2</sub>PO<sub>4</sub>, 2.2; Na<sub>2</sub>HPO<sub>4</sub>, 4.26; NaH<sub>2</sub>PO<sub>4</sub>, 1.98; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1). Bacteria were grown in MM for 5 to 7 days and in FPM for 24 to 48 h. All incubations were done at 20°C, and bacteria were harvested as actively growing cells.

### **Electron microscopy (EM)**

#### **Negative staining**

All electron microscopy specimens were mounted on formvar coated copper grids and negatively stained by floating grids on drops of an unbuffered saturated solution of ammonium molybdate in deionized water (Sleytr *et al.*, 1988a; Stewart *et al.*, 1986). Cells in suspension were mounted by placing 5  $\mu\text{l}$  of the bacterial suspension on a grid, allowing 1 to 5 min. for cells to settle and adhere to the film. The remaining

suspension was then removed from the grid with a piece of filter paper. Specimens were also mounted by directly touching, with the coated side of a copper grid, the surface of an A450 isolated colony. After staining grids were air dried and observed in an EM 300 Phillips Transmission Electron Microscope, using an accelerating voltage of 80 kV.

### **Immunogold labeling of thin sections for transmission EM**

Bacterial cells were processed following the general recommendations of Beesley (1989). Briefly, cells were fixed at room temperature for 2 h., in filtered 4% freshly depolymerized paraformaldehyde and 0.1% glutaraldehyde in phosphate buffered saline (PBS) (NaCl 8 g, KCl 0.2 g, KH<sub>2</sub>PO<sub>4</sub> 0.2 g, Na<sub>2</sub>HPO<sub>4</sub> 1.15 g, pH 7.4). Fixed cells were pelleted and enrobed in 0.3% agarose in PBS prior to dehydration in a graded series of ethanol, and final embedding in LR White resin (London Resin, Surrey, England). Embedding was conducted in straight LR White overnight at room temperature, followed by two 2 h. changes of fresh resin. Blocks were hardened at 60°C for 20-24 h., under a nitrogen atmosphere. Thin sections (50-60 nm) were cut in a Reichert ultramicrotome and mounted on nickel grids. Post-embedding labeling was carried out using a modification of the procedure of DeMey (1983). Mounted sections were blocked with 1% powdered skim milk in IG buffer (20 mM TRIS, 150 mM NaCl, pH 8.2 containing bovine serum albumin to a final concentration of 0.1%), washed in IG buffer and floated on drops of polyclonal rabbit anti-A-protein serum diluted 1:500 in IG buffer. After an overnight incubation at 4°C, sections were washed 3X in IG buffer, and floated, for one hour at room temperature, on a 10 nm protein A-gold conjugate probe (Janssen Biotech, NV, Olen, Belgium) diluted 1:50 in IG buffer. Three final washes in IG buffer preceded a final fixation of the labeled sections on drops of 4% glutaraldehyde in IG buffer, before staining with 2% aqueous uranyl acetate pH 4.5, and 0.2% neutralized lead citrate. The anti-A-protein serum was provided by J. Winchester, Department of Biochemistry and Microbiology, University of Victoria, Canada.

### **Computer simulation**

A model of the A-layer p4 symmetry array was drawn in a Macintosh II computer using the program Macdraft™. This model was drawn using the subunit sizes and the mass distribution of the A-layer tetrameric morphological unit, according to Dooley *et al* (1989). Each tetrameric unit was joined to neighbour units in a type II

array, with a theoretical lattice constant of approximately 14 nm, and displaying the hand normally seen in actual micrographs. This array was copied to produce a second identical array which was then superimposed to the first one in the four possible in-register forms.

Negative staining was simulated by drawing the spaces between tetrameric units (pores) and filling them in black. The staining of the tetrameric units themselves was based on the processed images of negatively stained A-layers reported by Stewart *et al* (1986) and Stewart and Beveridge (1988). Due to the A-layer topography, the putative inner surface of the linker domains appears as four white lobes when facing the observer, and the core unit is always stained in the center due to the depression created by the association of the four more massive A-protein domains into a funnel shaped core (Dooley *et al*, 1989). Finally, it is known that the putative inner face is more sculptured than the outer one (Dooley *et al*, 1989) and therefore, staining of units that present this face to the observer is predominant. I carefully considered these structural features in my simulation. Arrays of "negatively stained" units and spaces, as well as their corresponding superimpositions were generated by the computer as described above.

### **DOC extraction method for A-protein**

A-layers are co-isolated with outer membranes (OM) when these are prepared by extraction with sodium lauryl sarcosinate (Kay *et al.*, 1981; Phipps *et al.*, 1983; Trust *et al.*, 1980). OM prepared in this way were subjected to serial extraction with sodium deoxycholate (DOC) as follows: 4.5 ml of an OM preparation, 4.0 ml of 10 mg/ml lysozyme, and 31.5 ml of 20 mM TRIS pH 8.0 with 5 mM EDTA, were mixed and agitated for 30 min. at room temperature (21 to 25°C). Forty ml of 4% DOC in 0.5 M NaCl with 5 mM EDTA were added and the preparation was incubated for 30 min. at 30°C. The extracted preparation was separated by centrifugation at 10000 X g for 10 min. at room temperature, and washed with 20 mM phosphate buffer, pH 7.3. The DOC extraction steps were serially repeated up to 4 times (procedure carried out by J. Winchester, Department of Biochemistry and Microbiology, University of Victoria, Canada).

### **Treatment of whole cells or purified A-layers with EDTA or EGTA**

A450 cells grown in TSB were separated by centrifugation and resuspended in 100 mM HEPES, pH 7.0, with 25 or 500 mM EDTA or EGTA (D/G buffer). Cells were incubated at room temperature for up to 5 h. Alternatively, blocks of TSA, each containing a single isolated colony of A450, were immersed in D/G buffer, and incubated at room temperature for up to 5 h. Also, whole cells or purified A-layers were mounted on copper grids and observed. Clean, isolated reference sloughs of A-layer were localized and micrographs taken. Grids were then removed from the microscope and floated on drops of D/G buffer, deionized water, or 100 mM HEPES, pH 7, for up to 4 h. After treatment, reference sloughs were re-localized and photographed.

### **Treatment of altered A-layers with divalent cations**

A450 was grown in FPM with 5 mM EGTA. Cells were separated by centrifugation and resuspended to the original volume in 50 mM imidazole buffered saline, pH 7.0, containing  $\text{Ca}^{2+}$ , or  $\text{Sr}^{2+}$  or  $\text{Mg}^{2+}$ , to a final concentration of 25 mM, or  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , or  $\text{Sr}^{2+}$  and  $\text{Mg}^{2+}$ , each at a final concentration of 12.5 mM. Plain imidazole buffered saline was used to resuspend control cells. Cell suspensions were incubated at room temperature for a minimum of 3 hours.

### **Image processing**

Electron micrograph images were analyzed by densitometry with an Eikonix Model 1412 camera system (Eikonix Corporation), as arrays of 1024 X 1024 or 1536 X 1536 pixels, at a pixel size of 15.1  $\mu\text{m}$ , corresponding to 0.44 nm at the specimen level. Image processing was performed using the SEMPER system (Saxton *et al.*, 1979). Image subframes were extracted, appropriately masked to isolate areas of projected A-layer, and subjected to correlation averaging (Saxton and Baumeister, 1982) (Procedure carried out by B.M. Phipps, at the Max Plank Institute for Biochemistry, Martinsreid, Germany).

## CHAPTER II

### A SINGLE STRUCTURAL TYPE IN NORMAL A-LAYERS

#### INTRODUCTION

*Aeromonas salmonicida* appears to be a prolific exporter of a variety of large cellulytic proteins which are also required for effective pathogenesis (Ellis *et al*, 1981; Fyfe *et al*, 1987, Price *et al*, 1989). S-layers of mesophilic eubacteria would permit, on average, free passage for molecules of approximate molecular weights of 10,000-15,000 (Sleytr *et al*, 1989). Thermophilic eubacteria and archaeobacteria seem to have S-layers with larger pores, but even some of the most porous among these arrays should have a molecular weight cut-off of less than 37,000 (Baumeister *et al*, 1989). It is not apparent then, how such an efficient protein export apparatus could function in *A. salmonicida*, in view of the presence of the contiguous A-layer (Kay *et al*, 1981; Phipps *et al.*, 1983). One suggestion has been that this layer is capable of adopting an alternate conformation with enhanced porosity (Stewart *et al*, 1986). Evidence for this unusual behavior was the discovery of two morphologically distinct patterns, called type I (with an associated closed conformation) and type II (with an associated open conformation), revealed by image enhancement techniques in two dimensional (2-D) mass distribution projections of negatively stained A-layer preparations (Stewart *et al*, 1986; Stewart and Beveridge, 1988). Subsequently, a three dimensional (3-D) reconstruction of the type II pattern was accomplished by Dooley *et al* (1989). The released A-layer sheets that were used in such reconstruction consisted of two identical A-layers, in opposite orientation, superimposed in perfect register, and with a type I-like 2-D projection (Dooley *et al*, 1989). This suggested the possibility that type I patterns may be formed from superimposed A-layers. Therefore, this possibility was examined and it was concluded that the type I pattern occurs routinely as an artifact of superimposition and staining.

## MATERIALS AND METHODS

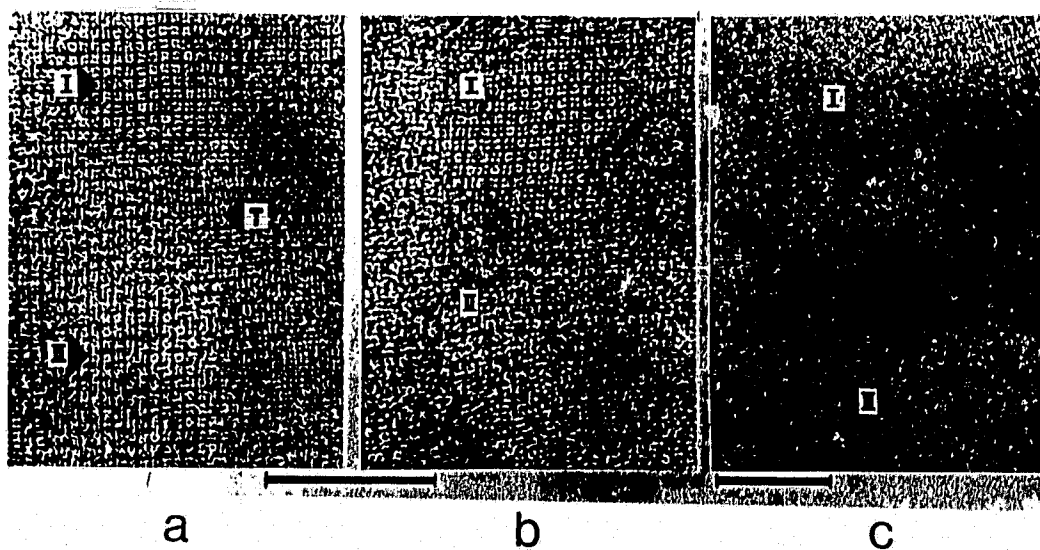
Refer to chapter I.

## RESULTS AND DISCUSSION

In well-contrasted micrographs of negatively stained preparations the two morphological A-layer patterns, type I and type II, are easily distinguished (Fig. 3). The non-handed type I morphology is more prominent, and it is not difficult to see that it is composed of two alternating, distinct morphological units. Type II morphology resembles a weaved basket work and is handed. When the two patterns are found together on a darkly stained area, there is a transition pattern between them (Fig. 3a). As noted by Stewart *et al* (1986), there is a continuity of the lattice lines, of both neighbour patterns, all across the transition zone. This transition zone is not usually present in between lightly stained type II patterns and perfect, dark type I patterns (Fig. 3b).

Invariably, the lightest zones of A-layer sloughs always showed a typical type II pattern, whereas darker zones displayed type I patterns (Fig. 3b and c). However, it was possible to occasionally observe type II patterns in dark zones, as in Fig. 3a. In other words, type I patterns were exclusive of darkly stained areas whereas type II patterns were found on lightly or darkly stained areas. However, type II patterns (either on light or dark areas) were always observed to be of the same hand, an observation previously reported (Stewart *et al*, 1986), suggesting that the A-layer preferentially attaches to the formvar film by only one of its faces or sides.

During preparation of negatively stained specimens, large portions of film-bound A-layer are presumably torn from whole *A. salmonicida* cells by physical stress, leaving a slough of A-layer presented to the observer by its inner face, as shown in Fig. 4a (sequence 1-2). The type II 3-D reconstructed model reported by Dooley *et al*, (1989), was used to discern the layer side involved in binding (i.e. the outer side of the layer). A simple comparison of the hand of its 2-D projections with that of actual micrographs was sufficient to determine that the A-layer interacts with the supporting film by the linker mass units, confirming that the side defined by the more massive core mass units is the inner side of the layer, the one in contact with the cell surface.

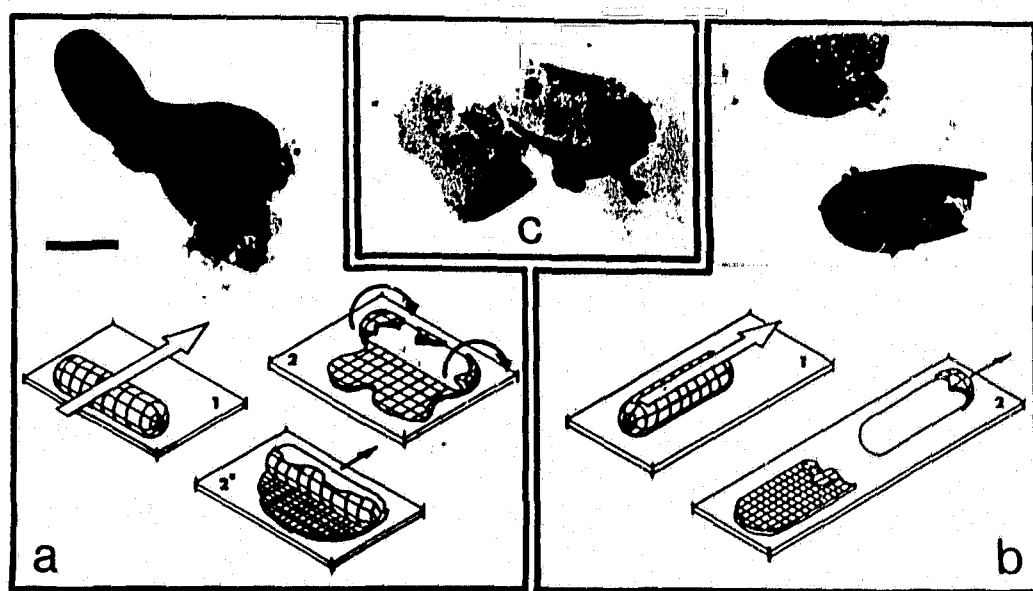


**FIGURE 3.** Electron micrographs showing the two morphologies in which negatively stained A-layer sloughs may appear. Notice the presence of a transition zone when neighbour type I and type II patterns are both darkly stained (a), as well as its absence when the type II pattern is lightly stained (b). (c) Normally, type I patterns appear darkly stained on a lightly stained background of type II patterns. I = Type I pattern, II = Type II pattern, T = Transition zone between patterns. Bars represent 0.1  $\mu\text{m}$ .

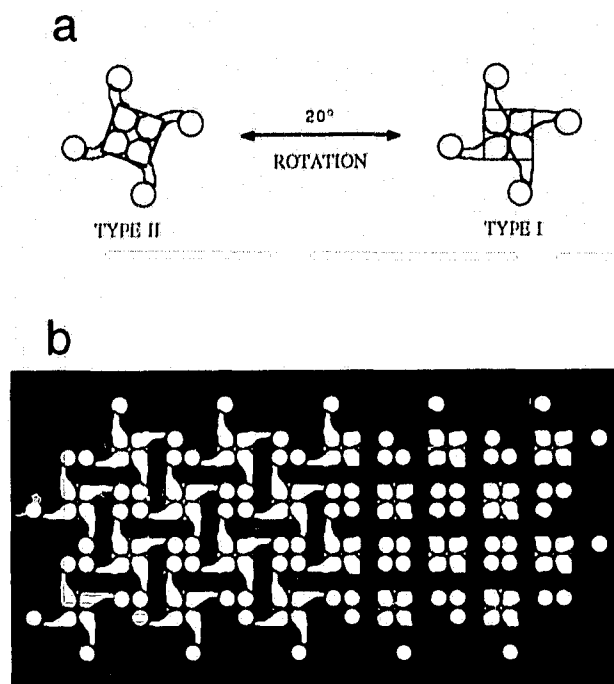
During sloughing, or during sac formation (Fig. 4a, sequence 1-2\*, and Fig. 4b) A-layers have abundant opportunity to bind to each other, fold over or upon themselves, and to present a superimposed structure to the observer. When this occurs the superimposed layers invariably appear darker than single layers (Fig. 4c). Type I patterns always showed up on darkly stained areas, therefore their appearance was naturally suspected to be associated with superimposition of layers. However, superimposition would also have to account for the appearance of type II patterns in dark zones and their conserved handedness, even though type II patterns were only occasionally observed in these zones.

It is at first reasonable to conceive of the existence of two A-layer conformations which could be readily arrived at, by a  $20^\circ$  rotational change in the arrangement of the tetrameric morphological units (Stewart *et al*, 1986, and illustrated in Fig. 5a); however on closer inspection, it can be seen that it is impossible to obtain a non-handed type I pattern this way (left side of Fig. 5b), without resorting to removal of the linker arms between the core and linker domains (right side of Fig. 5b). The accumulation of stain between two superimposed layers would easily account for the selective disappearance of linker arms. To test this hypothesis I artificially produced different superimpositions of two interacting type II patterns. Assuming that one of the interacting layers is fixed to the supporting film by the linker domains, two type II layers could form the four different in-phase interactions depicted in Fig. 6. Interaction (a) produced enhanced type II patterns, as those observed on darkly stained areas; interaction (b), a type I-like pattern with a single morphological unit, very similar to the one observed in Fig. 3a; and interaction (c) produced perfect type I patterns, as those in Fig. 3b and c. In Fig. 6d, the parallel running linker arms could accumulate some stain, revealing a type I-like image just as that produced in the right hand side of Fig. 5b. It has not been possible to determine if all interactions normally occur in A-layer preparations. However, Fig. 3a provides evidence for the existence of interactions (a) and (b), and Dooley *et al* (1989) observed a superimposition identical to interaction (c) which was negatively stained as a perfect type I pattern.

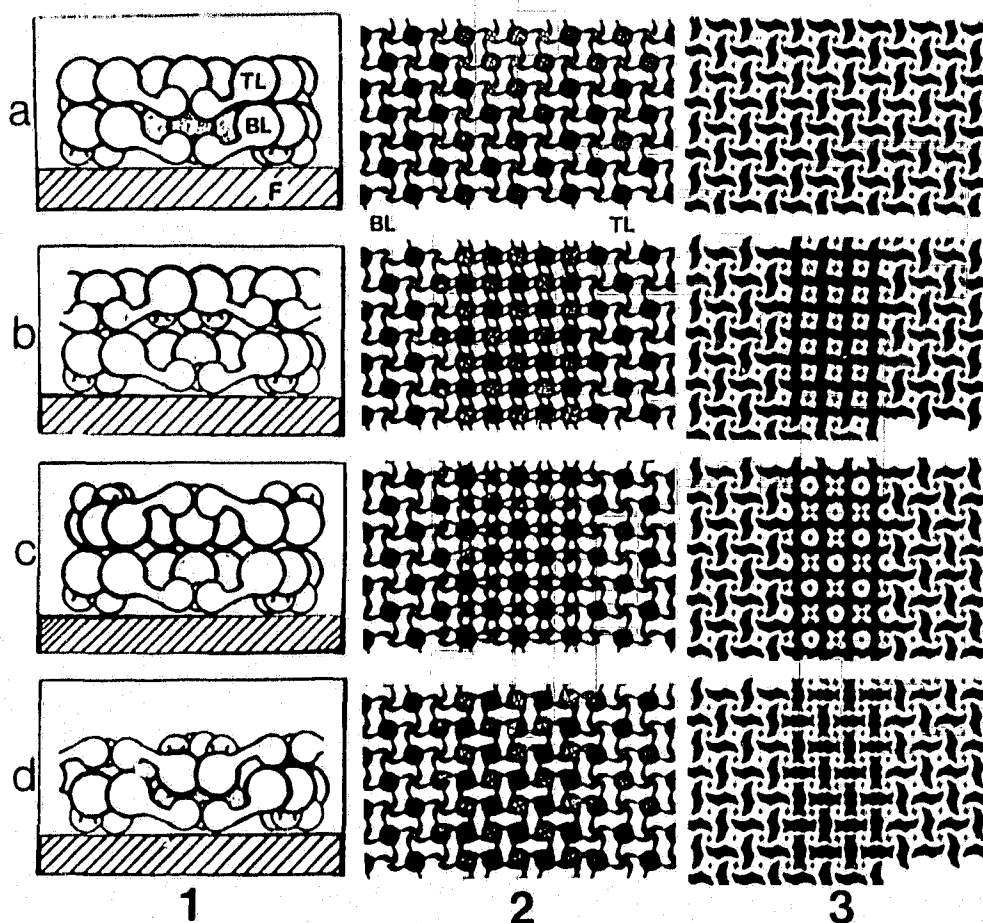
Superimpositions not only account for the occurrence of both patterns in darkly stained zones, as well as for the hand preservation in those superimpositions that produce type II patterns, but also predict the prevalence of type I patterns in them.



**FIGURE 4.** Explanatory model of how cells may shed their A-layers. A-layer sloughs (sequence 1-2 in "a"), lateral sacs (sequence 1-2\* in "a"), or terminal sacs (b) are usually formed. The top electron micrographs show the actual appearance of the negatively stained structures whose formation is represented in the drawings. Large white arrows indicate the direction of physical stress applied to the cell. Small black arrows indicate the proposed movement of the cell as a result of the physical stress applied. (c) Electron micrograph of a negatively stained, broken and folded sac, sloughed from an *A. salmonicida* cell. Notice how the superimposition of layers increases the darkness of the image. Bar represents 1.0  $\mu\text{m}$  for all micrographs.



**FIGURE 5.** Handedness of the A-layer lattice arrangement. (a). Schematic models of A-protein tetrameric units, from type II and type I patterns, showing the  $20^\circ$  rotational change required to go from one pattern to the other. Fig. III.3b is a drawing of an array of type I tetrameric units, according to the morphology presented in (a). Notice that this array still keeps its handedness (left side). In the right side of Fig. III.3b the linker arms of the array have been erased (selectively stained) to produce a type I-like pattern.

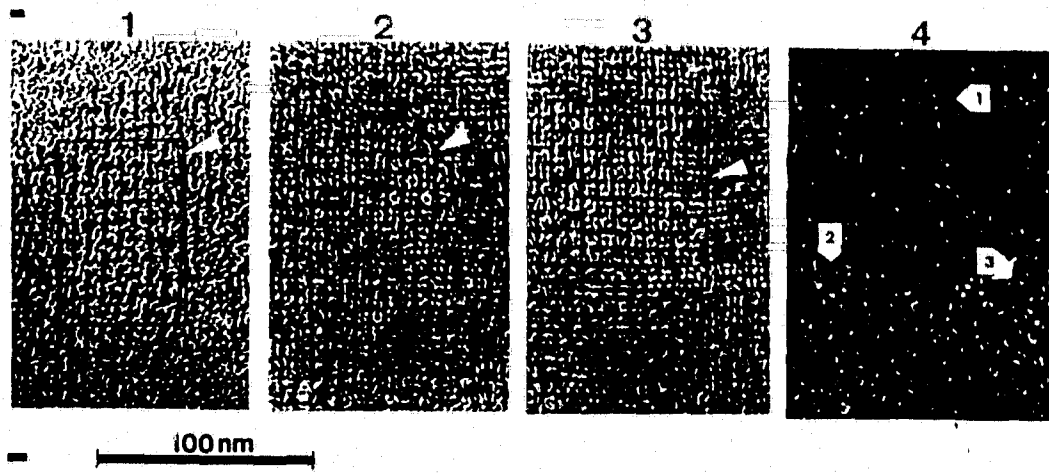


**FIGURE 6.** A-layer morphological types produced by superimposition and differential staining. Superimposition of two type II A-layers in four (a, b, c and d) of the eight possible in-phase interactions are shown. The bottom layer is fixed to the supporting film by the linker domains. Column 1 shows side views of the proposed A-protein subunit arrangements of the two interacting layers. Column 2 represents a top view of the two interacting layers. Layers actually interact only in the central zone. External zones represent single layers (bottom layer in black and top one in grey) in order to appreciate their orientation and the interacting mass units (cores or linkers). Column 3 is the simulated negative staining of the corresponding interacting layers depicted in column 2. Tetrameric units now appear in white and the stain filled pores in black. For details on negative stain simulation refer to "Materials and methods used in structural studies", in chapter II. BL = Bottom layer, F = Supporting formvar film, TL = Top layer.

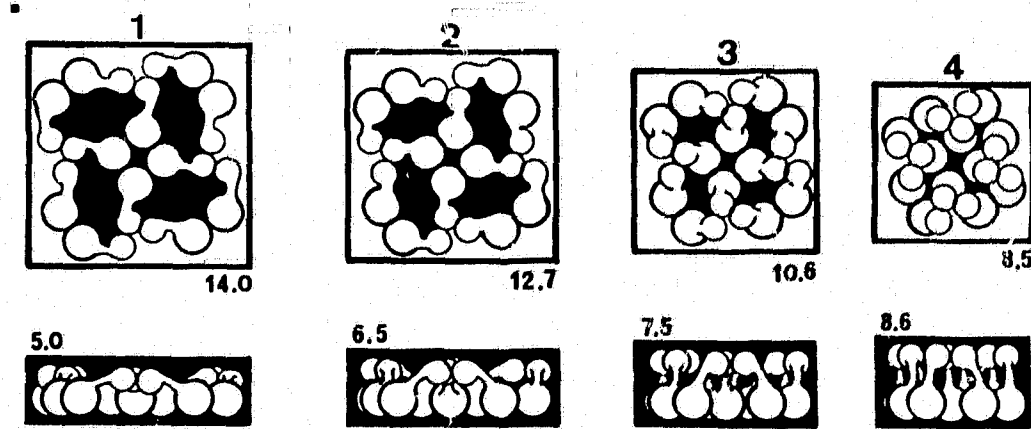
From modeling, 75% of the in-phase superimpositions give type I patterns (Fig. 6).

Some support for the legitimate existence of both type I and II patterns is the observed difference in their lattice constants (Stewart *et al.*, 1986). However, frequent lattice constant variations were found within both patterns (Fig. 7), and others (Dooley *et al.*, 1989) also have reported large variations in the spacing of type II patterns, which indicates that it is not reliable to exclusively associate a pattern with a specific lattice spacing. Nevertheless, it still remains true that smaller lattice constants prevail in type I patterns. Variations in lattice constant could be explained as a flattening artifact produced either by the traumatic process of layer detachment during slough formation (Bingle *et al.*, 1987) and/or adherence to the formvar film and air drying (Sleytr *et al.*, 1988, and references within; Wildhaber *et al.*, 1985). As presented in Fig. 8, an increase in lattice spacing would be necessarily accompanied by a proportional decrease in the depth of the layer. Presumably due to closer packing, double layers which produce type I patterns would more efficiently resist flattening, accounting for the smaller spacing associated with them.

It seems that confusion has been introduced by applying the same nomenclature to two disparate situations. The terms type I and type II were originally used to describe two morphological types of the A-layer in the context of negative staining. Unfortunately, the application of these terms has been generalized to include the description of two different structural conformations (open and closed, respectively) that could be adopted by the same morphological type, and not necessarily involving a structural change of the lattice array. The explanation that the two morphological units in the A-layer are formed by a single polypeptide chain partially found in one morphological unit and partially in the other (introduced by Stewart *et al.*, 1986), is in agreement with the data provided by the 3-D reconstruction analysis by Dooley *et al.* (1989). I advanced this concept in figures 5a, 6, and 8; where each tetrameric morphological unit is formed by four A-protein subunit domains, the core mass unit being formed by the more massive domains, and the linker mass unit by the lighter domains (Dooley *et al.*, 1989). The linker arms, which connect both the two A-protein domains and the two different morphological units, are strong determinants of the handedness of such structural arrangement. Therefore, if type I and type II patterns are two legitimate conformations of a single layer, both having similar morphological units (Stewart and Beveridge, 1988), one should not expect a loss of handedness in the type



**FIGURE 7.** Lattice constant variation in A-layer sloughs. Negatively stained type II (micrographs 1, 2 and 3) and type I patterns (micrograph 4), with different lattice constants. As measured on these micrographs the average lattice constants of the highlighted areas are: 1=12.6 nm, 2=10.9 nm, 3=10.3 nm, 4.1=9.6 nm, 4.2=10.6 nm, 4.3=11.6 nm. It is important to note that spacing variations occur in both morphological patterns.



**FIGURE 8.** Modelling of the lattice constant variations in A-layer sloughs. Top and side views of arrangements of A-protein tetrameric units in arrays with different lattice constant. The quantitative relationship between pore size, lattice constant and depth of the layer is evident. Numbers under the squares showing top views, indicate the theoretical lattice constant of each array (in nm). Numbers on the rectangles showing side views, indicate the depth of each array (in nm). The size of the A-protein subunit used to construct these arrangements was taken from Dooley *et al* (1989).

II to type I transition (Fig. 5). From a strict structural point of view, both patterns should remain handed unless i) one of the two morphological units dissociates, or ii) A-protein subunits suffer a major conformational change and the linker arms virtually disappear. The first alternative [which happens upon removal of divalent cations from the layer (refer to chapter III)] renders a very slightly handed pattern, but with a single morphological unit. Because type I and type II patterns are constituted by two distinct morphological units, this alternative is ruled out. The second alternative would require an unlikely gross modification of the A-protein molecule which has been amply demonstrated to be unusually stable under a variety of conditions (Phipps *et al* 1983; Phipps, 1988). Therefore, the most feasible possibility is one that strictly does not involve any structural change in the lattice array, i.e. distinct staining of two superimposed layers. This accounts for the preservation of lattice structure, as well as the two distinct morphological units in both patterns. Therefore, the type II pattern is the only A-layer structural type (that can adopt a closed or an open conformation) in normal A-layers, and type I pattern is an artifact of superimposition and distinct staining. I propose the use of the terms type I and type II to exclusively describe the two *morphological patterns* that appear in negatively stained preparations, and the open and closed nomenclature to describe the possible *different conformations* that layers of a single structural type (i.e. type II) can adopt.

Because it is certain that open conformations predominate in released material, and closed conformations in association with a normal cell surface, it has been suggested that type II patterns only occur in released material such as A-layer sloughs, cell ghosts, or A-layer sheets released by lipopolysaccharide (LPS) deficient mutants; and that perhaps type I patterns are the final conformer resulting from LPS associations on a normal cell surface (Dooley *et al*, 1989). This possibility cannot be rigorously excluded at this point. Nevertheless, based on experimental measurements of the A-layer thickness, that will be discussed below, the model depicted in Fig. 8 predicts that, in association with intact cells, an A-layer should have a fairly deep conformation, (Fig. 8-3). This type II deep conformation, with a predicted lattice constant of 10.6 nm, would have been previously associated with a type I pattern (i.e. a closed conformation). Interestingly, this predicted lattice spacing is in agreement with experimental measurements: 8.8-11.1 nm (Stewart *et al*, 1986) or  $11.0 \pm 0.2$  nm (Dooley *et al*, 1989). I therefore propose that, either in association with the normal cell surface or with sloughed material, the A-layer has a single structural type that acquires a

closed conformation (with increased depth) when associated with intact cell surfaces, and a more open conformation (with decreased depth) when sloughed from the cell surface.

Finally, it is relevant to consider the possible existence of double layers *in vivo*, in association with an intact cell surface. As demonstrated by Dooley *et al* (1989), free A-layers released into the culture medium by LPS-defective strains readily form stable sheets (consisting of two exactly superimposed layers) that can not be easily separated. However, these free layers typically interact through their inner faces which in intact cells would eliminate the polarity of the layer, presumably an undesirable circumstance *in vivo*. Although the double layer interactions (which preserve polarity) illustrated in Fig. 6a and b could theoretically occur *in vivo*, there is abundant evidence to the contrary: the measured depth of a single layer from its 3-D reconstruction (Dooley *et al*, 1989), the measured depth of the A-layer in freeze fractured specimens (Stewart *et al*, 1986) or in thin sections of intact chemically- or cryo-fixed *A. salmonicida* cells (Graham *et al*, 1991; Ishiguro *et al*, 1981), as well as theoretical calculations of A-protein yield and disposition (Phipps *et al*, 1983); all of which supports the contention that native A-layer exists *in vivo* as a *monolayer* of A-protein subunits. As mentioned before, this monolayer should have a fairly deep conformation of approximately 8 nm.

With a single closed *in vivo* A-layer conformation, it is now even more perplexing to satisfactorily account for the copious production of extracellular proteins by this bacterium. One possible explanation is enzyme release via outer membrane vesicularization seen in negatively stained as well as in thin sectioned whole *A. salmonicida* cells (not shown). Alternatively, enzymes may escape through lattice defects in the A-layer. Lastly, there is a distinct possibility that cells which express an A-layer do not, in reality, export proteins beyond it. The extracellular proteins commonly found in supernatants of liquid *in vitro* cultures, or the variety of enzymes and aggressins which diffuse in agar plates of differential media (giving clear halos or lytic zones), might have been released from lysed cells. Protein export beyond the A-layer remains to be rigorously shown.

## CHAPTER III

### NOVEL STRUCTURAL PATTERNS IN DIVALENT CATION-DEPLETED A-LAYERS, A- LAYERS FROM ENERGY STARVED CELLS OR A-LAYERS FROM MUTANTS DEFECTIVE IN A- LAYER ASSEMBLY

#### INTRODUCTION

Knowing that the A-layer is somewhat flexible, possessing a single type of lattice capable of adopting closed or open conformations, it was important to determine how the integrity of this flexible structure is maintained. Previous observations by Phipps (1988) suggested the involvement of calcium in either the formation of arrays or in attachment of the A-layer to the cell surface.

Extraction of S-layer proteins by chelating divalent cations, replacing a divalent cation with a non-functional substitute, or displacing divalent cations with a high concentration of monovalent cations indicate the involvement of divalent cations in the structure of S-layers and point, in some cases, to the specificity of this requirement (Beveridge, 1979; Beveridge and Murray, 1976b, 1976c; Koval and Murray, 1983; Thorne *et al.*, 1975; Watson and Remsen, 1969). Moreover, reassembly of monomers into S-layers on cell wall templates is often dependent on the presence of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  or  $\text{Sr}^{2+}$  (Austin and Murray, 1990; Beveridge and Murray, 1976a, 1976b; Bingle *et al.*, 1984; Buckmire and Murray, 1973; Kist and Murray, 1984; Thorne *et al.*, 1975; Tsuboi *et al.*, 1982), providing further evidence for the role of these cations in the assembly of S-layers. Accordingly, the prevalence of cation-mediated bonding in S-layers is consistent with the acidic nature of S-layer proteins. Specifically, the role of calcium in the assembly and organization of some S-layers has been well documented for *Aquaspirillum serpens* VHA (Buckmire and Murray, 1976; Kist and Murray, 1984; Koval and Murray, 1985), other *Aquaspirillum* species (Beveridge and Murray, 1976a, 1976b, 1976c) and *Azotobacter vinelandii* (Bingle *et al.*, 1984, 1986, 1987a; Doran *et al.*, 1987).

This chapter presents and discusses the experimental results obtained by studying the role of divalent cations in A-layer integrity. Also, the possible mechanisms involved in the formation of novel structural A-layer patterns, which resulted from energy or  $\text{Ca}^{2+}$  limitation, or  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  extraction with chelating agents, are discussed.

## **MATERIALS AND METHODS**

Refer to chapter I.

## **RESULTS**

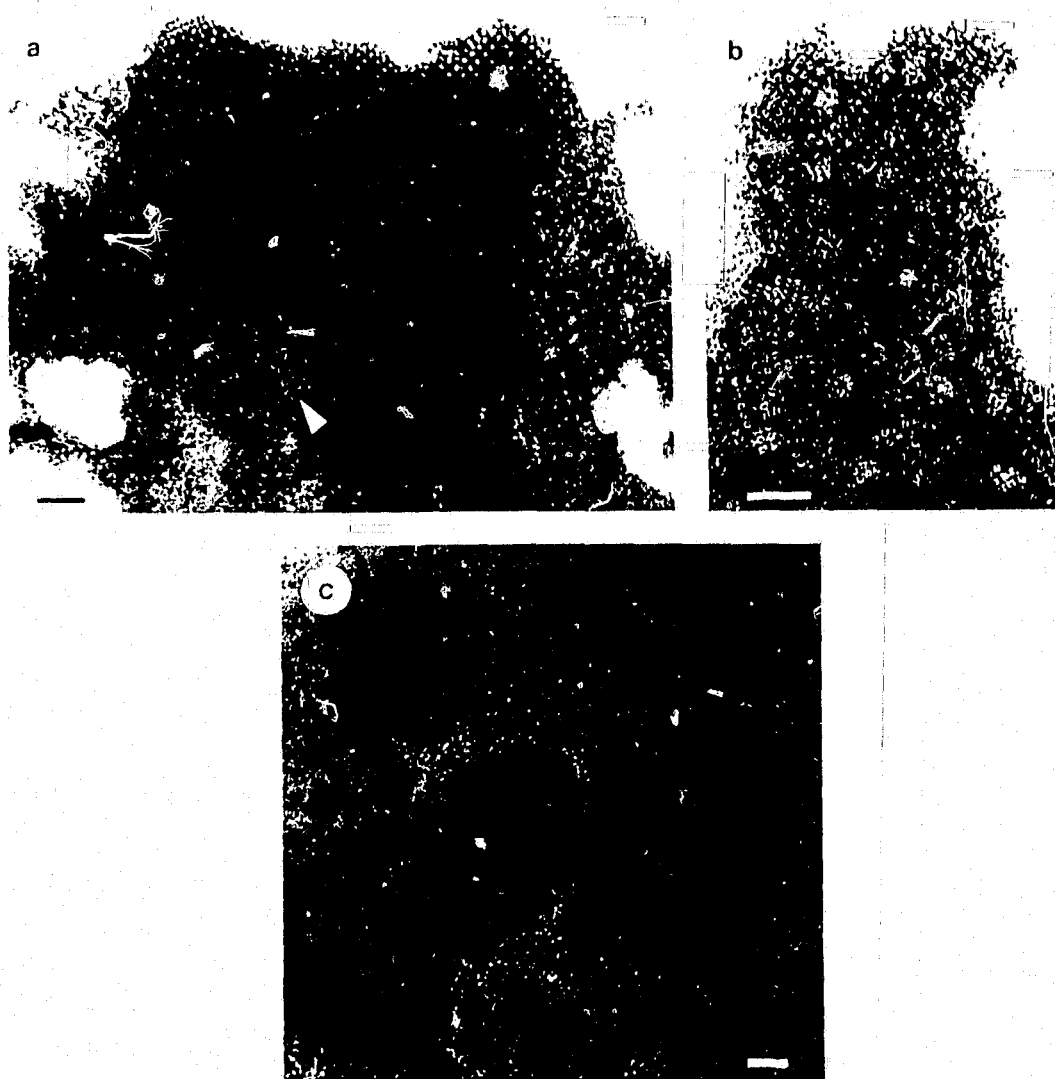
### **Review of biochemical studies on A-protein subunit interactions**

A-protein can be isolated in monomeric form, from whole cells, by treatment with 0.2 M glycine-HCl, pH 2.15 at 0°C (Phipps, 1988; Phipps and Kay, 1988). A 0.2 M alanine-HCl buffer of the same pH was equally effective indicating that pH, and not some specific effect of the glycine itself, was responsible for releasing A-protein. Extraction at pH 2.3 gave less than one third of the A-protein yield obtained at pH 2.15, and at pH 2.6, virtually no extraction took place, indicating that this process had a sharp pH cut-off. It was consistently observed that 5 to 20% of the total A-protein could not be released from cells by low pH extraction, even after a prolonged exposure (Phipps, 1988; Phipps and Kay, 1988).

Using a series of chemical extractions on outer membranes prepared by sarcosinate extraction, Phipps (1988) also demonstrated that the positively charged chaotropic agent guanidine-HCl, at a concentration of 6M, released A-protein from OM preparations nearly quantitatively (as estimated by SDS-PAGE), whereas the non-charged chaotrope urea, was totally ineffective (Phipps, 1988). Moreover, A-layer was relatively resistant to extraction with a variety of non-ionic or cationic detergents (both temperature and detergent concentration being important), ruling out the existence of hydrophobic interactions as the main force responsible for A-layer integrity (Phipps, 1988). Full solubilization of A-protein could be achieved by treatment with 0.5-1.8%

SDS at 60°C, which also solubilized most OM proteins with the exception of porin ( $M_r$  42,000). Outer membrane preparations extracted with DOC (provided by J. Winchester, Department of Biochemistry and Microbiology, University of Victoria, Canada) contained, upon inspection by electron microscopy (EM), numerous fragments of intact A-layer and what appeared to be free ring-shaped units (Fig. 9). The number of intact fragments decreased in proportion to the number of extractions and a proportional increase in the amount of free ring-shaped units was observed (Fig. 9a). Also, in DOC-extracted layers, I observed areas displaying a different morphological pattern, apparently formed by the grouping of free ring shaped units (Fig. 9b). This pattern was called "BS" (for "big squares").

Concentrating (on ultrafiltration membranes) cell-free supernatants from cultures of the A451-70 strain (containing free A-layer sheets), Phipps (1988) showed that the secreted A-layers formed small clear sheets, similar to cellophane sheets in appearance, which fractured, upon drying, into small pieces (~1 mm in length and variable width) with a characteristic curvature in one direction. These appeared to be semicrystalline in nature as they were rigid, refractive and tended to fracture along straight lines when subjected to physical stress. In EM specimens prepared from sonicated semicrystalline sheets I observed a substantial amount of arrayed A-layer fragments (Fig. 9c), suggesting that these semicrystalline sheets were composed of stacked A-layers. Crystallization only occurred at pH 7 or higher, and was also culture medium dependent. Medium dependence was assumed to be related to the presence of calcium, since casamino acids (derived from casein) were likely to contain the calcium level of TSB. Desalting of medium prior to concentration prevented crystallization, clearly indicating the involvement of ions in the process. Purified A450 A-protein also formed semicrystalline sheets (constituted by arrayed layers) when it was mixed with fresh casamino acids medium and concentrated, indicating that A-protein has the ability to self-assemble *in vitro* in the absence of a cell template or other *A. salmonicida* secreted factor. On the other hand, extensive concentration of purified A-protein in the divalent cation-deficient TEPA buffer induced precipitation of A-protein into an amorphous mass, suggesting that divalent, or other, cations were necessary for array and semicrystalline sheet formation.



**FIGURE 9.** Regular arrays found in detergent-extracted A-layers, or in semicrystalline sheets formed upon concentration of A-protein on ultrafiltration membranes. (a) Normal A-layer fragments and ring-shaped units in a preparation extracted three times with sodium deoxycholate (DOC). (b) Big squares pattern in a preparation extracted once with DOC. (c) One of the many large fragments of A-layer found in a preparation of sonicated semicrystalline sheets. Bars represent 50 nm in (a) and (b), and 0.1  $\mu\text{m}$  in (c).

### **EDTA or EGTA altered the A-layer structure**

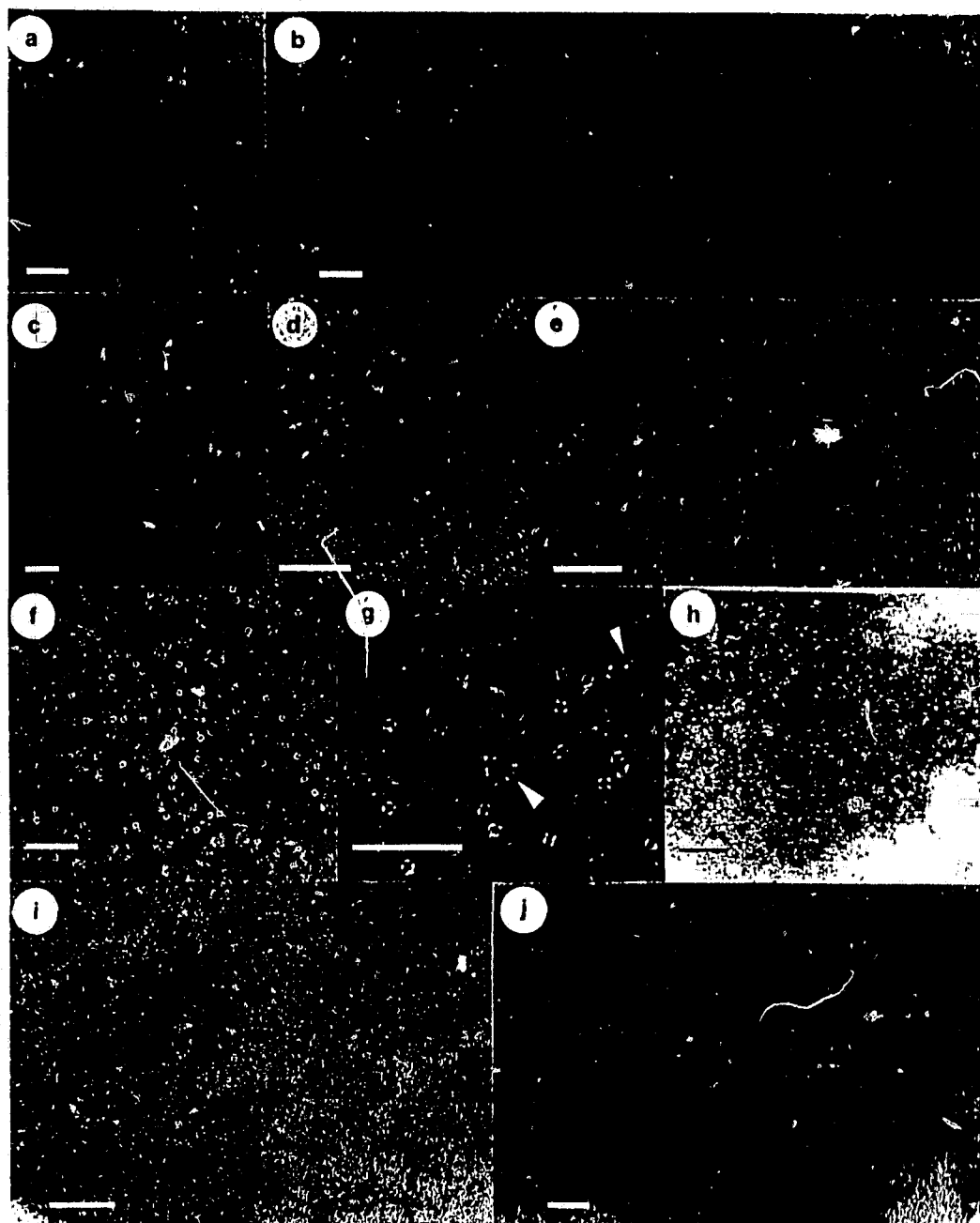
Extraction with 20 mM EDTA was ineffective in removing A-protein from OM (Phipps, 1988). Even when OM preparations were extensively dialyzed against 10 mM EDTA or EGTA in TEPA at 4°C and examined by SDS-PAGE, all the A-protein remained associated with the OM pellet (not shown). The possibility that this OM-associated A-protein existed in a disorganized state was investigated through electron microscopy.

The A-layer of cells treated with 25 mM EDTA showed areas with a different morphological pattern that I called "WD" (for "white dots"). The normal tetragonal array was not totally lost in these areas, but a second regular pattern, formed by rounded units which displaced the stain, was observed running at 45° with respect to the orientation of the normal array (Fig. 10a). Some other small areas displayed the BS pattern, first observed in the A-layers prepared by DOC extraction. Specimens treated with deionized water or HEPES buffer alone possessed normal A-layers. Direct treatment of *A. salmonicida* colonies with 25 mM EGTA rendered more distinguishable BS patterns (Fig. 10c). Treatment of colonies with 0.5 M EDTA or EGTA, produced the same effects but to a greater extent, suggesting a concentration-dependent process. Interestingly, in specimens treated with 0.5 M EGTA, it was also possible to distinguish free ring-shaped units, similar to those first observed in the A-layers prepared by DOC extraction. I called these structures "FR units" (for "free ring-shaped units"). The number of FR units in purified A-layer preparations increased upon treatment with 0.5 M EGTA, and were clearly visible (Fig. 10f). Layers bound to formvar films prior to treatment with 0.5 M EDTA or EGTA, showed no change at all, indicating that physically constrained layers were incapable of forming altered patterns.

Following EDTA or EGTA treatments, the A-layer remained cell-associated and interestingly, never showed extensive structural modifications. Therefore, the effect of calcium limitation on growing cells was investigated as an alternative to divalent cation extraction from normally pre-assembled layers.

### **A-layers of calcium deprived cells**

When cultured in LB broth containing up to 0.1 M EDTA, *A. salmonicida* cells grew as long filaments (Norris *et al.*, 1991) incapable of forming normal septa. The A-layers of these cells presented the same general feature of WD patterns associated with



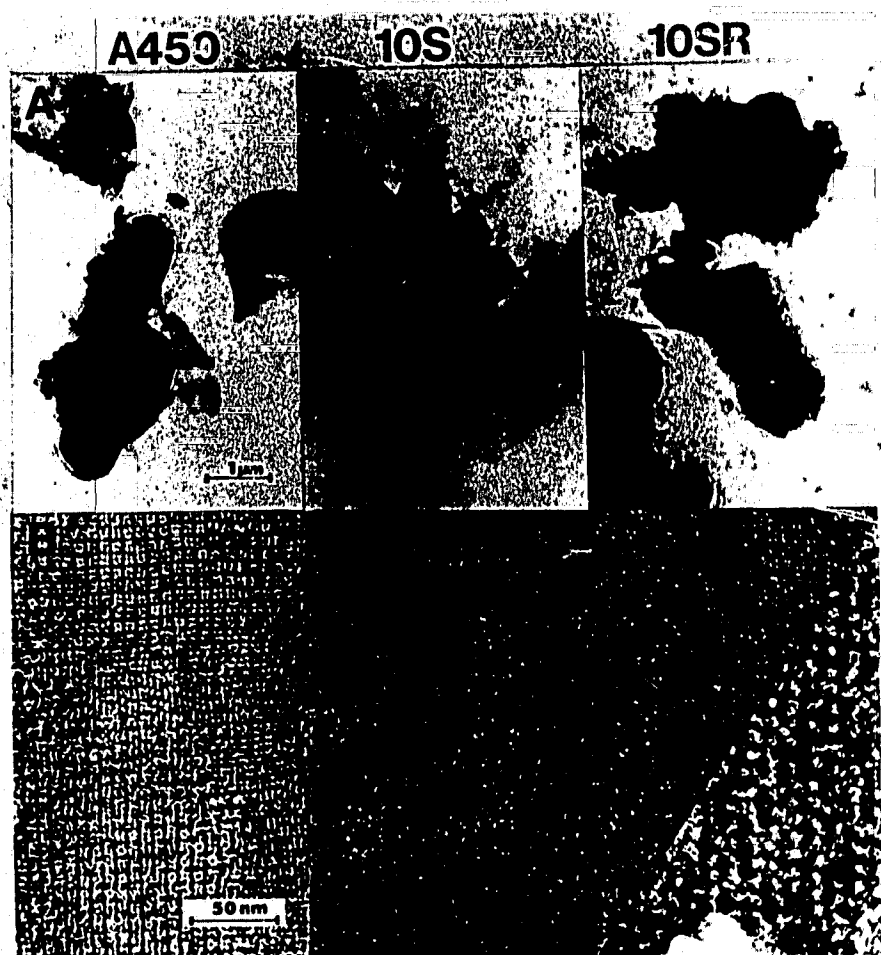
**FIGURE 10.** Altered A-layer patterns. Representative micrographs of the white dots pattern (a and b), big squares pattern (c to e), free ring-shaped units (f to h), intermediate patterns (i), and disorganized A-layers (j). Specimen from whole colonies treated with 25 mM EDTA in suspension (a). Specimen from whole colonies treated with 25 mM EGTA (c). Specimen from a purified A-layer preparation treated with 0.5 M EGTA (f). Specimens from cells grown in MM with 1.5 mM  $\text{Ca}^{2+}$  (b), MM (d and i), or FPM with 5 mM EGTA (e and j). Specimens from altered A-layer sloughs of cells grown in FPM with 5 mM EGTA, to which  $\text{Ca}^{2+}$  (g) or  $\text{Sr}^{2+}$  (h) were added. All bars represent 50 nm.

EDTA treatment. On the other hand, cells from cultures grown in LB with 0.1 M EGTA, did not produce filaments and their A-layers appeared normal.

*A. salmonicida* cells in MM (or MM with 1.5 mM calcium) were nutritionally and/or energetically limited, since only a single subculture could be achieved. These cells also appeared morphologically affected, with some filamentous and many coccoid forms. A-layers from cells grown in MM had prominent WD patterns. Also, BS patterns (Fig. 10d) and intermediate morphologies between WD and BS patterns (Fig. 10i) were observed. On the other hand, *A. salmonicida* cells grew without difficulty in FPM, and produced A-layers of poor order, which prominently displayed the BS pattern. Addition of 5 mM EGTA to this medium produced further A-layer disorganization, yielding small patches of arrayed layer and numerous FR units. Specimens of A450-1 cells grown in FPM with 5 mM EGTA, showed large areas of the grid covered with poorly ordered BS patterns (Fig. 10e), or disorganized free A-layers (Fig. 10j). The SDS-PAGE profile of proteins and LPS from cells grown in FPM did not show significant changes (results not shown).

### **The WD pattern is not exclusively associated with limitation of divalent cations**

Interestingly, cells grown in MM with 1.5 mM calcium also had prominent WD patterns (Fig. 10b); suggesting that environmental conditions different from  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  limitation may produce lattice alterations in the A-layer. EM of cells of the attenuated mutants A450-10S and A450-10SR (Thornton *et al.*, 1991), demonstrated that they are slightly smaller and are, in general, coccoid in form (Fig. 11a). Fig. 11b represents a negative stain of the A-layers from these strains as compared with the wild type strain A450. The layer from the 10S strain, showed regions displaying prominent WD patterns. The A-layer from the 10SR strain displayed a normal morphology (upper split frame), but older colonies, showed what I interpreted as A-layer aggregation (stacking of layers). The A-layers appeared quite dark, with rows of white dots showing some regularity (lower split frame). Fig. 11c & d shows the results from the immunogold labeling of thin sectioned cells. A450-10S was uniformly labeled at the cell periphery, as was (although to a lesser extent) A450. However, A450-10SR showed frequent gaps of more than 600 nm between neighbor gold particles (Fig 11d), as well as a reduced number of gold particles per cell, suggesting that covering of the cell surface with A-layer is incomplete, a finding compatible with A-layer aggregation.



**FIGURE 11.** Cell and A-layer morphologies of the surface disorganized mutants A450-10S and A450-10SR, as compared with the wild type strain A450. (a) Electron micrographs of negatively stained whole cells. (b) Representative micrographs showing the different A-layer morphologies found. (c)(next page) Immunogold labelling of thin sections showing the surface distribution of A-protein subunits. Scale bars for (a), (b), and (c), are shown in the first column (corresponding to A450). (d) (next page) Quantitative analysis of the distribution of gold particles over the cell surface. Analysis was performed on micrographs similar to those presented in (c) comprising 25 cells from each strain.

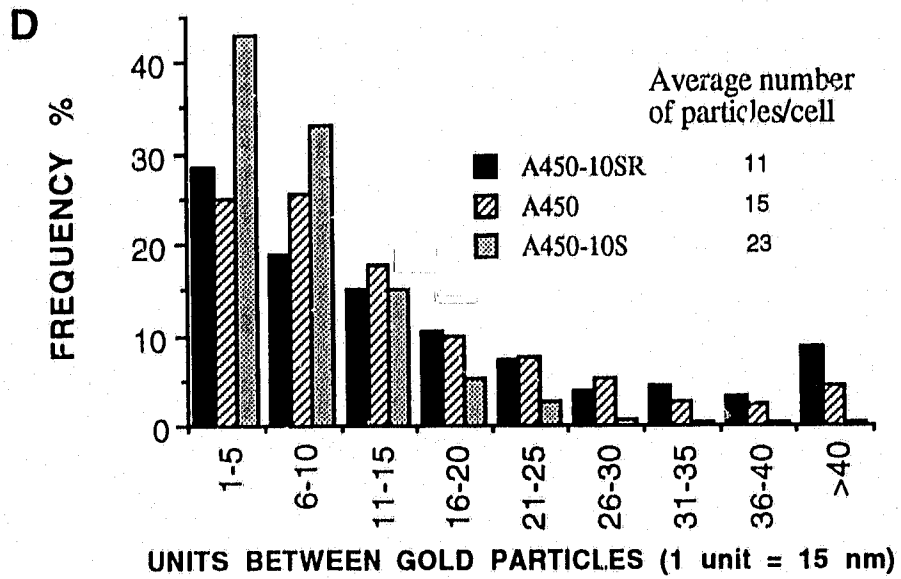
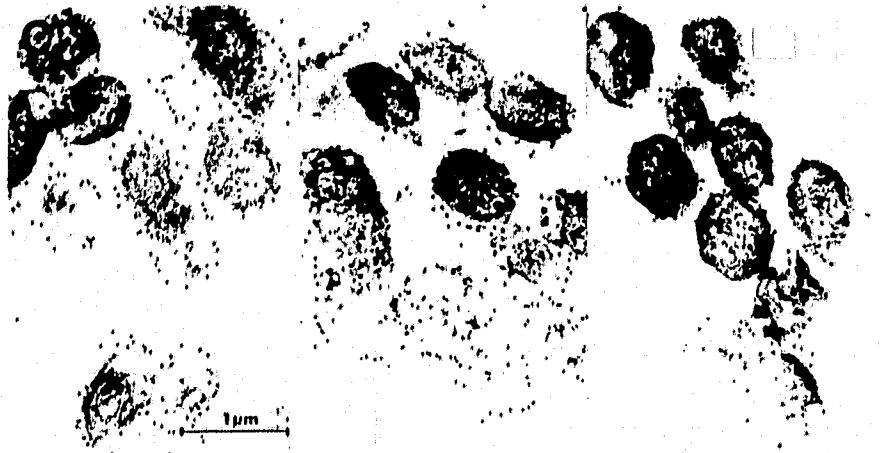


FIGURE 11. Continued from previous page.

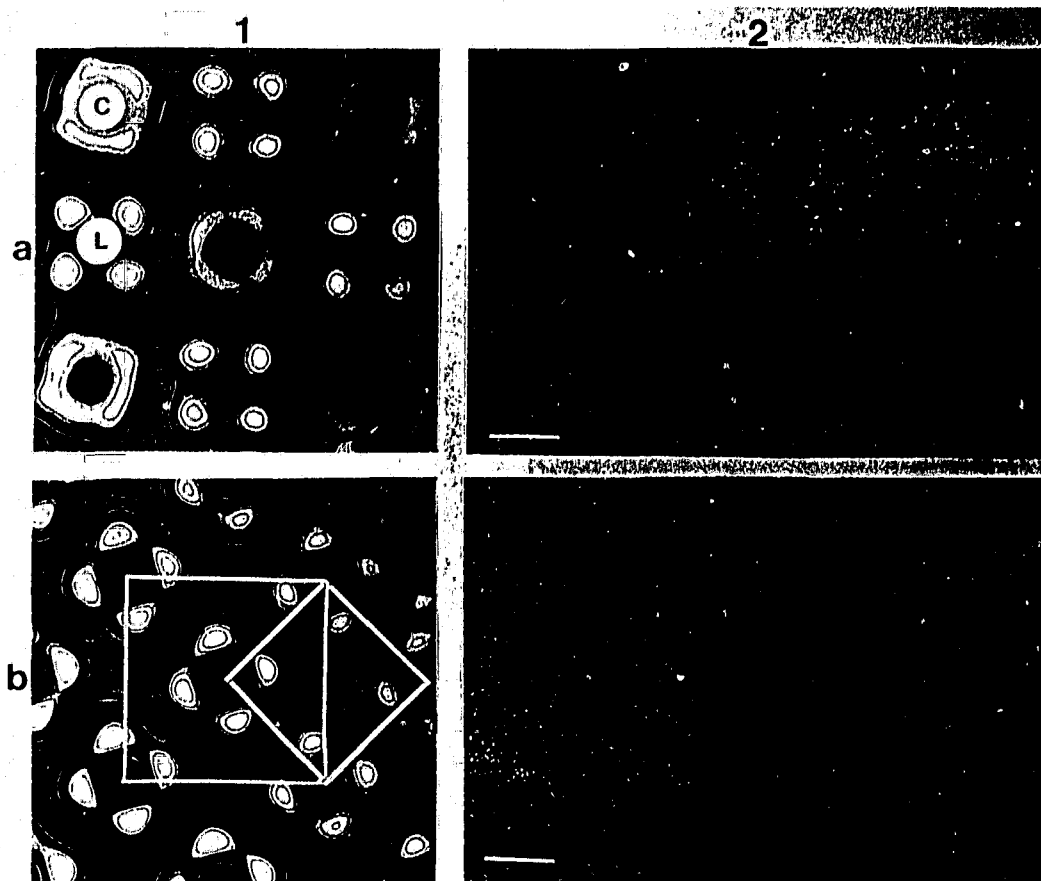
Also, cells kept in TRIS buffer for extended periods of time (~16 h) possessed A-layers with WD patterns (not shown).

### **Addition of divalent cations to altered A-layers**

The layers from cells grown in FPM with 5 mM EGTA were used for reconstitution because they showed the most extensive and uniform alterations. Since calcium could not be added directly to FPM due to precipitation and/or gelling of the medium, washed FPM grown cells were resuspended in 50 mM imidazole buffered saline containing the corresponding divalent cations used for reconstitution. Addition of  $\text{Ca}^{2+}$  or  $\text{Sr}^{2+}$  yielded a large number of FR units, either dispersed (Fig. 10g) or forming non-regular masses (Fig. 10h). Interestingly, extensive, highly ordered normal arrays could also be found. The addition of magnesium did not cause significant changes compared with the control, and the simultaneous addition of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , or  $\text{Sr}^{2+}$  and  $\text{Mg}^{2+}$ , did not improve the reconstitution process.

### **The BS pattern is formed by a single morphological unit**

Correlation averaging of well ordered BS patterns, such as the one shown in Fig. 12b-2, indicated that they were apparently composed of a weakly handed, single morphological unit (formed by four large domains grouped around a major 4-fold symmetry axis) with little or no mass connecting adjacent units (Fig. 12b-1). This is in contrast to projections through normal A-layers, which exhibit two distinct morphological units (representing the two A-layer domains) with clear connections between them (Fig. 12a-1). Averaging of other BS patterns rendered similar images, but with slight variations in lattice spacing. The lattice spacing of BS patterns was approximately equal to the average lattice spacing of normal A-layer divided by  $\sqrt{2}$ , suggesting the possibility that the core and linker A-layer domains were still present but had been altered to become similar in appearance. This situation, in which only alternating morphological units are equivalent, is represented by the large unit cell in Fig. 12b-1 (lattice spacing = 10.8 nm). The small unit cell (lattice spacing = 7.6 nm) would apply if all morphological units are in fact identical. The average shown in Fig. 12b-1 was obtained by averaging over alternating correlation peaks, on a lattice corresponding to the large unit cell, in order to detect any minor differences between adjacent units. No differences were apparent, consistent with the fact that correlation peak heights formed a continuum of values rather than partitioning into two



**FIGURE 12.** Comparison of the normal and the BS A-layer patterns. Correlation averages of normal (a-1) and BS (b-1) A-layer patterns, aligned to their corresponding micrographs (a-2 and b-2). The normal layer (a) shows two distinct morphological units representing the core ("C" in Fig. 4a-1) and linker ("L" in Fig. 4a-1) mass units. Lattice spacing is 10.4 nm. Number of unit cells included in the average = 250. The BS pattern (b) is from cells grown in FPM. Averaging was performed over alternating correlation peaks, i.e. adjacent morphological units were averaged separately. Number of unit cells included in the average = 520. The overlaid boxes in Fig. 4b-1 show the two possible unit cells: either i) all morphological units are equivalent (small box = 7.6 nm spacing) or ii) only alternating morphological units are equivalent (large box = 10.8 nm). Both averages were subjected to 4-fold rotational symmetrization. Both averages are presented at the same scale; their dimensions being 22 x 22 nm. Bars represent 50 nm. Correlation average and imaging was performed by B.M. Phipps at the Max-Planck Institute for Biochemistry, Martinsreid, Germany.

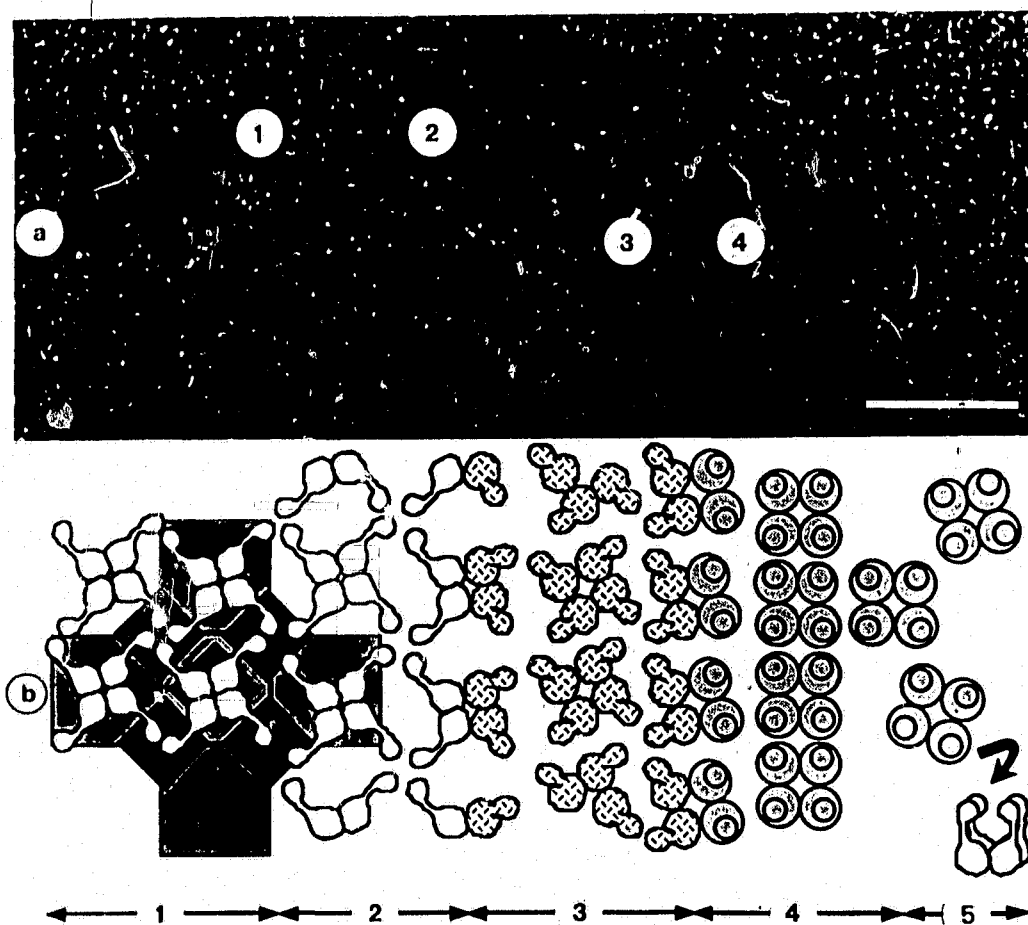
classes. It thus appears that only a single type of morphological unit is present in BS arrays. This is supported by the observation that BS patterns frequently lie at an angle of  $45^\circ$  to patches of normal A-layer, which is what one would expect if only one of the two types of A-layer domains were disrupted while the remaining domains packed more closely together.

## DISCUSSION

The organization of the *A. salmonicida* A-layer is clearly dependent on the availability of divalent cations, in particular  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  limitation compromised the order of the A-layer and altered its structure in a defined way. The following observations strongly suggested that A-protein subunit interactions were largely ionic and that divalent cation bridges were involved: i) only guanidine-HCl [a positively charged chaotrope capable of displacing cations (Koval and Murray, 1984)] effectively released monomeric A-protein from the outer membrane, ii) the *in vitro* crystallization process was both pH and salt-dependent, iii) there was a precise correlation between the pKas for glutamate and aspartate carboxylic groups and the pH required for A-protein extraction, and iv) divalent cation depletion or growth under calcium limitation produced A-layer structural alterations.

Furthermore, the following indicated that divalent cation bridges are predominantly restricted to one of the two A-layer domains (core or linker), and are not involved in association with the cell surface: i) the novel altered regular A-layer patterns produced by  $\text{Ca}^{2+}$  depletion were found to run at  $\sim 45^\circ$  to the normal orientation of the A-layer lattice (Fig. 10a, b and d; Fig. 13a); ii) monomeric A-protein was not released from the cell surface upon divalent cation depletion (Phipps, 1988); iii) tetrameric FR units were released upon physical disruption after divalent cation depletion (Fig. 10f); iv) serial DOC extractions solubilized most of the outer membrane components with a substantial preservation of intact A-layers (Fig. 9a); v) semicrystalline sheets were formed from LPS-free preparations of purified A-protein; and vi) A451-70 and A450-1 (both  $\text{A}^+$ ,  $\text{O}^-$  mutants) were capable of forming large free A-layer sheets (not shown).

The appearance of the structurally altered WD, BS and intermediate patterns, as well as the appearance of FR units, could be conceived as representing a sequence of rearrangements, as depicted in the hypothetical model of Fig. 13. Loss of divalent



**FIGURE 13.** Hypothetical model showing the proposed structural rearrangements within the A-layer. (a) Micrograph of an A-layer slough subjected to divalent cation removal by 0.5 M EDTA, in which the following elements are distinguished: (1) Small patch of a normal type I pattern (refer to chapter III), (2 and 3) intermediate patterns, (4) BS pattern. Note the lattice lines of normal and altered patterns running at  $45^\circ$  to each other. Bar represents 50 nm. (b) Schematic hypothetical representation of the changes in A-layer organization observed in 'a'. The normal tetragonal array (1, and black filled squares) is disrupted by calcium removal causing the dissociation (exaggerated in the drawing) of one of the two A-layer domains (in this case the linker domain), with the consequent formation of independent tetrameric units (2, and gray filled squares in 1), which upon rearrangement (3) may either be packed to form well-ordered BS arrays (4) or dispersed to form free rings (5). One of the free tetrameric units is presented in its side view (arrow in 5) to explain the appearance of "U" shaped free units (arrowheads in Fig. IV.3g). In the dissociation process, the WD pattern may appear as an intermediate morphology between normal arrays (1) and independent units (2).

cations would affect the integrity of one of the two A-layer domains. At first, this would produce a slight mass concentration at the unaffected centre and a consequent displacement of negative stain, accounting for the appearance of the WD pattern (Fig. 13a-1 to 13a-2). Complete disruption of the affected centre would lead to the appearance of intermediate patterns formed by independent A-protein tetramers (Fig. 13a-2 to 3, and 13b-2), which partially rotate out of the A-layer plane (Fig. 13b-3), allowing them to diffuse together into a close ordered packing (Fig. 13a and 13b-3 to 4), accounting for the formation of BS patterns. The lattice constant of this new array would vary with the degree of packing; a minimum determined by the size of the A-protein tetramers, and being similar to the lattice spacing of the normal A-layer in its closed conformation, divided by  $\sqrt{2}$  (compare square sizes of Fig. 13b-1). Physical stress or further loss of divalent cations would release FR units (Fig. 13b-5), implying that the A-protein tetramers in BS patterns interact weakly. Some of these tetramers might be presented to the observer in side view (Fig. 13b-5, arrow), accounting for the "U" shaped units observed in negatively stained preparations (Fig. 10g, arrows). Thus, according to this hypothetical model the FR units consist of A-protein tetramers still interacting at the unaffected A-layer domain. Results from the low pH extraction, which releases monomeric A-protein, suggest that the A-protein interactions at this unaffected domain (i.e. the one that holds together the morphological units of the BS pattern as well as the FR units) could also be ionic, but their nature has not yet been elucidated. While this model is somewhat speculative, it does account for all the biochemical and structural data presented herein. In principle, it should be possible to test parts of this hypothesis by 3-D reconstructions of the altered A-layers, particularly of the BS and WD arrays. However, since these arrays are products of structural modifications, it has so far been impossible to consistently obtain patches of these arrays which are sufficiently large and well ordered enough to warrant a 3-D reconstruction. In any case (even if the presented model would not stand further testing) the conclusion that the A-layer possesses significant structural plasticity, in which  $\text{Ca}^{2+}$  plays an important role, remains valid and unaffected.

Alternate explanations for the appearance of altered patterns (particularly the BS pattern), include superimposition of A-layers, and the occurrence of major conformational changes in the A-protein. The former was ruled out, since the only superimposition that produces a pattern with a single morphological unit possesses a different morphology to that of BS patterns (Fig. 6b in chapter II). Also, the degree of

staining observed for BS patterns, their poor order and their weak but significant handedness, make it unlikely that they were formed by superimposed layers. The second possibility implies that the core and linker domains of the normal array are structurally retained, but A-protein subunits have gone through a significant conformational change, such that the morphologies of the two domains can no longer be distinguished at low resolution. This is very unlikely for several reasons. Firstly, it is highly improbable that these distinctive structures would become so similar in appearance. Secondly, it has been amply demonstrated that A-protein is unusually stable under a variety of conditions (Phipps, 1988; Phipps *et al.*, 1983). Thirdly, if this were true, BS arrays would be "in line" with adjacent areas of normal A-layer, rather than at an angle of 45°. Also, the poor order sometimes associated with BS patterns should not be expected to occur in arrays formed by interlocked subunits.

Nevertheless, it is also true that irreversible minor conformational changes in the A-protein seem to be involved, as indicated by the failure of *in vitro* added Ca<sup>2+</sup> in restoring to normality abnormal layers assembled *in vivo*. Upon *in vivo* assembly in the absence of Ca<sup>2+</sup>, A-protein subunits may present carboxylate groups in a different orientation (possibly defined by the lowest A-protein conformational energy), preventing subsequent formation of calcium bridges. Doran *et al.* (1987) have shown that *in vitro* reassembled *A. vinelandii* S-layers do not acquire the *in vivo* state and that an association with the OM achieved during *in vivo* assembly is important to S-layer stability. It was also suggested (Bingle *et al.*, 1987a) that detachment of the *A. vinelandii* S-layer from the normal cell surface produces a subtle, presumably irreversible alteration in its structure. Since calcium has been observed to enhance A-layer-OM interactions (Phipps, 1988), it may be required for A-protein assembly proficiency [as for the *Aquaspirillum serpens* VHA array protein (Koval and Murray, 1984, 1985)] and/or for its proper interaction with LPS. Further support for this view comes from the observation that altered A-layers (e.g. from cells grown in calcium limited media) are shed from the cell surface more readily than those from normal cells. The same extensive sloughing of A-layer has also been observed with the mutant A450-10S, whose A-layers have prominent WD patterns.

Since binding of calcium to the A-layer seemed to be very tight (as judged from the high EGTA concentrations required to produce significant alterations in pre-assembled A-layers, and by the fact that cells grown in LB with 0.1 M EGTA had normal A-layers), the greater efficiency of EDTA in producing WD patterns was

possibly related to a selective extraction of  $Mg^{2+}$  from the A-layer, implying that  $Mg^{2+}$  has a specific role in the A-layer integrity. BS patterns were prominently produced under  $Ca^{2+}$  limitation in the presence of  $Mg^{2+}$  (i.e. in FPM) and, interestingly, they were disrupted upon addition of  $Ca^{2+}$ . These results are best explained by surmising that  $Mg^{2+}$  promotes the packing of A-protein tetramers into ordered BS patterns, and that  $Ca^{2+}$  is not functional in this role. Experimental evidence to support this hypothesis was obtained by growing *A. salmonicida* cells in MM or FPM without  $Mg^{2+}$ . These cells were incapable of assembling regular patterns (normal or altered), and produced large amounts of FR units grouped in masses similar to those shown in Fig. 10h. It is important to note that while calcium has been reported to be involved in linkages between S-layer subunits (Kist and Murray, 1984), between subunits and a cell wall component (Sára and Sleytr, 1987), or in both kinds of linkages within the same S-layer (Bingle *et al.*, 1987b; Doran *et al.*, 1987), a requirement for magnesium has only been documented for S-layer assembly in *Sporosarcina ureae* (Beveridge, 1979) and *Bacillus brevis* (Tsuboi *et al.*, 1982).

Finally, it is important to emphasize that the appearance of the WD pattern is not an exclusive consequence of divalent cations extraction or deficiency. The attenuated mutant A450-10S, as well as cells kept in TRIS buffer, or grown in MM, also displayed prominent WD patterns. Experimental evidence suggesting a mechanism for this effect has not been found, but it is very interesting that a common factor for the 10S strain (J.C. Thornton, personal communication) and the cells kept in buffer, or grown in MM, is energy starvation. Perhaps the synthesis of A-protein subunits under low energy conditions, leads to defective A-layer assembly.

The appearance of regular altered patterns upon calcium (and perhaps energy) limitation seems to be a unique response of the A-layer, since other S-layers in which calcium bridges have been specifically implicated lose all regularity upon calcium depletion (Buckmire and Murray, 1976; Koval and Murray, 1985; Beveridge and Murray, 1976c; Doran *et al.*, 1987). It is important to note that the facultatively intracellular pathogenic species of the genus *Yersinia* respond to low levels of calcium and increased temperature by inducing a complex programmed response (Cornelis *et al.*, 1989; Straley, 1991), apparently mediated by a two-component regulatory system (Forsberg *et al.*, 1991). Interestingly, I have observed that altered A-layers displaying the BS pattern exist *in vivo* and are capable of mediating a very efficient association of *A. salmonicida* with salmonid macrophages (refer to chapter IX). Perhaps, more than a

mere *in vitro* induced effect, the appearance of regular altered patterns may be an indication of the A-layer's ability to respond to environmental signals, implying that it may possess more than one functional organizational state.

## CHAPTER IV

### FUNCTIONS OF THE A-LAYER OF *Aeromonas salmonicida*

Functionally, the A-layer is extraordinarily versatile (Kay and Trust, 1991). Soon after its discovery it was shown that the A-layer was essential for virulence (Ishiguro *et al.*, 1981; Kay *et al.*, 1981); the LD<sub>50</sub> of A<sup>-</sup> mutants being four orders of magnitude greater than their corresponding A<sup>+</sup> parental strains. The A-layer is neither the only nor necessarily the major virulence determinant of *A. salmonicida*, since there are totally avirulent A<sup>+</sup> strains (Thornton *et al.*, 1991). The specific virulence mechanisms associated with the A-layer are still being elucidated, and constitute the core of the work presented in the remaining section of this dissertation.

As proposed for S-layers in general, the A-layer has a definite role as a physical barrier. It protects *A. salmonicida* cells against bacteriophages that use surface molecules, especially different portions of the LPS molecule, as receptors (Ishiguro *et al.*, 1981 & 1983). Since most receptor molecules are shielded by the A-layer, bacteriophages (which cannot penetrate the A-layer's pores) have a highly reduced ability to infect A<sup>+</sup> cells. It also confers resistance to predation by *Bdellovibrio spp.* (Koval and Hynes, 1991). Although the mechanism for this effect is not fully understood, it is very likely that the A-layer either hides a receptor molecule from making contact with *Bdellovibrio* cells, acts as a barrier against enzymatic attack or possibly strengthens the outer membrane to avoid breakage by physical attack. The protective action of the A-layer against killing by fresh serum was one of the first specific virulence related functions discovered (Munn *et al.*, 1982). A<sup>+</sup> *A. salmonicida* are able to withstand complement attack even in the presence of immune serum. However, A-layer is not the only protective barrier against killing by complement. Smooth LPS is also protective as demonstrated by the fact that O<sup>-</sup> strains with core region LPS are extremely sensitive to normal serum. Interestingly, fresh trout serum appears to be more bactericidal than rabbit or human serum against *A. salmonicida* (Munn *et al.*, 1982; J.C. Thornton and R.A. Garduño, unpublished observation). Furthermore, it has been shown that in its native arranged form, the A-layer is resistant to the action of proteases (Chu *et al.*, 1991; Phipps, 1988). By withstanding

proteolysis, the A-layer is able to protect underlying surface molecules from proteases, which importantly contribute to the non-oxidative killing mechanisms inside phagolysosomes (Lehrer and Ganz, 1990).

An intriguing protective effect of the A-layer against reduced oxygen species (ROS) has been observed (Karczewski et al., 1991). This protective effect will be the subject matter of chapter VII. Apparently, as in the case of serum resistance, the A-layer is not exclusively responsible for protection against ROS; production of the enzyme superoxide dismutase was also related to protection against superoxide (Karczewski et al., 1991).

The A-layer is associated with survival of *A. salmonicida* in the freshwater environment (Sakai, 1986). A<sup>+</sup> cells were able to survive for more than 15 weeks in the presence of diluted humic acid (10 mg/l), tryptone (10 mg/l), and cleaned river sand. A<sup>-</sup> cells incubated under the same conditions, and A<sup>+</sup> cells kept in saline without nutrients, only survived for 7 days at the most. Sakai (1986) proposed, based on experimental data, an electrostatic mechanism of adsorption to sand particles, where adsorbed A<sup>+</sup> cells could slowly utilize co-adsorbed nutrients. The role of the A-layer appeared to be the conferring of a net negative charge to A<sup>+</sup> cells; A<sup>-</sup> cells displayed a net positive charge.

Surprisingly, the A-layer has been demonstrated to be incredibly good at binding host molecules. It is capable of binding porphyrins (Kay *et al.*, 1985), a capability that has been speculated to be involved in an iron uptake mechanism (Kay *et al.*, 1985 & 1988), or, as it will be discussed in chapters V & VI, in increased penetration of host cells. Enhanced invasiveness after hemin binding also has been shown for the facultatively intracellular pathogen *Shigella flexneri* (Daskaleros and Payne, 1987). A<sup>+</sup> *A. salmonicida* cells were found to specifically bind immunoglobulins; rabbit IgG, with a K<sub>d</sub> of 1 μM, and human IgM, with a K<sub>d</sub> of 3.3 μM (Phipps and Kay, 1988). Intact IgG was requisite for binding, since both Fab and Fc fragments were not bound. It has been hypothesized that the complete immunoglobulin molecule is accommodated in the pit formed by the massive, funnel-shaped, A-layer core unit. Some support for this view comes from the facts that the average binding stoichiometry is approximately 1 immunoglobulin molecule to 4 A-protein monomers, and that the arrayed A-layer, but not monomeric A-protein, is required for binding (Phipps and Kay, 1988). Moreover, the A-layer has been demonstrated to specifically bind the host basement proteins fibronectin, laminin (Doig

*et al.*, 1992) and collagen IV (Trust *et al.*, manuscript submitted). The binding constants for laminin and fibronectin were 1.52 nM and 6.6 nM, respectively. Binding of collagen IV was irreversible, making the measurement of the equilibrium constant difficult; an estimation being 27 nM. This unique ability of the A-layer to bind host basement proteins may reflect an A-layer-mediated, specialized mechanism of host colonization. Instead of exclusively interacting with host cell surfaces, *A. salmonicida* cells may alternatively bind to and colonize extracellular matrices. Also, binding of extracellular matrix proteins to the bacterial cell surface may subsequently enhance the ability of *A. salmonicida* to colonize certain tissues or gain access to specific cell types, exploiting host extracellular basement protein receptors. Elevated fibronectin levels may contribute to enhanced tissue adherence of pathogens (Hook *et al.*, 1989), including *A. salmonicida*. Finally, the unique abilities of the A-layer to bind immunoglobulins and other host molecules perhaps reflect another A-layer-mediated mechanism of host defense avoidance. Non-immune binding of immunoglobulins may overthrow the purpose of the humoral immune response, and further shield the bacterium from other defense mechanisms like opsonic phagocytosis by neutrophils. In the case of hemin and soluble fibronectin, binding to the A-layer may shield underlying bacterial surface antigens from normal immunological recognition (a case of molecular mimicry), again, subverting the purpose of the humoral and cellular immune responses. Specifically, soluble fibronectin has been identified in rainbow trout plasma and in the supernatant of rainbow trout gonad (RTG-2) cell line cultures (Lee and Bols, 1991). Interestingly, cortisol induced an increase in fibronectin synthesis by this cell line (Lee and Bols, 1991).

The A-layer promotes adhesion to host cells. It has been shown that A<sup>+</sup> cells were two to three times more active than their A<sup>-</sup> counterparts in their ability to associate with macrophages (Trust *et al.*, 1983) or with non-phagocytic cell lines of mammalian (baby hamster kidney, BHK-21) or piscine (RTG-2) origin (Parker and Munn, 1985). At first it would seem suicidal for *A. salmonicida* cells to associate with macrophages, since these are professional phagocytes capable of killing bacteria through oxidative and non-oxidative mechanisms. However, there is evidence indicating that A<sup>+</sup> *A. salmonicida* cells are able to survive killing by macrophages (Graham *et al.*, 1988; Olivier *et al.*, 1986). Therefore, it is reasonable to hypothesize that the macrophage constitutes an unsuspecting accomplice in the rapid spread of the

infection. This, and the issue of *A. salmonicida* as a facultatively intracellular pathogen, will be discussed in more detail in chapters V, VI, VIII and XI.

With this remarkable plethora of functions, it seemed important to determine the specific role of the A-layer in some specific processes, and to examine the mechanisms involved. Indeed, the explanation of why the A-layer is absolutely required for virulence is being unraveled.

## MATERIALS AND METHODS USED IN FUNCTIONAL STUDIES

### Buffers

Sterile phosphate buffered saline (PBS) pH 7.4, was used as the main general buffer (especially in bacteriological work). It contained 8 g of NaCl, 0.2 g of KCl, 0.2 g of  $\text{KH}_2\text{PO}_4$ , and 1.15 g of  $\text{Na}_2\text{HPO}_4$ , dissolved in one liter of deionized water. Filter sterilized Hanks balanced salt solution (HBSS), pH 7.6, was used as the main buffer for tissue culture (especially for piscine cells). HBSS was comprised of NaCl, 8 g; KCl, 0.4 g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.185 g;  $\text{KH}_2\text{PO}_4$ , 0.06 g;  $\text{Na}_2\text{HPO}_4$ , 0.05 g (or  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.09 g);  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g;  $\text{NaHCO}_3$ , 0.35 g; D-Glucose, 1.0 g; and deionized water to one liter. Extra buffering capacity was obtained by adding HEPES at a final concentration of 2.38 g/l. Millonig's Phosphate Buffer ( $\text{NaH}_2\text{PO}_4$ , 55.2 g/l; pH 7.4) was used to control the pH of fixatives and saline washes, during preparation of some specimens for electron microscopy.

### Bacterial strains

*A. salmonicida* strains A450, A450-1, A450-10S, and A450-10SR, were described in chapter I, "Materials and methods used in structural studies". The following strains were also used in functional studies: *A. salmonicida* **A450-3**, an A-avirulent mutant selected by incubating a culture of A450 at 30°C (Ishiguro *et al.*, 1981). It contains high molecular weight LPS with intact O-polysaccharide chains (O<sup>+</sup>); *A. salmonicida* **A451**, a wild type A<sup>+</sup>, O<sup>+</sup> strain isolated in France (C. Michel's strain TG51/79) (Kay *et al.*, 1981); *A. salmonicida* **MT26**, a wild type, highly virulent strain isolated from diseased salmon in the Canadian west coast (a Microtek R. & D. strain, B.C., Canada); *Escherichia coli* ATCC 11775; and clinical laboratory strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (obtained through the

Microbiology Teaching Lab, Department of Biochemistry and Microbiology, University of Victoria, Canada).

### **Growth conditions and preparation of bacterial cells**

*A. salmonicida* strains were routinely grown, as needed, in LB, TSB, FPM, or plates of TSA, as described in chapter I, "Materials and methods used in structural studies". *E. coli*, *S. aureus*, and *P. aeruginosa* were routinely grown on TSA plates at 37°C for 24 h. *A. salmonicida* strains used in association or cytotoxicity assays with macrophages or other host cells were cultured at 20°C, for 48-72 h, on TSA, or supplemented TSA plates, as needed: TSA/Congo Red (30 µg/ml) was prepared as described previously (Ishiguro *et al.*, 1985). TSA/hemin plates were supplemented with hemin at a final concentration of 10 µg/ml. Hemin was first dissolved in a 1:1 mixture of DMSO:Tween 20 and further diluted in deionized water, filter sterilized, and used as needed. TSA/blood plates contained 10% (v/v) aseptically collected, heparinized fresh trout blood. Hemin and blood supplements were added to the TSA base at 50 °C, before pouring the medium into plates.

Routinely, bacteria grown in liquid media were prepared by pelleting them in a centrifuge (1,500 x  $g_{av}$ . for 5 min.) and washing them 2 times with PBS. Alternatively, colonies of bacteria grown on plates were scraped off the plate's surface, suspended in PBS, pelleted by centrifugation, and washed under the same conditions described for cells grown in liquid media. Then, bacterial cell suspensions with a final cell density of 1 OD. (absorbance read at 650 nm), were prepared, as needed, in PBS, HBSS or tissue culture medium. These bacterial cell suspensions were used in further experiments, either directly, or after proper dilution. Several viable cell counts of 1 OD<sub>650</sub> suspensions of the different bacterial strains used, were made by the standard dilution-plate method (see below: "Staining and counting methods"), and the average number of CFU/ml unit determined (0.75-1.0 x 10<sup>9</sup> CFU/ml of a 1 OD. bacterial cell suspension). Dilutions of 1 OD. suspensions were routinely made to adjust the bacterial cell density as needed.

### **Murine macrophages (mØs)**

The murine mØ cell line P388D1, derived from the mouse strain DBA/2, was used for those experiments described in chapter V. MØs were initially obtained from J.L. Kluffinger and R.W. Hancock, University of British Columbia, to perform

preliminary experiments. Later, a stock culture of P388D<sub>1</sub> mØs was obtained from T.W. Pearson, University of Victoria.

MØs were kept growing at 37°C and 5% CO<sub>2</sub>, in 40 ml tissue culture flasks (Falcon Laboratories) with 10 ml of complete RPMI-1640 medium, comprised of incomplete medium (see below) supplemented with 5% heat inactivated fetal bovine serum (Sigma Heart Line). They were passaged every 48 h to be used in experiments, and every 96-120 h for maintenance. MØs were routinely detached by repeated washing. Incomplete RPMI-1640 consisted of basal medium (Sigma) without sodium bicarbonate or glutamine, 450 ml; glutamine (30 mg/ml), 5 ml; 2-mercaptoethanol (3.5 µl/ml), 0.55 ml; HEPES (1 M), 5 ml; NaHCO<sub>3</sub> (7.5%), 13.35 ml; pH 7.4.

### **Isolation and culture of rainbow trout (*Oncorhynchus mykiss*) mØs**

#### **Materials.**

**Surgical tools:** blunt nosed scissors, forceps, scalpel, sterile scalpel blades, and spatulas; all made of stainless steel. These were kept immersed in 95% ethanol. Prior to use, tools were preferably flamed or alternatively, left to air dry in a clean laminar flow hood. It was convenient to have a dissection board on which to keep the fish in a correct position, and into which wire hooks, used as retractors, were fixed.

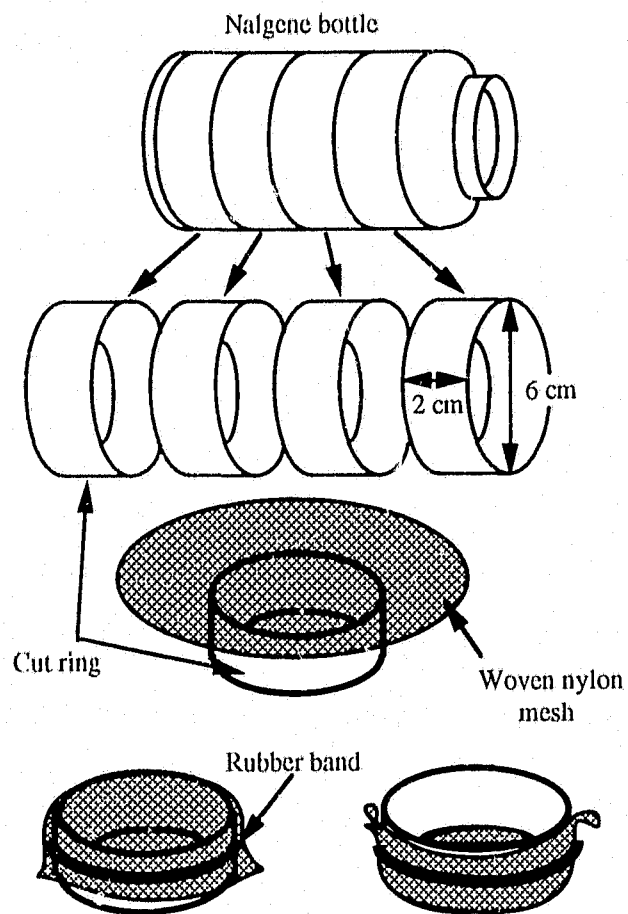
**Plasticware:** Sterile Nalgene™, 10 ml, 16 x 64 mm, round bottom, screw capped, polycarbonate tubes, used for high speed centrifugation (up to 50,000 x g); sterile, graduated, screw capped, 17 x 120 mm, 15 ml polystyrene conical centrifuge tubes; sterile polystyrene Petri dishes, 100 x 20 mm; tissue culture polystyrene dishes, 35 x 10 mm; tissue culture 24-flat well polystyrene plates, with lid; Nalgene™ 500 ml bottle top filters (with a 50 mm cellulose acetate membrane of 0.2 µm pores), to fit bottles with 45 mm neck size; sterile syringes (different volumes); needles for bleeding (18 gauge x 25 mm), and syringe filters 0.2 µm pore size.

**Glassware:** Long Pasteur pipets (9 inches); glass bottles (bottle neck size 45 mm) of 500 and 100 ml capacity, used for storage of tissue culture media and buffers; glass pieces of 10 x 10 x 3 mm to be used as supports for culture coverslips; square coverslips No. 1 thickness (0.13-0.17 mm), 22 x 22 mm; glass rods (7 mm diameter) with bulged flat ends, used to squash the head kidney tissue.

**Culture media:** Leibovitz L-15 tissue culture medium was routinely used. Basal L-15 was prepared from a powdered stock (GIBCO BBL). Incomplete L-15

consisted of the basal L-15 formulation supplemented with 0.4% glucose. This could be stored at 4°C for up to 2 months in tightly closed glass bottles. Freshly made glutamine (filter sterilized with a syringe filter) was added to incomplete L-15, immediately before use, to a final concentration of 150 mg/l. Freshly made L-15/Heparin (10 heparin units/ml) was used to initially suspend the cells from squashed head kidney tissue. Frozen 300 µl aliquots of a sterile heparin solution at a concentration of 1000 units/ml were kept at -20°C. Each thawed aliquot made 30 ml of L-15/heparin, which was the volume usually required per isolation batch. Complete L-15 was comprised of 89 volumes of incomplete L-15, 10 volumes of heat inactivated bovine calf serum, and 1 volume of the antibiotic-antimycotic stock solution (see below). Calf serum was a good substitute for fetal bovine serum. There were no noticeable differences observed in trout mØ cultures kept in either complete L-15 prepared with 5% fetal bovine serum or with 10% bovine calf serum. Very good results were obtained using trout serum as a substitute for bovine serum. In this case, complete L-15 was prepared with 95 volumes of incomplete L-15, 4 volumes of fresh trout serum, and 1 volume of the antibiotic-antimycotic stock solution. Serum was routinely obtained from the same trout used as source of head kidney tissue. Usually, the volume of blood was proportional to the number of mØs obtained, so that the volume of serum recovered was enough to supplement the culture medium. Before use, fresh serum was filtered through a 0.2 µm pores syringe filter. The use of trout serum was avoided in those cases where contaminating hemoglobin was undesirable. The antibiotic-antimycotic stock solution was prepared from lyophilized product (SIGMA). The reconstituted stock solution was stored at -20°C. This contained 100X the needed concentration of penicillin (10,000 U/ml), streptomycin (10,000 U/ml), and amphotericin B (25 µg/ml). For convenience, I kept the antibiotic-antimycotic stock frozen as 1.0 ml aliquots. Each thawed aliquot supplemented 100 ml of complete L-15. Gentamicin/L-15 (5 µg of gentamicin/ml) was used in assays where bacterial contamination control was required, but the presence of antibiotics inside mØs was not desired; i.e. intracellular bacteria were not exposed to the antibiotic. This was prepared using 9.6 volumes of incomplete L-15, 0.4 volumes of serum, and 0.01 volumes of a 5 mg/ml stock solution of gentamicin. Gentamicin was stored at 4°C.

**Other materials:** Sieving cups (Fig. 14) were used to disperse the head kidney tissue into a cell suspension. Circular pieces (12 cm in diameter) cut from a woven nylon mesh, 20 µm pore size (Macroporous Filter™, obtained in 30 x 30 cm



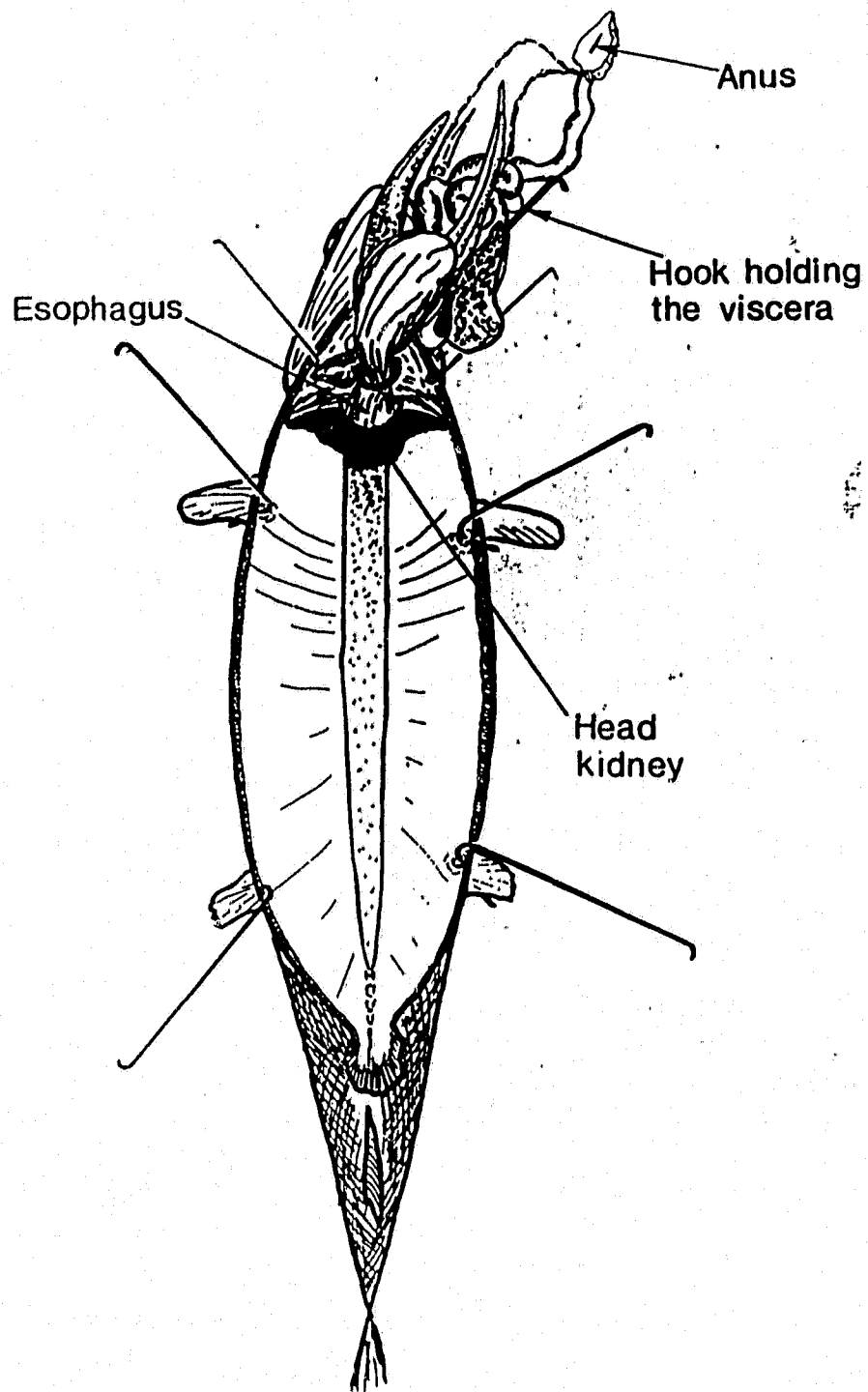
**FIGURE 14.** Manufacturing of laboratory-made sieving cups. An autoclavable 250 ml bottle (Nalgene, polypropylene) was cut to obtain plastic rings of the indicated dimensions. Rubber bands were used to keep the woven nylon mesh in place, covering the bottom of each ring. Cups were autoclaved before use. After use, the nylon mesh was removed from the ring, thoroughly washed with water, and reattached using new rubber bands.

sheets from Spectrum Medical Industries Inc.) were used to make sieving cups as illustrated in Fig. 14. A hemacytometer (Bright Line™, from American Optical), was used to count cells in suspension. A pipet-filler pump (Drummond), was used with 1, 5 and 10 ml serological pipets in the laminar flow hood.

#### **Method.**

*Dissecting the head kidney.* A trout weighing 300-500 g was stunned on the head with a wooden stick and immediately bled through the caudal vein. Between 5 and 8 ml of blood were usually obtained. The blood was transferred to a 15 ml conical tube for clotting at room temperature. The trout was placed on the dissection board with the ventral side up and the mucus covering the ventral skin was scraped off with a spatula. The ventral skin was then disinfected with 70% ethanol and a scalpel was used to carefully cut around the anus (intestine was not cut or perforated). Once the muscle and skin around the anus were separated, the blunt nosed scissors were used to cut the abdomen (from the anus to the gill level), exposing the internal cavities. Hooked wires were used to retract the abdominal walls and keep them pulled to the outside, fixed to the dissection board. The viscera were moved to the front, pulling from the anus, and held at the front by means of a blunt-ended wire hook passed around the esophagus and fixed to the dissection board. All the organs were kept intact, especially the digestive tract, so that their contents were not spilled in the internal cavities. The kidney was entirely exposed (Fig. 15). The membranes covering the head kidney were cut with a sterile scalpel blade, and gently pulled with fine forceps. A spatula was then used to scoop out all the head kidney tissue.

*Processing the head kidney tissue.* The dissected head kidney was placed in a sieving cup inside a Petri dish containing 10 ml of Heparin/L-15, and was squashed with the flat end of a glass rod. The bottom of the Petri dish was kept on ice to assure that during squashing, the tissue was constantly bathed with cold Heparin/L-15. The cell suspension produced had a dark brownish color and contained a total number of cells (excluding erythrocytes) approximately equal to  $10^6$  times the trout's weight in grams. The volume of the cell suspension was adjusted to 24 ml with cold Heparin/L-15 and the suspension split into six 4 ml aliquots. Each aliquot was loaded in a 10 ml Nalgene™ centrifuge tube containing 4.2 ml of stock isotonic Percoll™ (SIP). Under these conditions the number of cells did not exceed the limit of  $8 \times 10^7$  per gradient (i.e. per tube).

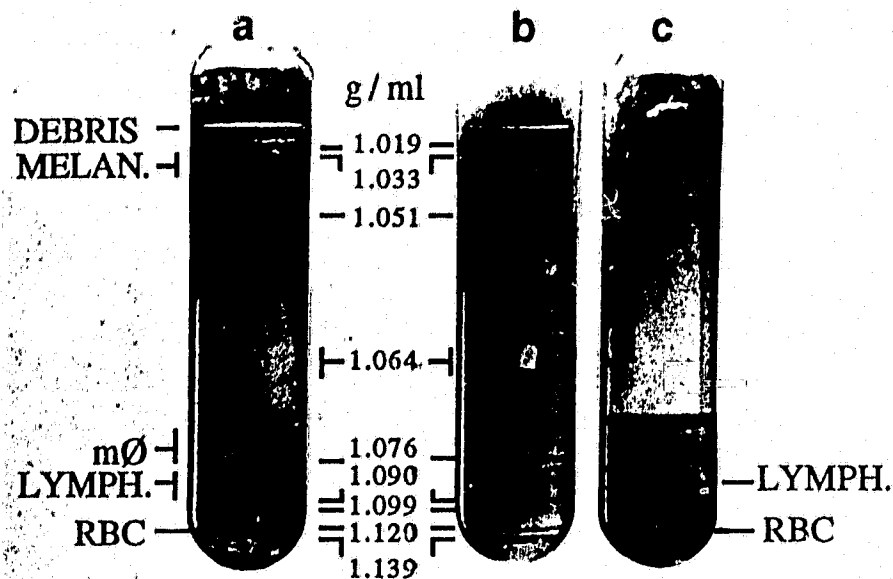


**FIGURE 15.** Representation of a dissected trout (top view of the abdominal cavity) just before removing the head kidney. Notice the position of the wire retractors fixed to the dissection board, and the position of the internal organs.

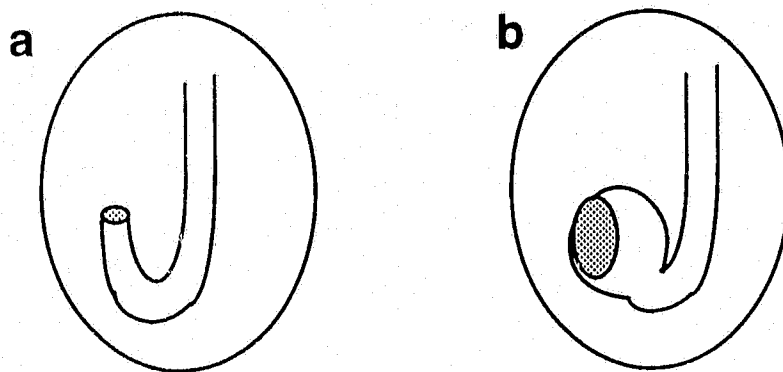
**Separating mØs.** Percoll™ (Pharmacia, Uppsala, Sweden) consists of colloidal silica particles, 15-30 nm in diameter, which have been coated with polyvinylpyrrolidone. Coating renders the silica particles non-toxic and significantly increases their stability in suspension. Percoll™ has a very low osmotic pressure and viscosity, even at high densities (up to 1.3 g/ml), and it is able to form self-generated gradients in a short time (10-30 min.), which make it useful in cell separations by isopycnic centrifugation. Before use, Percoll™ was made isotonic by mixing 9 volumes of Percoll™ with 1 volume of sterile 1.5 M NaCl. Routinely, batches of 100 ml of stock isotonic Percoll (SIP) were prepared, so that the bulk of the product was kept undiluted in its original bottle.

By mixing 4 ml of the head kidney cell suspension in Heparin/L-15 with 4.2 ml of SIP a suspension with an initial density of 1.065 g/ml was obtained. Upon centrifugation in an angle-head rotor (JA-20 Beckman™) for 20 min., 20,000  $\times g_{av}$ , at 5°C, a continuous density gradient was symmetrically distributed around this initial density of 1.065 g/ml, and the head kidney cells were distributed along the gradient in discrete bands as indicated in Fig. 16a. The top band was formed by dead cells, cell clumps and a gummy, viscous, dark debris. The second band from the top usually appeared lumpy and had a brownish color; it was mainly formed by melanocytes. The third band, with a density distribution of 1.069-1.075 g/ml was the one enriched in mØs. Immediately below, were the lymphocytes, sometimes separated into discrete bands but usually appearing as a single band as in Fig. 16a. The fifth band consisted of red blood cells. Finally, a black pellet of melanin granules was formed against the tube's wall. The first and second bands from the top were taken out of the tube and discarded. The third band was then recovered from beneath, by means of a "U-shaped" Pasteur pipet (Fig. 17). Recovery from beneath was always cleaner than recovery from the top with straight pipets (Fig. 16c).

**Sequential discontinuous and continuous Percoll™ density gradients.** When highly purified preparations of head kidney mØs were required, it was possible to combine the use of discontinuous and continuous density gradients. I applied first a modification of the method of Braun-Nesje et al. (1981), followed by a continuous density gradient purification. Discontinuous gradients were prepared in 15 ml conical centrifuge tubes. The bottom layer consisted of 6 ml of a Percoll™ suspension prepared by mixing 30 ml of SIP and 19.7 ml of HBSS (density = 1.077 g/ml). The middle layer consisted of 3 ml of a Percoll™ suspension prepared by



**FIGURE 16.** Distribution of head kidney cells in a continuous density gradient (initial density 1.065 g/ml). Five cell bands were distributed as shown in panel (a). Density was tracked by colored density marker beads (Pharmacia), as shown in panel (b). In panel (c), the macrophage band (mØ) has been recovered from the tube shown in panel (a). Notice the undisturbed lymphocyte band left (LYMPH.). MELAN. = Melanocyte band, RBC = Red blood cell band.

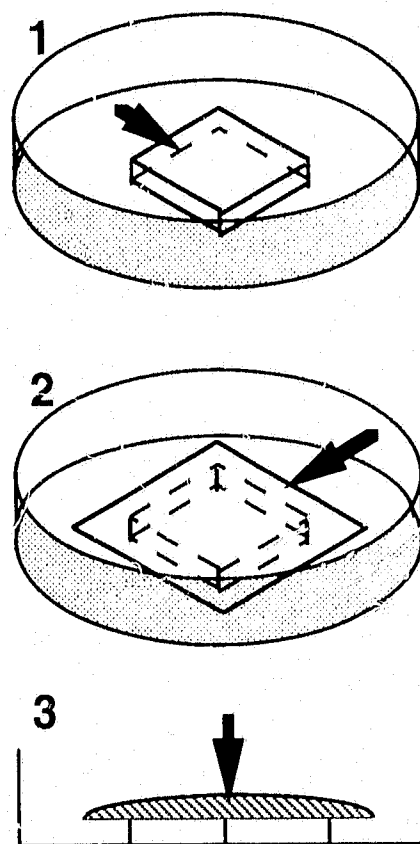


**FIGURE 17.** Tips of the modified Pasteur pipettes used in the isolation of trout mØs. (a) The "U-shaped" pipette is used to recover macrophages from continuous density gradients. (b) The "balloon" pipette is used to smoothly load the layers of discontinuous density gradients. The pressure at the tip of the balloon pipette is reduced, avoiding the usual turbulences produced by delivery of liquids through straight narrow pipette tips. Both modified pipettes are easily made by means of a Bunsen burner. In the case of the "balloon" pipette, the tip is first sealed and then blown until the balloon formed bursts, leaving a large lateral hole.

mixing 10 ml of SIP and 10.8 ml of incomplete L-15 (density = 1.066 g/ml). The middle layer was loaded onto the bottom one, by means of a modified "balloon" Pasteur pipet (Fig. 17). Since Pasteur pipets are not graduated, it was convenient to use the graduation on the tube to dispense the appropriate volume of each layer. The top layer consisted of 4 ml of a head kidney cell suspension in Heparin/L-15 (prepared as described above). This top layer was also loaded by means of a balloon pipet. Tubes were centrifuged at  $400 \times g_{av}$  for 40 min. at  $4^{\circ}\text{C}$  in a centrifuge with free-swinging rotor, and the cell band located between the middle and bottom layer (enriched in mØs) was recovered from each discontinuous gradient (with a U shaped pipet) and pooled. Pooled mØs were washed once by centrifugation ( $300 \times g_{av}$  for 10 min. at  $4^{\circ}\text{C}$ ) in L-15, resuspended in 16 ml of incomplete L-15, and equally divided in four 10 ml Nalgene™ centrifuge tubes containing 4.5 ml of SIP. After mixing, tubes were centrifuged in an angle-head rotor (JA-20 Beckman™) for 20 min.,  $20,000 \times g_{av}$ , at  $5^{\circ}\text{C}$ . After centrifugation, a top broad band of mØs (with basically the same density distribution of 1.069-1.075 g/ml) was clearly distinguished above several thin bands of lymphocytes and other contaminating cells. Sometimes an upper band of dead and/or clumped cells was formed near the meniscus. This was discarded prior to recovery of mØs by means of a U-shaped pipet.

**Washing and plating mØs.** Collected mØs were washed three times by centrifugation ( $300 \times g_{av}$  for 10 min. at  $4^{\circ}\text{C}$ ), with 10 ml of incomplete L-15. Before the last wash, cells in suspension were counted in the hemacytometer, after a 1:2 dilution in 0.4% solution of Trypan Blue in PBS. Trypan Blue was used to determine cell viability, since live cells exclude the dye, whereas dead cells take it up. Cell viability always was  $\geq 90\%$ . At this point, approximately 40-50% of the head kidney cells in suspension (excluding erythrocytes) were recovered. This yield was lower when consecutive discontinuous and continuous gradients were used.

Recovered cells were suspended in a single sterile bottle at a cell concentration of  $10^6/\text{ml}$ , and plated on supported coverslips (Fig. 18) at  $7.5 \times 10^5$  cells/coverslip or in 24-well plates at  $5 \times 10^5/\text{well}$ . Cultures were incubated at  $14^{\circ}\text{C}$  for two hours and non-adherent cells were washed away from the coverslips or wells. Adherent mØs were then covered with fresh complete L-15; cultures on supported coverslips used  $750 \mu\text{l}$  of medium/coverslip and cultures in 24-well plates, 1 ml/well. The yield of adherent mØs varied between 15-20% of the plated cells. This yield was higher for mØs purified by discontinuous-continuous gradients. The minimal final purity of mØs was



**FIGURE 18.** Preparation of mØ cultures on supported coverslips. Glass coverslips were raised from the bottom of 35 x 10 mm tissue culture dishes by means of a supporting glass piece. (1) Melted vaseline was used to attach the supporting piece to the bottom of the dish. (2) Also the coverslip was fixed to the supporting glass piece with vaseline. Coverslips are centered, so that no contacts occur between their edges and the walls of the dish. (3) Side view of a mounted culture covered with 750  $\mu$ l of tissue culture medium. Notice the shallow depth of the medium covering the culture, and its curvature at the free edges of the coverslip.

>95%. The main contaminant cells were lymphocytes, adherent granulocytes, and apparently immature cells with strongly basophilic cytoplasm.

The use of supported coverslips with free edges proved to have many quantitative advantages. Non adherent cells were easily and completely eliminated through washing of the supported coverslips. In contrast, it was more difficult to eliminate non-adherent cells from cultures kept in walled devices (multi-well plates or tissue culture dishes), owing to the fact that in these, a remnant of non-adherent cells always persisted at the edge formed by the walls and the bottom of the device. Moreover, also in contrast to walled devices, at the time of plating, the surface tension of the tissue culture medium in contact with the free coverslip edges, forced the cells away from the edges, preventing their accumulation in regions difficult to reach and observe. Adherent mØs, confined to the surface of the coverslip, were 100% observable and recoverable for counting purposes. Furthermore, in bacteria-mØ association assays, all added bacterial cells also remained confined to the surface of the coverslip and interacted exclusively with the mØs on the coverslip. Bacterial cells were presumably more available to mØs in coverslip cultures, due to the low depth of the liquid phase covering the culture. During assays, mØs on supported coverslips were maintained with 0.5 ml of buffer or tissue culture medium covering them.

***MØs in culture.*** The tissue culture medium was changed daily for mØs on supported coverslips and every 48h for mØs in tissue culture plates. Although it has been reported that head kidney mØs may stay in culture for up to one month (Braun-Nesje *et al.*, 1981), I usually used mØs after 24 h and before 72 h. in culture. In this way, complete adherence to the substratum was assured, and the risk of bacterial and/or fungal contamination kept low. However, it was possible, without difficulty, to keep mØs in culture for up to one week. Usually, no deterioration of mØs (commonly detected by rounding of the cells and/or the presence of granular inclusions) was observed during the first week of culture.

### **Fish cell lines**

The cyprinid cell line EPC (epithelioma papulosum cyprini) from common carp, *Cyprinus carpio*, and the Chinook salmon (*Oncorhynchus tshawytscha*) embryo line CHSE-214 (ATCC CRL 1681) were obtained from Microtek R. & D., B.C., Canada. Cells were maintained as monolayers in 75 cm<sup>2</sup> tissue culture flasks (Falcon) containing 50 ml of Minimal Essential Medium (MEM) (GIBCO BRL) with 10% fetal

bovine serum, at 15°C. Cells to be used in association assays with bacteria were transferred to 24-well plates as follows. Cells from one flask were trypsinized with 10 ml of a ready to use trypsin-EDTA solution (GIBCO), centrifuged to eliminate the trypsin (300 x g<sub>av</sub> for 10 min. at room temperature) resuspended in 48 ml of MEM/plates medium, dispensed in two 24-well plates (about 10<sup>6</sup> cells/well), and incubated at 15°C. MEM/plates medium was comprised of MEM supplemented with 2% fetal bovine serum, 10 mM HEPES, and antibiotic/antimycotic mixture (GIBCO) to achieve a final concentration of 1,000 U penicillin, 1 mg streptomycin and 2.5 µg amphotericin B per ml. Cells were used after they had completely spread, but before confluent growth was achieved.

### **Staining and counting methods**

Monolayers of mØs or fish cell lines were air dried, fixed for 5 min. in methanol, and stained with Wright or Giemsa stain (SIGMA). Bacteria were stained dark purple by these stains. MØs in suspension were counted in a Neubauer haemocytometer, after a 1:2 dilution with 0.4% Trypan Blue in PBS. The number of adherent host cells or bacteria per coverslip, the percentage of infected host cells, as well as the number of particles (i.e. bacteria or latex beads) per infected host cell, were determined by direct microscopy, following the procedures reported by Mallette (1969). Applying a 95% confidence level to the bacterial counts, and at the usual density of host cells per microscopic 100X objective field, 250 mØs/coverslip were routinely analyzed. This number could be reduced to 60-70 mØs/coverslip in highly infected preparations. The average number of bacteria per infected host cell was multiplied by the percentage of infected host cells, to obtain the number of bacteria per 100 cells.

Heat-fixed bacteria were stained by Gram stain, or with Crystal Violet for 1 minute, as needed. Bacteria, as well as latex beads, in suspension were counted in a Petroff-Hausser counting chamber (in 5 µl aliquots) or by using an internal standard of fungal spores (Mallette, 1969). For the latter, a suspension of spores from a puffball (*Calvatia* sp.) was prepared in FPA fixative (37% formalin, 5 ml; concentrated propionic acid, 5 ml; 70% ethanol, 89 ml; Tween 20, 1 ml). The suspension was placed in a bottle containing 1 mm glass beads previously washed in FPA fixative, and gently agitated overnight in a rotary shaker. The suspension of spores was standardized through 25 different counts of the number of spores/ml in five

independent samples (by using the Petroff-Hausser chamber). Normally, 20  $\mu$ l of the standardized fungal spore suspension were thoroughly mixed with 20  $\mu$ l of the bacterial sample and spread on a clean glass slide, air dried, and Gram stained for examination by light microscopy. Proportional counting and calculation of the number of bacteria were performed as described by Mallette (1969).

Viable bacterial cell counts were routinely made on TSA plates, by the standard dilution-plate method, using two or three plates per sample. When a high variability among plates of a single sample was observed, only the results from plates with a number of colonies between 30 and 300 were recorded. Congo Red (CR) plates (30  $\mu$ g/ml) were used to distinguish A<sup>+</sup> (which give red colonies) from A<sup>-</sup> bacteria (which give white colonies) (Ishiguro *et al.*, 1985). The dilution medium used for viable cell counts was LB without glucose, or nutrient broth, containing 0.5 % (v/v) of Tween 20 to reduce autoaggregation of A<sup>+</sup> cells.

## **Electron microscopy (EM)**

### **Negative staining.**

Negative staining with ammonium molybdate was performed as reported in "Materials and methods used for structural studies" (chapter I).

### **Scanning electron microscopy (SEM).**

SEM of mØs, or fish cell lines, was done by conventional methods. Briefly, cells in 24-well plates or attached to round (10 mm diameter) glass coverslips were fixed in 2.5 % glutaraldehyde for at least 2 h at room temperature or overnight at 4°C. Cells were washed with HBSS or Millonig's phosphate buffered saline and, for 24-well plates, the bottoms of the wells were cut with a cork boring machine. Dehydration of coverslips or cut bottoms was carried out in a graded series of ethanol (up to 100% ethanol), with a maximum dehydrating time of 40 min. Dehydrated specimens were critical point dried in a OMAR SPC-1500 drier (liquid CO<sub>2</sub> at 1200 psi, 40°C), and mounted on aluminum stubs using silver paste. Then, coverslips were gold coated for 2 min., in an Edwards Sputter Coater, at 40-100 milliamperes and an argon atmosphere at a pressure of 100 millitorrs. Specimens were examined with a JEOL JSM 35 scanning electron microscope at an operating voltage of 10 or 20 kV. Millipore™ membranes recovered from intraperitoneal chambers (see below) were also prepared for SEM, cut in half and mounted on aluminum stubs for gold coating. Thus, each aluminum stub had both sides of the membrane exposed.

**Transmission electron microscopy (TEM).**

Bacterial cells, or scraped mØs or fish cell lines were fixed, as needed, in different fixatives. Conventional fixation was carried out in 2.5% glutaraldehyde in HBSS (piscine cells) or Millonig's phosphate buffered saline (murine mØs). For detection of slime layers on bacteria, fixatives containing positively charged stains (traditionally used to enhance visualization of acidic polysaccharides) were used: i) Alcian Blue fixative contained 2.5% glutaraldehyde, 1.25% formaldehyde, 0.5% Alcian Blue stain and 0.03% tannic acid in HBSS, ii) Ruthenium Red fixative contained 2.5% glutaraldehyde, 0.075% Ruthenium Red stain and 50 mM L-lysine hydrochloride in HBSS. Cells were fixed for at least 2 h at room temperature (or overnight at 4°C), and then pelleted in a conical microcapsule (BEEM). Postfixation was conducted with 1% osmium tetroxide (Stevens Metallurgical Corp., New York, U.S.A.) in HBSS or Millonig's phosphate buffered saline, for 1 h at 4°C. After careful removal of the postfixative, the pelleted cells were overlaid with a thin layer of melted 0.5% agarose. Remaining fixatives were eliminated with three 5 min, HBSS or Millonig's phosphate buffered saline washes without agitation. Specimens were dehydrated in a graded series of ethanol (up to 100%), with a maximum dehydration time of 50 min. The agarose overlay was then removed and the cell pellet separated from the bottom of the microcapsule. Pellets were embedded in Epon 812 and blocks hardened at 60°C overnight. Thin sections (50-60 nm) were cut in a Reichert Ultramicrotome, mounted on clean copper grids, and contrasted with uranyl acetate/lead citrate stains. Thin sections were observed in a Phillips EM 300 at an accelerating voltage of 60 kV. EM chemicals were obtained (unless otherwise specified) from Marivac LTD, Halifax, Canada.

**Immunogold labeling of whole cells for negative staining.**

A modification of the immunonegative stain technique of Beesley (1989) was routinely used. Briefly, bacterial cells suspended in PBS were mounted on formvar coated grids. Grids were blocked with 1% skim powdered milk in PBS, washed in PBS, and floated on drops of rabbit anti-A-protein serum diluted 1:100 in PBS containing 1% bovine serum albumin (PBS/BSA). After a 2 h incubation at room temperature, grids were washed in PBS/BSA and floated for 1 h., at room temperature, on drops of protein A-gold probe (15 nm AuroProbe™ gold label protein A, Amersham International) diluted 1:50 in PBS/BSA. Grids were washed in PBS and fixed in Ruthenium Red fixative (see above) before negative staining with ammonium

molybdate. The rabbit anti-A-protein serum was provided by W.W. Kay, Department of Biochemistry and Microbiology, University of Victoria, Canada.

### **Survival curves**

The survival of *A. salmonicida* at 37°C, in PBS or LB, was determined by direct viable cell counting. MØs were also tested for survival in PBS. Aliquots of mØs suspended in PBS at 37°C, were counted in the presence of Trypan Blue. MØs stained in blue, unable to exclude the stain, were considered non-viable.

### **Purified A-layer and A-protein**

Outer membranes (OM) of *A. salmonicida* A450, prepared by sodium lauryl sarcosinate extraction, were subjected to serial extractions with sodium deoxycholate (DOC), as previously reported in chapter I "Materials and methods used in structural studies". The DOC extraction steps were serially repeated to obtain the corresponding 2X, 3X and 4X DOC preparations. A-protein from *A. salmonicida* A450, purified to homogeneity by previously published methods (Phipps *et al.*, 1983), was obtained from B.M. Phipps (Department of Biochemistry and Microbiology, University of Victoria, Canada). A-layer sheets from the A-layer secreting mutant A450-1 were purified from culture supernatants by centrifugation at 100,000X  $g_{av}$  for 1h, followed by a single DOC extraction.

### **A-layer reconstitution**

*A. salmonicida* strains A450-3 and A450-1 were co-cultivated in a mixed lawn on TSA plates. Suspensions of the two strains to an  $OD_{650\text{ nm}}$  of 1, were prepared in PBS, and mixed in an A450-3 to A450-1 ratio of 1.5:1. One hundred  $\mu\text{l}$  of this mixture were spread on a TSA plate and incubated for 72 h at 20°C. Bacterial growth was scraped off from the plate and washed 2X in PBS. Bacterial cell density was adjusted as needed. Reconstitution in liquid phase was similar to the method of Griffiths and Lynch (1990). Washed A450-3 cells suspended to 1  $OD_{650}$  in an A450-1 culture supernatant, were gently agitated for 3 h at room temperature. Alternatively, 0.4 mg of the 4X DOC A-layer preparation were added to 1 ml of the A450-3 cell suspension and gently agitated for 1 h at room temperature.

### **Coating of latex beads with A-layer or A-protein**

Latex beads of 3.1  $\mu\text{m}$  diameter (SERADYN, Particle Technology Division) were extensively washed with glycine buffered saline (0.1 M glycine, 0.85% NaCl, pH 8.4). The suspension of beads was adjusted to a density of 1% solids ( $\sim 5.6 \times 10^8$  beads/ml), and different A-layer preparations (1X DOC, 3X DOC and A450-1 layer), or purified A-protein, were added, to a final concentration of 50  $\mu\text{g}/\text{ml}$ . Coating was allowed to occur for 3 hours at room temperature, under continuous gentle agitation. Beads were then washed with PBS twice, and stored on ice. Confirmation of protein coating and purity was made by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of 25  $\mu\text{l}$  were boiled for 10 minutes with 9  $\mu\text{l}$  4X sample buffer. After centrifugation in an Eppendorff microcentrifuge, 20  $\mu\text{l}$  of the liquid beneath the floating beads were loaded on a 12% gel for SDS-PAGE.

### **Bacteri $\alpha$ -host cell association assays**

#### **Murine m $\phi$ s.**

*Assays with bacteria.* M $\phi$ s were plated in 35 mm tissue culture dishes, each containing a 22 x 22 mm glass coverslip attached to the bottom, at a ratio of 1 million m $\phi$ s/dish. Alternatively, 24-well tissue culture plates were inoculated with 0.5 million m $\phi$ s/well. Dishes or plates were incubated for up to 4 hours at 37°C, to allow attachment of m $\phi$ s, or overnight, to allow attachment and growth of m $\phi$ s on the substratum. Before the experiment, m $\phi$ s were washed twice with PBS and covered with 1 ml of fresh incomplete medium or PBS. Adherent m $\phi$ s were scraped from two dishes or wells, to determine their number. Suspensions of washed bacteria in PBS were adjusted, so that 100  $\mu\text{l}$  contained a number of bacteria equal to 50 times the number of m $\phi$ s per dish or well.

Assays were initiated by inoculation of the dishes or wells with 100  $\mu\text{l}$  of the corresponding bacterial suspension. In assays with 24-well plates, the inoculation of wells was done sequentially (at the corresponding sampling times), so that at the end of the experiment all the wells in a single plate were processed together.

Two 35 mm dishes were usually processed at every sampling time. Supernatants from the two dishes were removed (and sampled when necessary), and the dishes washed 3X with PBS. M $\phi$ s in one dish were lysed in 1 ml of deionized water for 10 min., with agitation. The m $\phi$  lysate (1 ml) was collected and the dish washed 3X with 3 ml of deionized water. Washes were collected with the lysate to

produce a 1:10 dilution. The second dish was air-dried and the coverslip stained and prepared for direct microscopy counts on monolayers. At the end of the experiments conducted in 24-well plates, supernatants from all the wells in a plate, were removed and the plate washed 3X with PBS using a wash bottle. Plates were air dried and stained. Well bottoms were cut with a cork boring machine and mounted on a glass slide for direct microscopy counts on monolayers. All association assays were performed at 37°C.

**Assays with coated latex beads.** Phagocytosis experiments with latex beads were done in PBS and using 24-well tissue culture plates as described in "Assays with bacteria". The bead to mØ ratio was adjusted to 5:1.

**Frustrated association/phagocytosis assay.** This assay was prepared as a regular bacteria-mØ association assay in PBS using 35 mm dishes, but with the following modifications. Half of the coverslips were coated with purified A-layer: a 1 ml suspension of 3X DOC A-layer (140 µg/ml) in deionized sterile water was allowed to dry at room temperature on each coverslip. Then coverslips were covered with 0.5 ml of an A-layer suspension in PBS (70 µg/ml), for 4 h. Control coverslips were similarly treated with deionized water and PBS. Coverslips were then washed with excess PBS, inoculated with  $5 \times 10^5$  mØs, and incubated for 4 h, prior to replacement of culture medium with 1 ml of an A450 suspension in PBS adjusted to give a bacteria to mØ ratio of 50:1. After 3 hours, dishes were washed in PBS and processed for direct microscopy counts in monolayers.

**A. salmonicida cytotoxicity to mØs.** Cytotoxicity was evaluated in adherent mØs through bacteria-induced morphological changes. MØs and bacteria were prepared in PBS as for "Assays with bacteria", but mØs were inoculated on 35 mm dishes with four circular coverslips (10 mm in diameter) attached to the bottom. Two different bacteria to mØ cell ratios were used: 500:1 and 2,000:1. At 2 and 4 h after the addition of bacteria, two coverslips were removed from each dish. One coverslip was air-dried, and processed for light microscopy and the other prepared for SEM.

Also, cytotoxicity towards mØs suspended in PBS was evaluated through mØ viability, as determined by the Trypan Blue exclusion assay (described in the section "Survival curves" above), using bacteria:mØ mixtures at a cell ratio of 100:1.

#### **Rainbow trout mØs.**

**Assays with bacteria.** MØs were gently washed 2X with HBSS and adherent cells from two supported coverslips stained and counted. Based on the mØ

count, bacterial cell suspensions were adjusted, so that 0.5 ml contained a number of bacteria equal to 50 times the number of mØs per coverslip. Supported coverslips were covered with 0.5 ml of the corresponding bacterial suspension in HBSS or in tissue culture medium. At each sampling time, coverslips were gently washed 3X with 3 ml of HBSS, and air dried. MØs were fixed and stained for examination by light microscopy.

In 24-well plates, the number of mØs per well was estimated according to the average yield of adherent to plated cells, previously determined by direct microscopy. MØs were gently washed 2X with HBSS and the removed tissue culture medium replaced with one ml of HBSS or incomplete L-15. Wells were inoculated with 100 µl of the corresponding 1 OD. bacterial cell suspension. Under these conditions the bacteria to mØ ratio was kept between 60 and 100 bacteria to one mØ. The inoculation of wells was done in duplicate and in sequence (at the corresponding sampling times) so that at the end of the experiment, all the wells in a plate were processed together by shaking off the well supernatants and washing the plate 2X with HBSS using a wash bottle. Plates were then air dried and mØs stained. Well bottoms were cut with a cork boring machine and mounted on a glass slide for examination by light microscopy.

**Assays with latex beads.** Phagocytosis experiments with latex beads were done in 24-well tissue culture plates using a beads to mØ ratio of 6:1, and following the protocol described above for "Assays with bacteria".

**Intracellular replication assay.** MØs in suspension ( $10^6$ /ml of incomplete L-15), recovered by the sequential use of discontinuous and continuous Percoll density gradients, were infected with *A. salmonicida* at a multiplicity of infection of 50, and centrifuged ( $500 \times g$  for 20 min. at  $4^\circ\text{C}$ ) to enhance, by pelleting, mØs-bacteria interactions. The pellet was resuspended, and divided into 1 ml aliquots (i.e.  $10^6$  cells), into a series of 14 ml polystyrene tubes (Falcon Laboratories), preconditioned overnight with 10% fresh trout serum in L-15, and washed with PBS, prior to the addition of the infected mØs suspension. After two hours at  $14^\circ\text{C}$ , non-adherent cells and free bacteria, were washed away from the tubes and the time zero samples were taken to determine the basal number of bacterial colony forming units (CFU), and the number of mØs per tube. Adherent infected mØs were then covered with 1 ml of gentamicin/L-15 medium. At two hour intervals, two tubes per tested strain were processed as follows: 9 ml of sterile deionized water were added per tube, vortexed vigorously for 21 sec. and remaining viable bacterial cells determined by the standard

dilution plate method. Extra tubes were sampled at longer intervals to monitor the number of mØs per tube. These were washed once in L-15, air dried, fixed in methanol and stained for cell counting, using light microscopy.

#### **Fish cell lines.**

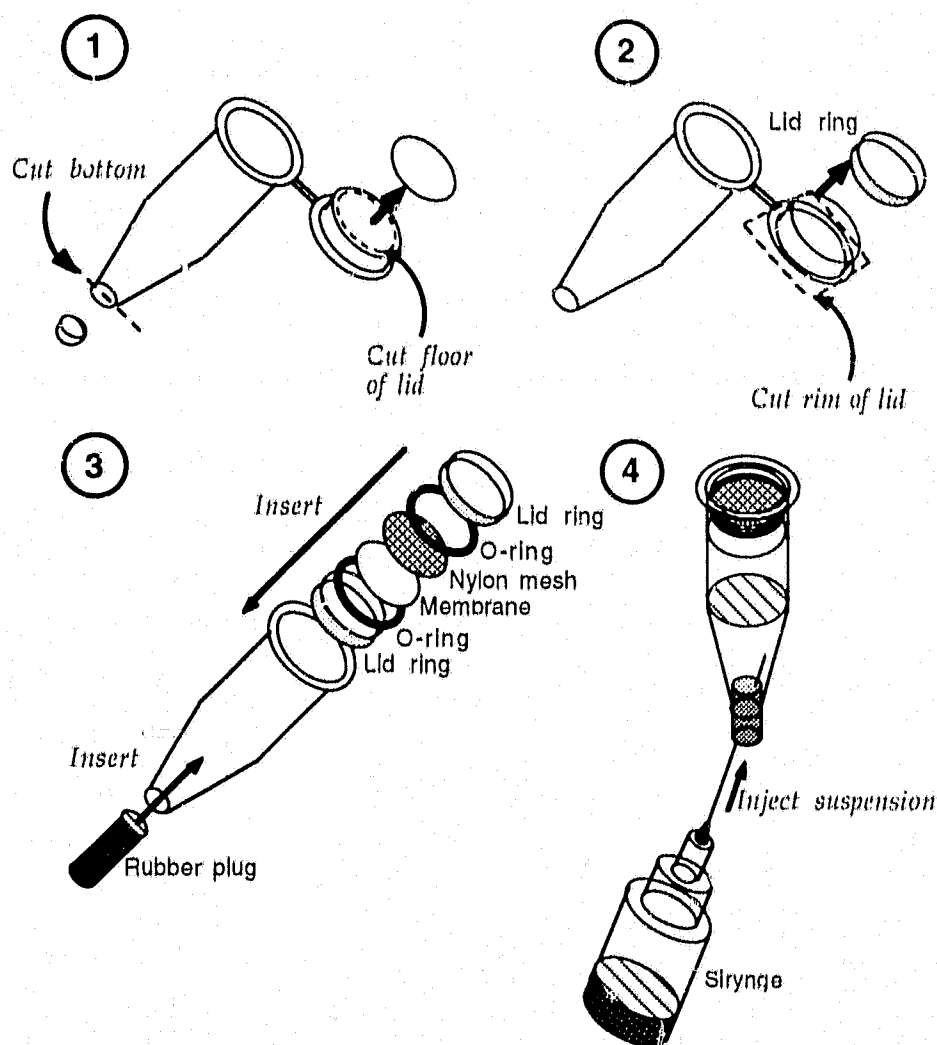
**Assays with bacteria.** The culture medium covering the EPC or CHSE cells in 24-well plates was replaced by 1 ml of HBSS, and 100 µl of the corresponding 1 OD. bacterial cell suspension were added in a sequential way, at the corresponding sampling times. Further processing was done as described above for trout mØs ("Assays with bacteria" in 24-well plates).

#### **Intraperitoneal chambers (IPC) used for *in vivo* growth of *A. salmonicida*.**

Microcentrifuge conical tubes (1.5 ml) (Sarstedt, Newton, U.S.A.), were cut and assembled into IPCs as illustrated in Fig. 19. Autoclaved IPCs were filled by injection of bacterial cell suspensions adjusted to different cell densities, as also illustrated in Fig. 19. The point of injection was disinfected by placing the chambers inside sterile tube caps (Bacti-capall™, Sherwood Medical Industries, England) filled with 70% ethanol in sterile deionized water. Before implantation IPCs were flamed to eliminate any remaining ethanol.

#### **Surgery of rainbow trout**

Rainbow trout of 300-400 g, anesthetized with neutralized M.S. 222 (Wedemeyer, 1969), were held (ventral side up) in a clean styrofoam box with wet sponges, and immediately intubated through the mouth. During surgery (performed inside a laminar flow hood) a gentle flow of cold water (<10°C), containing half strength anesthetic, was constantly passed through the gills with the aid of a peristaltic pump. After mucus removal and skin disinfection with 70% ethanol, a ventral incision of approximately 1.5-2.0 cm was made, and the filled IPC implanted in a horizontal position. Sterile, synthetic polyglactin 910, 3-0 suture (ETHICON Ltd., Peterborough, Canada) was used to close the incision. A solution of 14 µg/ml tetracycline in ethanol was then applied to the sutured incision to minimize post-surgical infections. The complete surgical process was performed in about 10-15 min. Trout were placed in clean aerated water for recovery (assessed by upright swimming) and then transferred to individual tanks receiving a continuous flow of dechlorinated



**FIGURE 19.** Sequential steps in the assembly of intraperitoneal chambers using microcentrifuge tubes. 1. The bottom of the tube was cut (dotted line), and the floor of the lid depression bored with a cork boring tool. 2. The lid's rim was cut (dotted plane), to obtain a lid's ring that tightly fits inside the tube. 3. A circle cut from a Millipore™ membrane, type HAWP 04700 of 0.45  $\mu\text{m}$  pores, and a similar circle cut from a 20  $\mu\text{m}$  pore size nylon mesh (Macroporous filters™, Spectrum Medical Industries Inc., U.S.A.) were sandwiched in between two silicone O-rings, using two lid rings as holders. The bottom of the chamber was then closed with a rubber plug cut from a stopper, by also using a cork boring tool. 4. An autoclaved chamber is being filled with a bacterial cell suspension, with the aid of a syringe. The chamber is held in a vertical position to displace, upon filling, all the air contained in it.

freshwater. Post-surgical fitness reduction was not detected and 24 h after surgery, trout fed normally and continued to feed thereafter. The survival rate after surgery was 100% and the overall post-recovery survival rate was approximately 95%. There were no cases of furunculosis associated with the implantation of IPCs containing the virulent strain A450.

### ***In vivo* grown *A. salmonicida***

*In vivo* grown cells of *Aeromonas salmonicida* were obtained from intraperitoneal chambers surgically implanted in rainbow trout (see above). To follow the *in vivo* growth curve, duplicate fish were sacrificed, at different time intervals, to remove the implants. The IPCs content was used to determine the number of colony forming units (CFU) per ml (by means of the standard plate dilution method) and to prepare specimens for electron microscopy. To routinely obtain *in vivo* grown cells to be used in further assays, IPCs were routinely recovered one week after implantation. Bacterial cells were then recovered by centrifugation, washed once, and resuspended to a cell density of 1 OD. in HBSS or PBS.

### **Challenges with serum and peritoneal fluid**

Bacterial cell suspensions in PBS (1 OD.) were diluted 1:5 in fresh trout serum or fresh trout peritoneal fluid. Samples were taken at different time intervals to determine the number of colony forming units (CFU) per ml by the standard dilution-plate method. Percentage of survival was calculated in reference to the initial number of CFU/ml.

### **Challenges with reduced oxygen species (ROS)**

#### **Liquid phase assay.**

Bacterial cell suspensions (1 OD.) were diluted 1:20 in nutrient broth (NB) (DIFCO). Commercial 3% hydrogen peroxide (Fisher Laboratories) was added to a final concentration of 1 mM to start the challenge. The xanthine/xanthine oxidase system was used to generate superoxide radical, following a modification of the method reported by Hassan (1984). Bacterial cell suspensions (1 OD.) were diluted 1:20 in superoxide buffer, and 3  $\mu$ l of a xanthine oxidase preparation (ICN, from bovine milk) were added to start the challenge. Under these conditions, the rate of superoxide generation was determined to be approximately 3  $\mu$ moles/min. (Hassan 1984).

Superoxide buffer was prepared by dissolving 3 mg of xanthine (ICN) in 10 ml of boiling 70 mM phosphate buffer, pH 6.5. Xanthine solution was cooled down to room temperature and EDTA to a final concentration of 0.1 mM was added.

Samples were taken at different time intervals to determine the number of CFU/ml by the standard dilution-plate method. Percentage of survival was calculated in reference to the initial number of CFU/ml.

The presence of an inducible response to ROS was assayed by exposing the bacterial cells to sublethal concentrations of  $H_2O_2$  (60  $\mu$ M) or  $H_2O_2$  and SNG (1  $\mu$ g/ml), in the presence or absence of 100  $\mu$ g/ml of chloramphenicol. After 1.5 hours a lethal challenge with ROS was conducted. The protective effect of superoxide dismutase (ICN, from bovine erythrocytes), at a final concentration of 80 U/ml, or catalase (SIGMA, from bovine liver), at a final concentration of 1000 U/ml, was also tested by adding, just before the lethal challenge, the corresponding enzyme. In some cases both enzymes were added.

#### **Disc inhibition assay.**

The effect of peroxide was also measured by a modification of the disc inhibition assay reported by Scheilhorn and Hassan (1988). Aliquots (0.5 ml) of a 1 OD bacterial cell suspension were mixed with 3 ml of melted nutrient agar (NA). This mixture was overlaid on freshly prepared 10 ml plates of NA. Sterile paper discs, of 6 mm diameter, cut from a sheet of chromatography paper (Whatman No. 1 CHROM), were placed on the test plates (6 discs per plate) and different amounts of  $H_2O_2$  added onto the discs. The dilution medium was sterile deionized water. Plates were incubated at 20°C for 48 h and the diameter of the inhibition zones measured and processed following the procedures of Cooper (1973).

### **Challenges with intracellular generators of superoxide**

#### **Disc inhibition assay.**

The procedure was basically the same described above for peroxide challenge on plates. In addition, NA supplemented with Congo red (CR), protoporphyrin IX, or hemin were used. In this case, also the melted agar mixed with the bacterial inoculum contained the same concentration of CR or porphyrins than the supporting NA layer. The two redox-active compounds used (SIGMA) were streptonigrin (SNG), and plumbagin (PG). SNG was solubilized in N,N-dimethylformamide (DMF) to produce a stock solution of 1 mg/ml. This was diluted to produce a working solution of 0.1

$\mu\text{g}/\mu\text{l}$  in 50% DMF in sterile deionized water. PG was solubilized in 95% ethanol to produce a working solution of  $5 \mu\text{g}/\mu\text{l}$ .

#### **Liquid phase assay.**

Only SNG was used for challenges in liquid medium. Bacterial suspensions (1 OD.), were diluted 1:20 in NB (approximately  $3.5 \times 10^7$  bacterial cells/ml), and SNG at a final concentration of  $1 \mu\text{g}/\text{ml}$  was added to start the challenge. Mixtures of 0.1 OD. bacterial cell suspensions of A450 and A450-3, or A450-1 and A450-3 in a 1:1 ratio were also challenged. At different times the number of CFUs/ml was determined by the standard dilution-plate method, and the percentage of survival calculated using the original CFUs/ml of the inoculum as 100%. The presence of an inducible response to SNG, and the protective role of superoxide dismutase and catalase, were assayed in the presence or absence of chloramphenicol, as described above for challenges with ROS in liquid phase.

#### **Cytochrome C reduction assay**

Cytochrome C reduction was done by a modification of the procedure of Johnston (1984), originally described for the measurement of superoxide secreted by monocytes and macrophages. Briefly,  $125 \mu\text{l}$  of a  $1.2 \text{ mM}$  filter sterilized solution of cytochrome C (SIGMA, from horse heart), was mixed, in a  $1.5 \text{ ml}$  rectangular semi-micro cuvette (HELLA), with  $300 \mu\text{l}$  of xanthine solution ( $7.6 \text{ mg}/10 \text{ ml}$ , in  $0.07 \text{ M}$  phosphate buffer, pH 6.5), and  $1075 \mu\text{l}$  of  $0.07 \text{ M}$  phosphate buffer containing  $0.1 \text{ mM}$  EDTA. Absorbance at  $550 \text{ nm}$  was recorded (until a stable reading was achieved) and  $1\text{-}3 \mu\text{l}$  of a liquid preparation of xanthine oxidase (ICN, from bovine milk,  $20 \text{ U}/\text{ml}$ ) were added. Absorbance at  $550 \text{ nm}$  was then recorded for a period of  $5 \text{ min}$ . To test the inhibition of cytochrome C reduction,  $5$  or  $10 \mu\text{l}$  of a superoxide dismutase (ICN, from bovine erythrocytes) solution ( $1 \text{ mg}/\text{ml}$ ,  $\sim 3500 \text{ U}/\text{ml}$ ), as well as different amounts of purified A-protein, or A-layer, were added to the reaction cuvette, before the addition of xanthine oxidase.

#### **Hemolysis assay**

Washed suspensions of erythrocytes, obtained from blood of different animal species (PML Microbiologicals, Tualatin, Oregon, U.S.A.), were prepared in PBS at a final density of 5% wet v/v. To test hemolysis these suspensions were diluted 1:5 in fresh trout serum or fresh trout peritoneal fluid, to a final volume of  $100 \mu\text{l}$ , in a 96-

well plate. Control wells were prepared diluting the erythrocyte suspensions 1:5 in PBS. Hemolysis was evaluated by a loss of turbidity and quantified in an automatic plate reader (Dynatech MR500).

## CHAPTER V

### A - LAYER - MEDIATED SPECIFIC INTERACTION OF *Aeromonas salmonicida* WITH MURINE MACROPHAGES

#### INTRODUCTION

It has been observed that virulent A-layer positive ( $A^+$ ) isolates of *A. salmonicida* readily associate with host cells (Parker and Munn, 1985; Trust *et al.*, 1983; Udey and Fryer, 1978). This was thought to be the result of a rather non-specific hydrophobic effect, which has been demonstrated to promote adherence between bacteria and host cells, and in some instances phagocytosis (Absolom, 1988; Magnusson, 1989). Peritoneal elicited exudate cells from rainbow trout, coho and masu salmon were also used to show that phagocytosis of  $A^-$  cells by salmonid mØs is rather poor and that opsonization of  $A^-$  bacteria with specific antibodies and homologous complement, increased phagocytosis (Sakai, 1984).

Despite the above observations there is considerable evidence to the contrary, suggesting that the A-layer is not involved in the bacterial-host cell interaction. Early observations indicated that virulent, strongly agglutinating strains of *A. salmonicida* (presumably  $A^+$ ) were less easily phagocytosed than non-virulent, non-agglutinating strains (presumably  $A^-$ ), by elicited or activated peritoneal mØs from brook trout (Olivier *et al.*, 1986). It was also reported that virulent,  $A^+$  strains of *A. salmonicida* resist phagocytosis by mØs, as compared to  $A^-$  strains (Sakai and Kimura, 1985). Furthermore, it has been suggested that A-layer was not essential to virulence and that a supposedly  $A^-$  mutant of *A. salmonicida* (virulent and autoagglutinating) adhered very effectively to monolayers of cells in culture (Ward *et al.*, 1985). Others suggested that the presence of A-layer did not correlate with enhanced adhesion to rainbow trout blood leukocytes or with virulence (Johnson *et al.*, 1985). In yet another study it was suggested that association of *A. salmonicida* with two different salmonid cell lines was charge-mediated and a role for the A-layer was excluded (Sakai, 1987).

Due to the unavailability of an established mØ cell line from fish, mØs have been isolated from widely different sources by different isolation procedures. To

complicate matters further, a variety of *A. salmonicida* isolates, not always well characterized, have also been used. Therefore, it was decided to use a well characterized murine mØ cell line (P388D<sub>1</sub>) to avoid possible differences in mØ functional competence, which accompanies the above variations, as well as an isogenic set of well-characterized mutants of *A. salmonicida*. This defined system was used experimentally to define the role of the A-layer in the association of *A. salmonicida* with mØs.

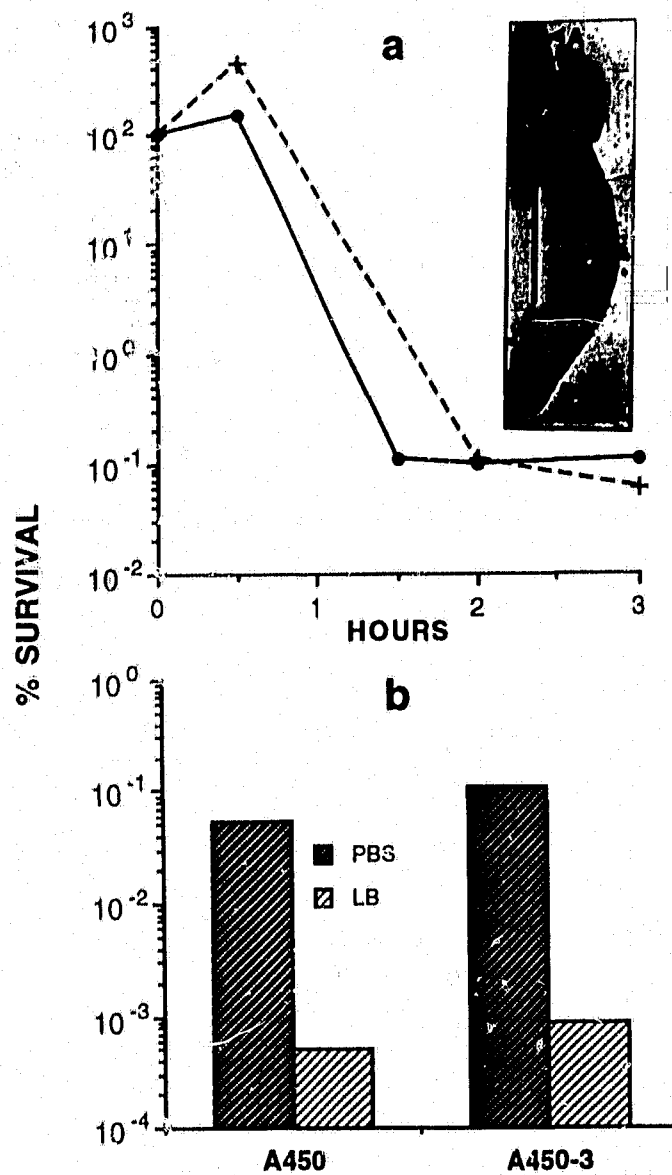
## MATERIALS AND METHODS

Refer to chapter IV.

## RESULTS

### **Effect of experimental assay conditions on murine mØs and *A. salmonicida***

P388D<sub>1</sub> mØs remained 90% viable when suspended in PBS for up to six hours (not shown). SEM showed that, when compared with control mØs kept in complete RPMI-1640, there were no major alterations in the gross morphology of mØs kept in PBS, except for a less spread appearance. On the other hand, incubation of *A. salmonicida* in LB at 37°C caused a rapid decline in viability (Fig. 20a). Microscopic examination of these heat stressed cultures showed that although most bacterial cells were elongated, A<sup>+</sup> bacteria frequently possessed A-layers in the form of caps at the cell poles (Fig. 20a, inset). Bacterial cells suspended in PBS were also killed at 37°C, but not to the same degree (Fig. 20b) as cells in LB medium. Electron microscopy (not shown) revealed that these cells were less elongated (or even not at all), and A-layers were found surrounding most A<sup>+</sup> bacteria. These observations ensured a valid experimental design in which the target cell, the mØ, remained viable and morphologically unperturbed and the adherent cell, retained its surface components under test.



**FIGURE 20.** Survival of *A. salmonicida* at 37°C. (a) Survival curves of A<sup>+</sup> and A<sup>-</sup> cells at 37°C in LB. (●—●), A<sup>+</sup> (A450); and (x---x), A<sup>-</sup> (A450-3). (Inset) Electron micrograph of negatively stained A450 cells incubated at 37°C in LB. A = A-layer cap at one end of the cell. Bar represents 5 μm. (b) Survival of A<sup>+</sup> and A<sup>-</sup> *A. salmonicida* after 24 h at 37°C. Survival was enhanced by keeping the bacterial cells in PBS.

## **Bacteria-mØ association in PBS.**

### **Normal assays.**

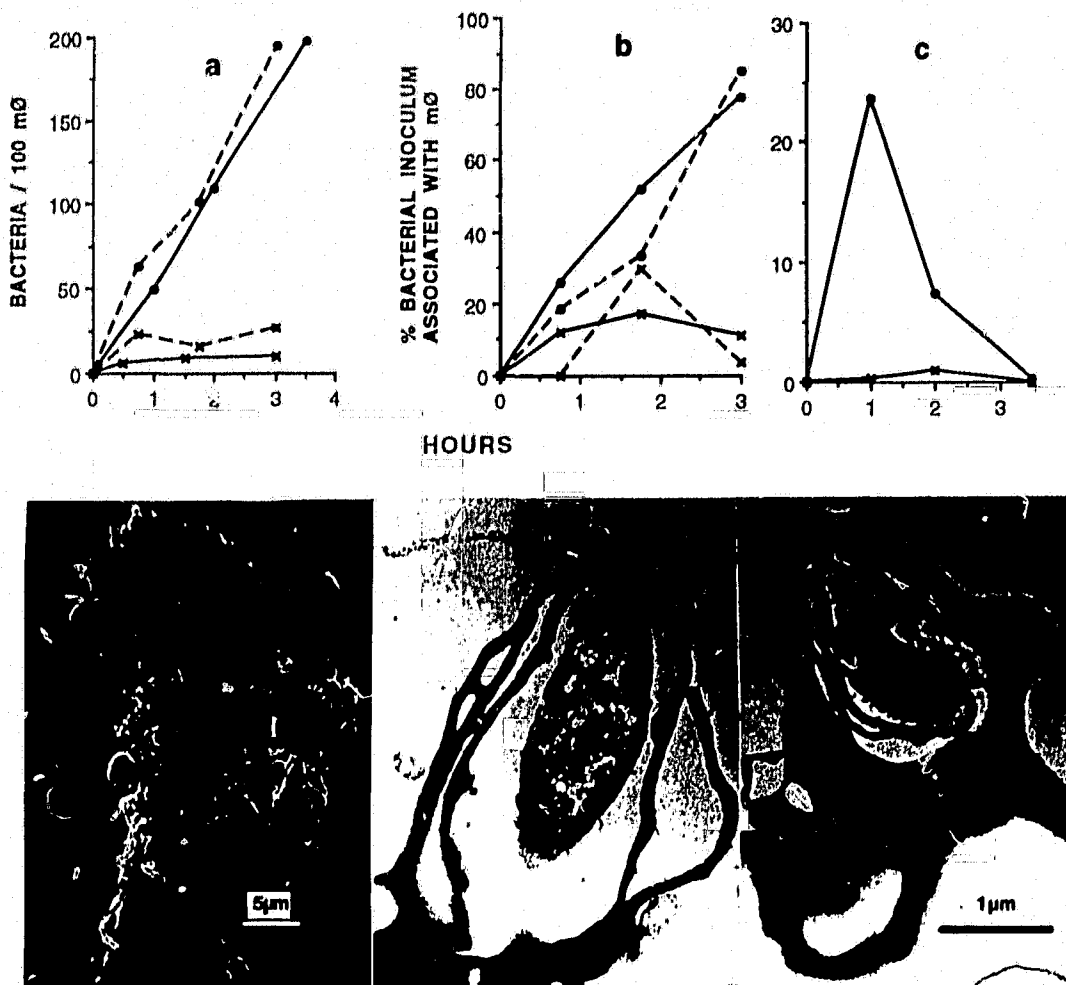
At all incubation times the association of *A. salmonicida* with mØs (as determined through different quantitative methods) was much greater for A<sup>+</sup> compared to A<sup>-</sup> strains (Fig. 21a to c). Specimens observed by SEM showed that A<sup>+</sup> cells readily adhered to mØs and sometimes covered the entire pseudopodial surface (Fig. 21d). TEM revealed that A<sup>+</sup> cells were also readily internalized by mØs in PBS (Fig. 21e and f), while it was not possible to identify any internalized A<sup>-</sup> cells.

### **Forced association.**

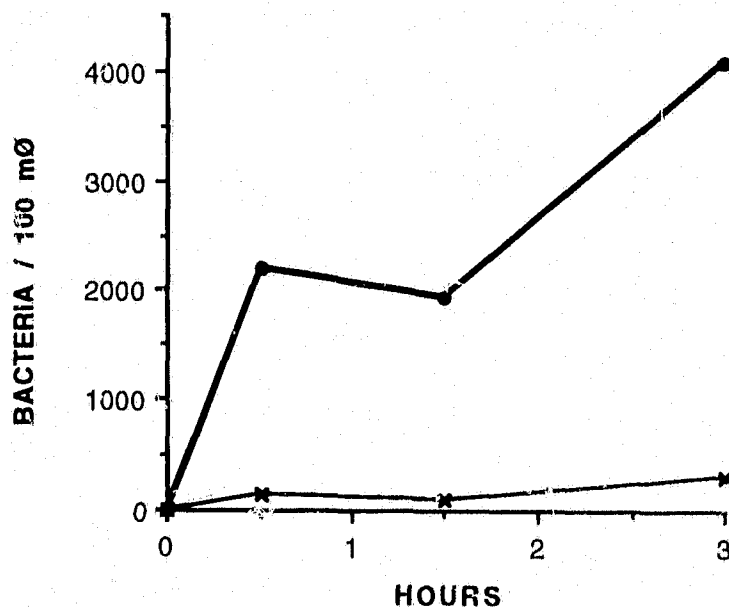
It is known that A<sup>+</sup> *A. salmonicida* cells in suspension, autoagglutinate and consequently sediment considerably faster than A<sup>-</sup> bacteria (Evenberg *et al.*, 1982; Udey and Fryer, 1978). In order to confirm that the higher association levels observed with A<sup>+</sup> cells were not an artifactual result of a faster sedimentation rate, both A<sup>+</sup> and A<sup>-</sup> cell suspensions were centrifuged onto the mØ monolayer and subsequently processed to determine their levels of association with mØs (Fig. 22). The degree of mØ association with A<sup>-</sup> cells was increased, but not nearly as dramatically as for A<sup>+</sup> cells. The overall quantitative levels of mØ association for both bacterial strains were nearly an order of magnitude greater by this procedure. Thus, the difference in association of A<sup>+</sup> and A<sup>-</sup> cells with mØs was not due to a cell availability artifact.

### **Selective adherence.**

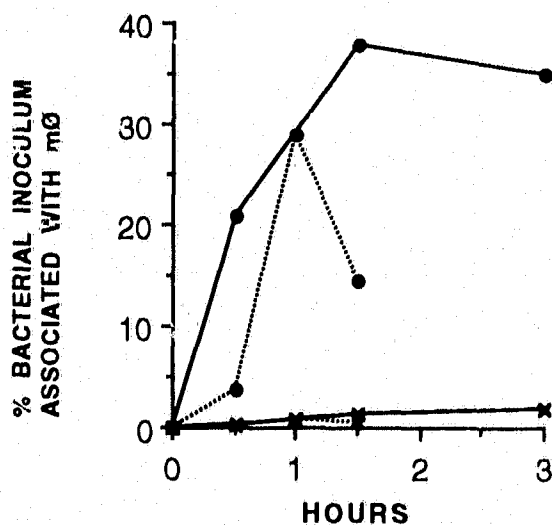
To examine the possibility that extracellular factors from A<sup>+</sup> cells or the association of A<sup>+</sup> cells with mØs, may activate them towards an enhanced association with A<sup>-</sup> cells, A<sup>+</sup>/A<sup>-</sup> coincubation experiments were carried out. Two co-infection experiments were conducted using a 50:1, and 500:1 A<sup>-</sup> to A<sup>+</sup> cell ratios (Fig. 23). Viable cell counts of mØ lysate samples, were used to monitor the level of bacterial association with mØs. A<sup>+</sup> and A<sup>-</sup> cells were discriminated on the basis of Congo Red binding. Due to the lethal effect of the incubation temperature upon *A. salmonicida*, the absolute value of these results can be regarded as an underestimate. Nevertheless, it was still evident that mØs in PBS were unable to associate with A<sup>-</sup> cells, whereas A<sup>+</sup> cells, even in the presence of a large number of A<sup>-</sup> cells, efficiently associated with these same mØs. Thus, the association of A<sup>+</sup> bacteria with mØs was not a cell density-determined artifact, nor did one cell type predispose mØs to associate with the other.



**FIGURE 21.** Association of  $A^+$  (A450) and  $A^-$  (A450-3) bacteria with mØs kept in PBS as determined by three different quantitative methods and EM. (a) Bacterial cells associated per 100 mØs, as determined by direct microscopy counts in mØ monolayers. Two separate experiments are shown: experiment 1 (—); experiment 2 (---);  $A^+$  (●);  $A^-$  (x). (b) Bacteria-mØ association, as determined by direct microscopy counts using the internal standard method. Data was obtained from samples of experiment 2 in 'a', and is expressed as percentages of the bacterial cells added: Counts done in mØ lysate samples (—); counts done in supernatant samples (---);  $A^+$  (●);  $A^-$  (x). (c) Bacteria-mØ association, as determined through viable cell counts. Data was obtained from mØ lysate samples taken from experiment 1 in 'a', and is expressed as percentages of the viable cells added.  $A^+$  bacteria (●—●);  $A^-$  bacteria (x—x). (d) to (f) SEM and TEM of mØs kept in PBS, with bound and internalized  $A^+$  bacteria.



**FIGURE 22.** Forced association of  $\Lambda^+$  ( $\Lambda 450$ ) and  $\Lambda^-$  ( $\Lambda 450-3$ ) *A. salmonicida* with mØs in PBS. Association assays were done in 24-well plates in which bacteria were centrifuged (500 X g<sub>av</sub> for 10 min) onto the mØ monolayer. At the different sampling times, rows of wells were washed three times in PBS, and the mØs kept in fresh PBS until the end of the experiment, when the plate was air dried and processed.  $\Lambda^+$  (●—●);  $\Lambda^-$  (x—x).



**FIGURE 23.** Macrophage association from mixtures of  $\Lambda^+$  ( $\Lambda 450$ ) and  $\Lambda^-$  ( $\Lambda 450-3$ ) strains of *A. salmonicida* at different  $\Lambda^-$  to  $\Lambda^+$  cell ratios. Association with mØs was determined through viable cell counts of mØ lysate samples, on Congo Red agar plates (refer to Fig. VI.2e).  $\Lambda^-$  to  $\Lambda^+$  cell ratio 500:1 (—);  $\Lambda^-$  to  $\Lambda^+$  cell ratio 50:1 (·····);  $\Lambda^+$  (●);  $\Lambda^-$  (x).

## Effect of complex media on bacteria-mØ interactions

When association assays were carried out in tissue culture medium, mØs were particularly found to associate more readily with A<sup>-</sup> bacteria than previously observed (Fig. 24a and b). These more competent mØs also internalized A<sup>-</sup> bacteria efficiently (Fig. 24c), whereas they were unable to do so in PBS. Moreover, there were no apparent qualitative differences in the mechanism by which mØs bound or internalized A<sup>-</sup> and A<sup>+</sup> bacteria. Under these experimental conditions, *A. salmonicida* was internalized by a conventional zipper mechanism (Silverstein *et al.*, 1977), as depicted in Fig. 25.

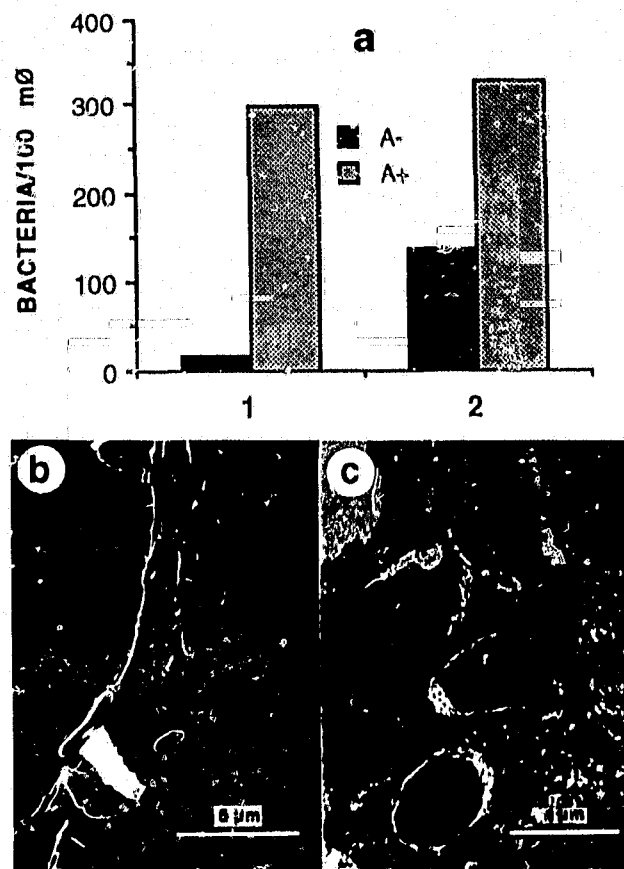
## A-layer or A-protein coated surfaces

### Coated latex beads.

A series of association assays with coated latex beads were conducted to evaluate the contribution of the A-layer alone, to the processes of adherence and internalization. All A-layer preparations used were able to enhance the interaction of latex beads with mØs (Fig. 26a), all the more appreciated considering that uncoated, hydrophobic latex beads are efficiently phagocytosed by mØs, and for this reason are commonly used in phagocytosis assays (Silverstein *et al.*, 1977). I attributed this enhancing effect to the regularly arrayed A-layer, since the uncoated beads and those coated with purified monomeric A-protein, behaved similarly. The A-layer prepared from A450-1 had, as determined by negative stain EM, the greatest degree of order (results not shown), and was the most effective at promoting adherence.

### Coated coverslips.

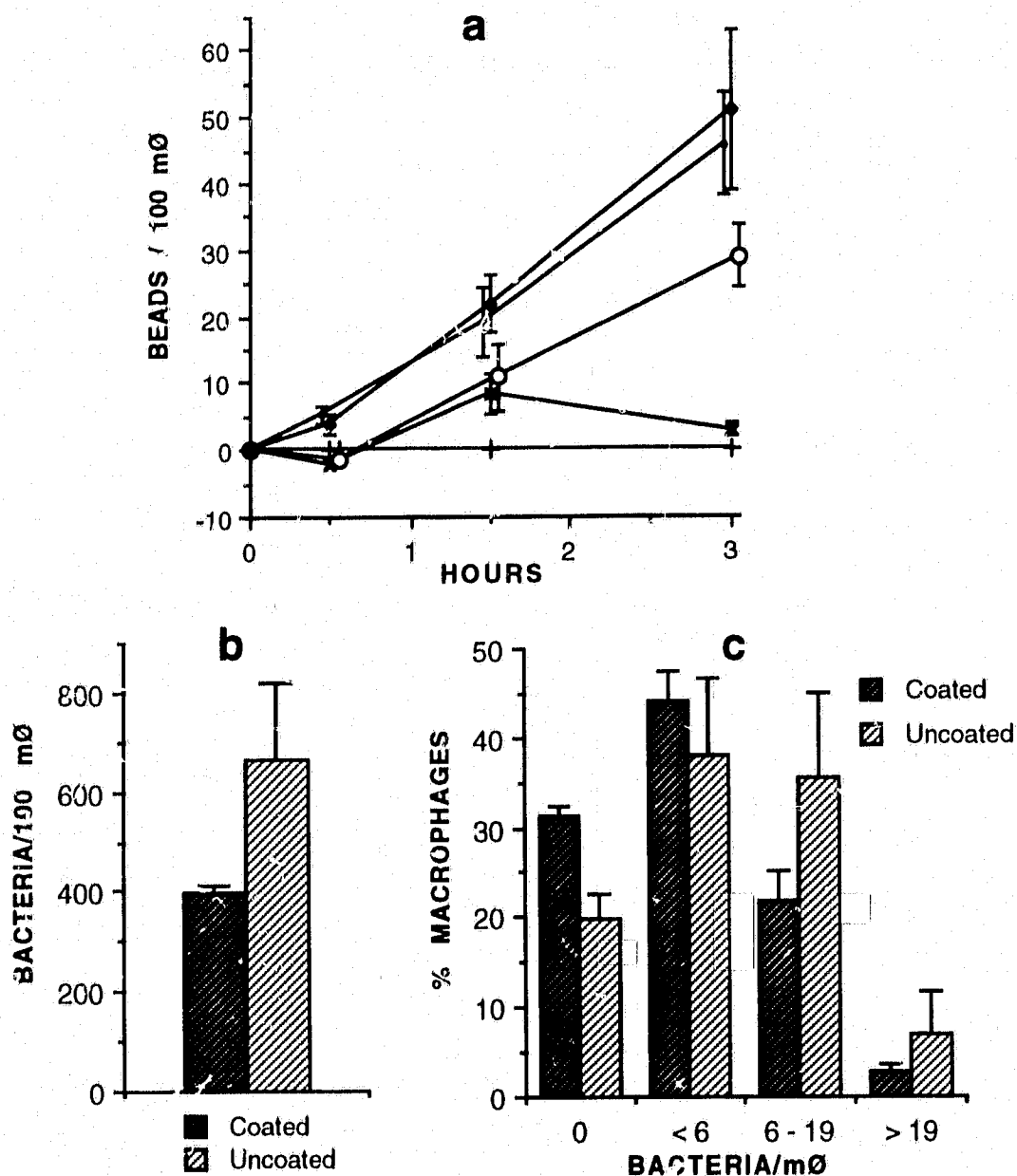
The possible existence of a specific adhesin-receptor interaction between the arrayed layer and the mØ surface was further examined in a frustrated phagocytosis assay. In this type of assay, the presence of putative receptors for a particular ligand is indirectly revealed by recruiting available mØ receptors on a ligand-coated substratum (down-modulation), thereby depleting available receptors and reducing subsequent bacterial cell interaction (Michl *et al.*, 1979; Speert *et al.*, 1988). The global capacity for binding A<sup>+</sup> cells, was reduced 40% in macrophages plated on A-layer coated coverslips (Fig. 26b). This reduction was accompanied by a clear shift in the association profile of A<sup>+</sup> cells to mØs (Fig. 26c) (Speert *et al.*, 1988), indicating that a specific receptor, presumably involved in the adherence process of A<sup>+</sup> *A. salmonicida* to mØs, was down-modulated by the presence of the A-layer coating.



**FIGURE 24.** Association of A<sup>+</sup> (A450) and A<sup>-</sup> (A450-3) *A. salmonicida* with mØs in tissue culture medium, RPMI-1640. (a) A<sup>+</sup> and A<sup>-</sup> bacteria-mØ associations were measured after one hour incubation in PBS (1), or RPMI-1640 (2). The bacteria to mØ cell ratio was 100:1. (b) and (c) SEM and TEM of mØs with bound and internalized A<sup>-</sup> bacteria from (a-2) above.



**FIGURE 25.** TEM reconstruction of the internalization mechanism of *A. salmonicida* by murine mØs in tissue culture medium, RPMI-1640. Bar represents 0.5 µm for the three micrographs.



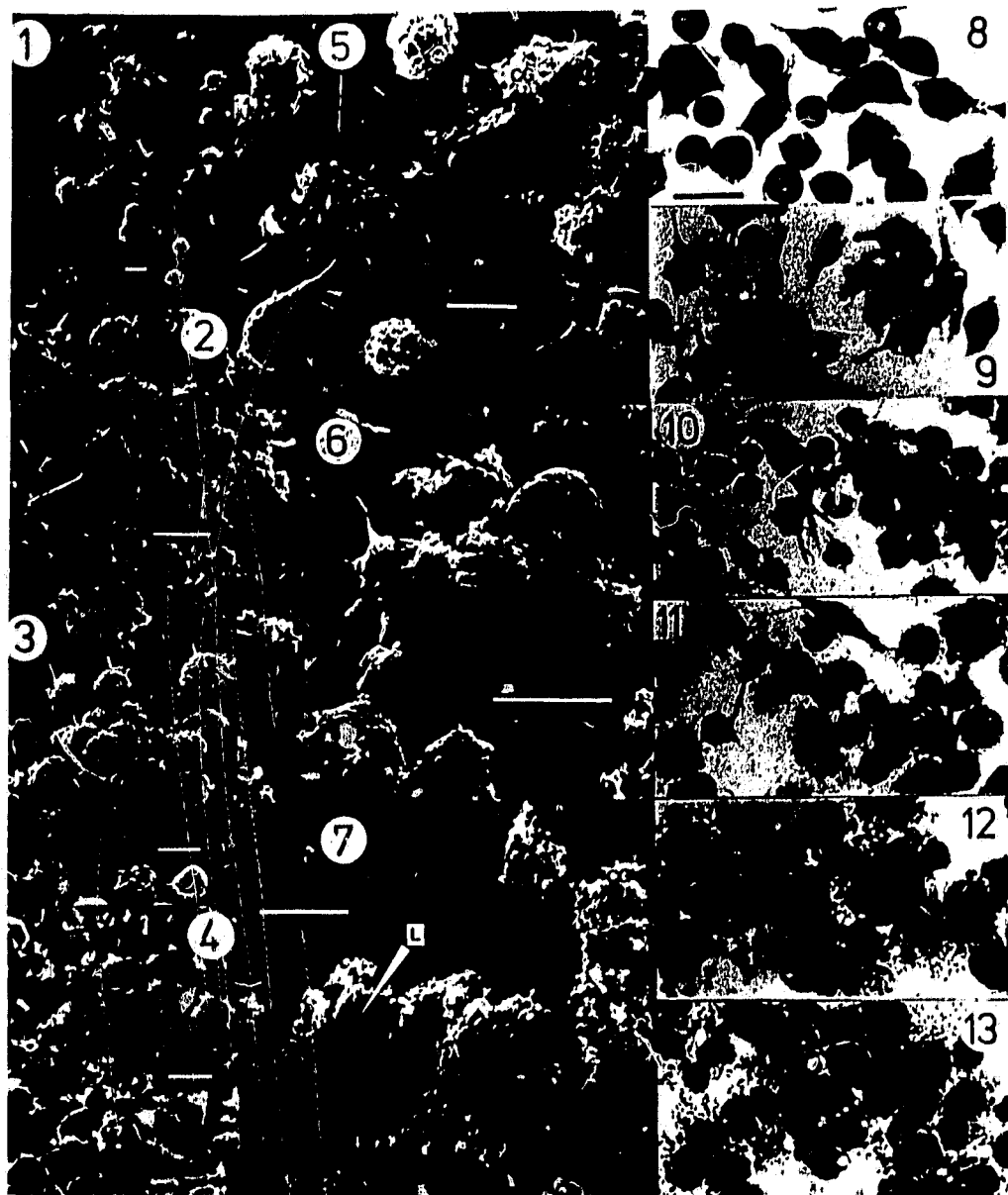
**FIGURE 26.** Macrophage association with particles or substrata coated with A-layer. (a) Association experiments with plain, uncoated latex beads (+), and beads coated with A-protein (x), 1X DOC A-layer preparation (O), 3X DOC A-layer preparation (●), and A-layer sheets isolated from strain A450-1 (◊). The number of uncoated beads/100 mØs was subtracted, at each sampling time, from the rest of the values. Each point represents the average of six independent counts from two separate experiments with triplicate samples. (b) and (c) Results from the frustrated phagocytosis experiment. Each column represents the average of three independent counts from a single experiment with triplicate samples. (b) Association of A<sup>+</sup> bacteria with mØs attached to glass coverslips coated with 3X DOC A-layer preparation, or to uncoated control coverslips. (c) Distribution of mØs according to the number of associated A<sup>+</sup> bacteria.

### ***A. salmonicida* cytotoxicity**

During the association assays, it was observed that mØs exposed to A<sup>+</sup> bacteria appeared more compact (darkly stained), and had a larger proportion of lysed cells, than those exposed to A<sup>-</sup> cells. These observations prompted an investigation of the possible cytotoxic effects of A<sup>+</sup> bacteria, and in particular, the role of the A-layer. Control mØs (bacteria-free) and mØs challenged with either A<sup>+</sup> or A<sup>-</sup> cells (whether they were previously grown in the presence or absence of hemin), contained approximately the same proportion of cells permeable to Trypan Blue after an 8 h challenge (data not shown). However, there were marked morphological differences among adherent mØs treated with the different bacteria used (Fig. 27). The marked effects of A<sup>+</sup> cells upon adherent mØs were confirmed by light microscopy (Fig. 27-12 and 13), in contrast to the less severe effects induced by A<sup>-</sup> bacteria (Fig. 27-8 to 11). Interestingly, SEM specimens indicated that A<sup>+</sup> bacteria induced a marked rounding of mØs and smoothing of their surface, presumably due to severe cytoskeletal modifications (Fig. 27-4 and 6). This effect was also noticeable in mØs exposed to A<sup>-</sup> bacteria (Fig. 27-5), but only at an unusually high bacteria to mØ cell ratio of 2,000:1, or at longer incubation times. Similar rounding and cratering effects were also seen when mØs were challenged with A<sup>-</sup> bacteria grown on TSA/hemin plates. However, the morphological changes induced by A<sup>-</sup> bacteria were never as extensive as those induced by A<sup>+</sup> bacteria, and most mØs exposed to A<sup>-</sup> bacteria remained intact (Fig. 27-3, 10 and 11). Only hemin coated A<sup>+</sup> bacteria induced massive lysis in some mØ monolayers. Macrophages that had not yet lysed in these monolayers, resembled golf balls, with "craters" on their surface, and were densely covered with bacteria (Fig. 27-6 and 7). Of special interest was the appearance of apparent "cytoskeleton ghosts", presumably consisting of a preserved cytoskeletal structure still attached to the substratum but devoid of a cell membrane ("CG" in Fig. 27, micrographs 5 and 7). There was a good correlation between the light microscopy images and the SEM detailed observations.

### **DISCUSSION**

The use of a well defined system to study the role of the A-layer in mØ association appears to be justified, on the basis that perhaps, the wide discrepancies in



**FIGURE 27.** Morphological changes induced by *A. salmonicida* upon adherent mØs, as detected through SEM (1 to 7), and light microscopy (8 to 13). Macrophages were challenged with different bacterial preparations, at different cell ratios and for different times. (1 and 8), control mØs kept in complete RPMI-1640 (note the prominent spreading); (2 and 9), control mØs kept in PBS for 3 h (mØs are more rounded and less spread, but still displaying a rough surface covered with microvilli, blebs and ruffles); (3 and 10), mØs in PBS exposed for 3 hours to A<sup>-</sup> cells (A450-3) at a bacteria to mØ ratio of 500:1; (4 and 12), mØs in PBS exposed for 3 hours to A<sup>+</sup> cells (A450) at a bacteria to cell ratio of 500:1; (5), detail of some mØ morphological changes induced by A450-3 after 3 hours in PBS, at a bacteria to mØ ratio of 2,000:1; (6, 7 and 13), mØs in PBS exposed for 3 h to A450 previously grown on TSA/hemin plates, at a bacteria to mØ ratio of 500:1; (11), mØs in PBS exposed for 3 h to A450-3 previously grown on TSA/hemin plates, at a bacteria to mØ ratio of 500:1 (note the larger proportion of lysed to normal cells, as compared with micrograph 10). Bars represent 10 µm in all micrographs. All light microscopy micrographs have the same magnification shown in micrograph 8. CG = cytoskeleton ghost, L = lysed mØ.

the literature regarding this role are due to differences in the source of mØs, isolation methods, culture conditions and bacterial strains used. A well characterized mammalian cell line and isogenic *A. salmonicida* strains were used to overcome these disparities, but a problem of incubation temperatures arose. The growth temperature for *A. salmonicida* lies between 20-25°C, or up to 30°C for A<sup>-</sup> bacteria (Ishiguro, 1988; Ishiguro *et al.*, 1981), whereas the optimal temperature for murine mØs is 37°C. A temperature of 37°C was used in all association or cytotoxicity assays to keep the mØs under more favorable conditions. At the same time, the continued presence of A-layer was confirmed for A<sup>+</sup> bacteria by electron microscopy and by SDS-PAGE. Moreover, the A-layer has proved to be structurally stable at temperatures of up to 50°C (Phipps and Kay, 1988), assuring that no significant compromise in the adherence of *A. salmonicida* would take place due to major alterations on its surface.

Using different approaches it was evident that the A-layer of *A. salmonicida* is involved in adherence to the mØ cell surface, and may lead to internalization. This effect, which is analogous to the role of some characterized adhesins of bacterial pathogens (Falkow, 1991; Isberg, 1991), was not merely the result of a non-specific hydrophobic effect, as demonstrated in the latex beads and frustrated phagocytosis experiments. Neither was it due to a cell density-mediated process, as demonstrated in the mixed strains assay; nor to a secondary artificial effect of bacteria or particle availability, as demonstrated in the mixed strains, the forced association, and the latex beads experiments; nor was it due to the intervention of bacterial components different from the A-layer, as demonstrated by the latex beads assay.

In the assays with latex beads, only the natively arrayed layer was competent in mediating enhanced adherence to mØs. Interestingly, specific binding of immunoglobulins by the A-layer also requires the presence of assembled A-layer (Phipps and Kay, 1988), suggesting, together with the results from the frustrated association assay (indicating the involvement of a receptor in A-layer binding), the possibility that the putative receptor which interacts with the A-layer may be a leukocyte adhesion molecule of the immunoglobulin superfamily (Albelda and Buck, 1990). Cell-adhesion molecules containing immunoglobulin-like domains (Anderson, 1990) have been identified as receptors for rhinoviruses (Greve *et al.*, 1989; Staunton *et al.*, 1989) and poliovirus (Mendelsohn *et al.*, 1989). Differences observed in the adherence of beads coated with different A-layer preparations suggest that specific properties of the assembled layer make it more or less suitable as an adhesin. These could include

high order (as in the A450-1 A-layer preparation), content of LPS O-polysaccharide, (higher in the 1X DOC preparation and absent in the A450-1 A-layer), or the presence of the novel BS A-layer conformational pattern (refer to chapter III) (observed in the 3X DOC preparation).

The best evidence for the adhesion role of the A-layer was obtained from experiments carried out in PBS, an energy poor medium, apparently unfavorable for the normal phagocytic process. The efficient internalization of A<sup>+</sup> bacteria inside multilayered membrane lined phagosomes (Fig. 21e and f), indicated the presence of an unusual, pathogen-directed mechanism that operates even in the absence of a normal phagocytic process; a situation similar to that observed for *Legionella pneumophila* (Halablab *et al.*, 1990) and *Salmonella* (Finlay and Falkow, 1989). If *A. salmonicida* uses a pathogen-directed internalization mechanism to penetrate macrophages, it may also be able to use it in the penetration of non-phagocytic cells, as has been demonstrated for some invasive intracellular pathogens (Gaillard *et al.*, 1991; Isberg, 1991; McGee *et al.*, 1988; Sansonetti, 1992; Weel *et al.*, 1991) and suggested for *Salmonella* (Finlay and Falkow, 1989). Preliminary results with non-phagocytic fish cell lines, support this contention (refer to chapter XI). In respect to this, the unique ability of the A-layer to bind hemin (Kay *et al.*, 1985) may be related to penetration of non-phagocytic cells as it has been demonstrated for *Shigella flexneri* (Daskaleros and Payne, 1987). Observations here support the view that hemin coated bacteria interact more efficiently with murine mØs, producing a more marked cytotoxic effect.

A<sup>-</sup> *A. salmonicida* cells were fairly efficiently internalized by competent mØs kept in RPMI-1640 medium. Under these favorable conditions, A<sup>+</sup> and A<sup>-</sup> bacterial cells were similarly internalized following a conventional zipper mechanism (Fig. 25), presumably carried out as a lectinophagocytosis process (Ofek and Sharon, 1988). The lack of a complete A-layer in elongated A<sup>+</sup> bacteria kept in RPMI-1640, probably accounted for this similarity. Importantly, internalization of A<sup>-</sup> bacteria, lacking the A-layer adhesin, exclusively depended on this conventional phagocytic mechanism, which only occurred efficiently in competent mØs. These results may be interpreted to imply that *in vivo*, both A<sup>+</sup> and A<sup>-</sup> *A. salmonicida* cells are efficiently internalized by phagocytic cells; a view supported to some degree by infection kinetic studies in fish (Kay *et al.*, 1986). Thus the fact that A<sup>+</sup> bacterial cells are able to survive in the *in vivo* environment, whereas A<sup>-</sup> cells are rapidly cleared (Kay *et al.*, 1986), suggest that the A-layer assists *A. salmonicida* survival within the phagocytic cell, as it has been

also reported by Graham *et al.* (1988) and Olivier *et al.* (1986). The A-layer is known to resist proteases, some of which would likely be encountered within the phagolysosome (Kay *et al.*, 1986). It also has been shown to be involved in resistance to oxidative killing (Karczewski *et al.*, 1991; and results presented in chapter VII).

Despite the increased alterations in m $\phi$  morphology produced by A<sup>+</sup> bacteria, several observations indicated that the A-layer itself was not cytotoxic. During phagocytosis of uncoated latex beads, as well as A-protein and A-layer coated ones, there were no observed differences in m $\phi$  morphology. Also, in the frustrated phagocytosis assay, m $\phi$ s in direct contact with an A-layer coated substratum did not exhibit any apparent morphological differences, neither was there evidence of a larger number of lysed cells, when compared to m $\phi$ s plated on control coverslips. Two well characterized adhesins of pathogens, the invasins from *Yersinia pestis*, and internalin from *Listeria monocytogenes*, have also been demonstrated to be independent of cytotoxic activities (Gaillard *et al.*, 1987; Kuhn *et al.*, 1988; Rosqvist *et al.*, 1990). For *A. salmonicida*, the cytotoxic factor could be one or several of its cytolysins (Ellis, 1991). Since A<sup>-</sup> bacteria still produce these cytolysins (Lee and Ellis, 1990; Price *et al.*, 1989; J.C. Thornton, personal communication), the observed difference in cytotoxic efficiency between A<sup>+</sup> and A<sup>-</sup> bacteria was clearly associated with adherence and perhaps, a more focused targeting of cytolysins to the surface (Ofek *et al.*, 1990; Rosqvist *et al.*, 1991) or the intracellular region of the m $\phi$  (Clerc *et al.*, 1987; Cluff *et al.*, 1990; Mouallem *et al.*, 1990). The great difference in cytotoxicity observed between attached and suspended m $\phi$ s, may be explained by the fact that attachment of phagocytes, as well as the type of substratum they attach to, profoundly affect their functions (Ginis and Tauber, 1990; Laurent *et al.*, 1991), including opsonic (Newman and Tucci, 1990) and non-opsonic phagocytosis (Kluftinger *et al.*, 1989).

In summary, I have demonstrated that the A-layer acts as a specific adhesin positively implicated in binding to and penetration of murine m $\phi$ s, as well as in targeted cytotoxicity. Owing to the lethal effect that the incubation temperature had upon *A. salmonicida*, further experimentation on survival inside macrophages or behavior of internalized bacteria was prevented. Therefore, to continue this line of investigation it was necessary to translate the present findings to a more relevant system, i.e. the use of salmonid macrophages with no compromise in incubation temperature.

## CHAPTER VI

### A - LAYER - MEDIATED INTERACTION OF *Aeromonas salmonicida* WITH RAINBOW TROUT MACROPHAGES

#### INTRODUCTION

The results presented in chapter V demonstrated that the A-layer is capable of mediating attachment to and penetration of murine macrophages (mØs), through a specific mechanism. Furthermore, only the assembled layer was effective, yet different preparations of assembled A-layer varied in efficacy, suggesting that specific properties of the A-layer (amount of associated LPS O-chains, proportion and type of structural arrays present, and layer's orientation) may modulate its adhesin-like activity.

Besides introducing a mØ system more relevant for studies with *A. salmonicida*, it was important to study the effect that some surface structural modifications, especially those affecting the A-layer, have on the ability of *A. salmonicida* to associate with rainbow trout mØs.

#### MATERIALS AND METHODS

Refer to chapter IV.

#### RESULTS

##### Isolation and culture of trout mØs

A method was developed to isolate tissue mØs, from the head kidney of rainbow trout, by using continuous density gradients of Percoll™. The method was described in detail in chapter IV. Isolation of mØs from head kidney tissue, by the original method of Braun-Nesje *et al.* (1981) (or modifications of it) is well established (Secombes, 1990). The cells routinely isolated were adherent, capable of

phagocytosing bacteria, remained in culture for up to one week, had a cell morphology very similar to that reported by Braun-Nesje *et al.* (1981) and Secombes (1985), and therefore were confidently considered to be mØs. The use of supported coverslips with free edges proved to have quantitative advantages (chapter IV). Importantly, adherent mØs, confined to the surface of the coverslip, were 100% observable and recoverable for counting purposes, as were the added bacterial cells.

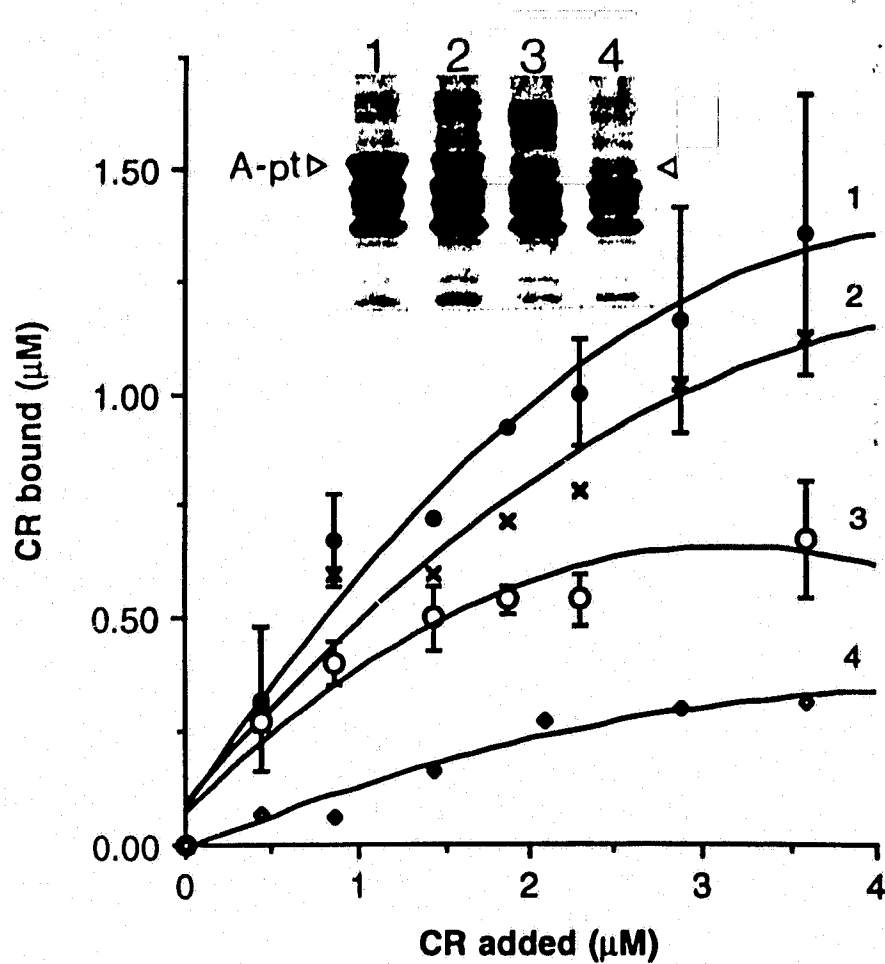
### **Reconstitution of an A-layer onto A<sup>-</sup>, O<sup>+</sup> cells.**

A functionally competent A-layer was successfully reconstituted onto the surface of A450-3 by a novel technique of co-culturing on agar plates. A450-1 produces A-layer sheets but cannot tether them to its cell surface due to the lack of LPS O-chains. These A-layer sheets, formed and released as the culture grew, attached to neighbor A450-3, A<sup>-</sup>, O<sup>+</sup> cells, whose surface acted as an immobilized reconstitution template, producing a mixture of A<sup>-</sup> and reconstituted A<sup>+</sup> bacteria. The presence of cell associated A-layers in reconstituted bacteria was confirmed by the appearance of a distinct band at the A-protein position on SDS-PAGE gels (inset in Fig. 28). The reconstituted A<sup>+</sup> mixture, was shown to be still competent in Congo Red binding assays (Kay *et al.*, 1985) (Fig. 28). Co-culturing the two strains in liquid medium did not provide an effective reconstitution, as judged by SDS-PAGE (not shown).

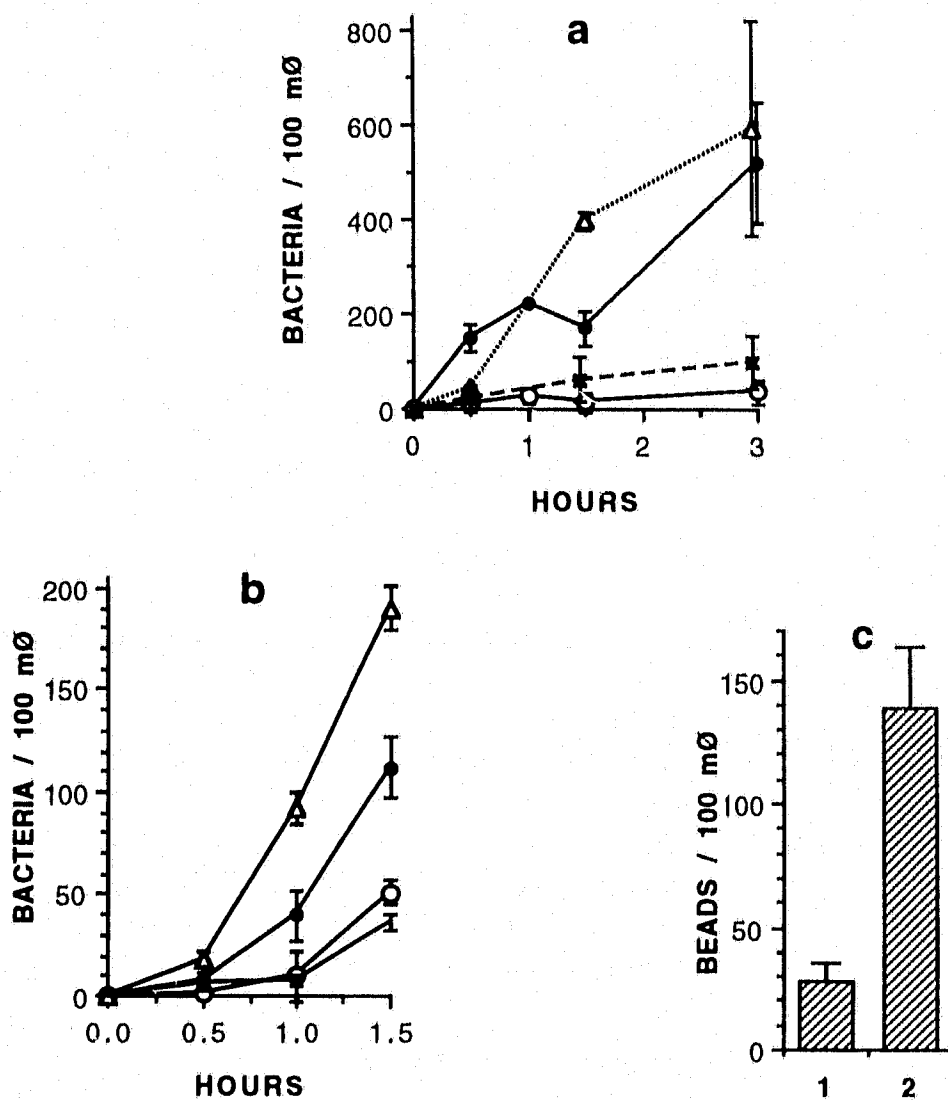
### **A. *salmonicida* surface changes and mØ association**

The use of different *A. salmonicida* strains, each with a particularly different cell surface, permitted the evaluation of the role of predominant surface structures in the bacteria-mØ interaction.

mØ association with wild type A<sup>+</sup>, O<sup>+</sup> bacterial cells, or with co-cultured A-layer reconstituted cells, was significantly greater than that of the A<sup>-</sup> strains A450-1 and A450-3 (Fig. 29a and b). Phenotypically, A450-1 behaves as an A<sup>-</sup> strain since it is unable to tether the A-layers it produces. A<sup>-</sup>, O<sup>+</sup> cells (A450-3) reconstituted in liquid phase with supernatant from an A450-1 culture, were still unable to associate with mØs (Fig. 29b). In contrast, reconstitution in liquid phase with the 4X DOC A-layer preparation resulted in the appearance of a cell associated A-protein band in SDS-PAGE (not shown) and a slightly enhanced mØ association (Fig. 29b). A mixture of A450-3 and A450-1 cells (mixed in a 1.5:1 ratio just before the association assay) still displayed a low association level (Fig. 29b), demonstrating that the enhanced



**FIGURE 28.** Congo Red binding of the A<sup>+</sup> reconstituted co-cultured mixture, as compared with wild type A450, and the separate reconstitution partners A450-1 and A450-3. Curves represent the average of three independent assays. For clarity, standard deviation bars are shown for only two of the curves. (Inset) Whole cell lysate SDS-PAGE analysis of the strains used in the CR binding assays. Lanes in the inset and curves in the graph are similarly numbered for direct identification: 1, A450 (●—●); 2, Reconstituted mixture (x—x); 3, A450-1 (O—O); 4, A450-3 (◊—◊). A distinct band at the A-protein position (A-pt) is observed in the reconstituted bacteria.



**FIGURE 29.** Association of trout mØs with different strains of *A. salmonicida* and  $\Lambda$ -layer or  $\Lambda$ -protein reconstituted cells or latex beads. (a) Macrophage association of the  $\Lambda^+$  reconstituted co-cultured mixture ( $\Delta$ --- $\Delta$ ), as compared with wild type  $\Lambda$ 450 ( $\bullet$ — $\bullet$ ), and the separate reconstitution partners  $\Lambda$ 450-1 (x - - x) and  $\Lambda$ 450-3 (O—O). Curves represent the average of two independent experiments run in duplicate. (b) Effect of different  $\Lambda$ -layer reconstitution methods upon mØ association.  $\Lambda$ -layer reconstitutions of  $\Lambda$ 450-3 were: co-culturing with  $\Lambda$ 450-1 on TSA plates, as in 'a', ( $\Delta$ — $\Delta$ ); reconstitution in liquid phase using the 4X DOC  $\Lambda$ -layer preparation ( $\bullet$ — $\bullet$ ); reconstitution in liquid phase using the supernatant from an  $\Lambda$ 450-1 culture (+ — +). A control consisting of a 1:1.5 mixture of the  $\Lambda$ 450-1: $\Lambda$ 450-3 strains (O—O) was included. (c) Results from a 3 h mØ association assay with plain uncoated latex beads (Column 1), as well as beads coated with  $\Lambda$ -layer sheets from  $\Lambda$ 450-1 (Column 2). Each column represent the average of three measurements in a single experiment.

association of the reconstituted mixture with mØs was specifically due to the reconstitution of an A-layer and not to the mere presence of both cell types. Furthermore, coating latex beads with A-layer sheets from A450-1, resulted in a 5-fold increase in adherence, when compared with plain uncoated beads (Fig. 29c).

### **Effect of A-layer structural modifications**

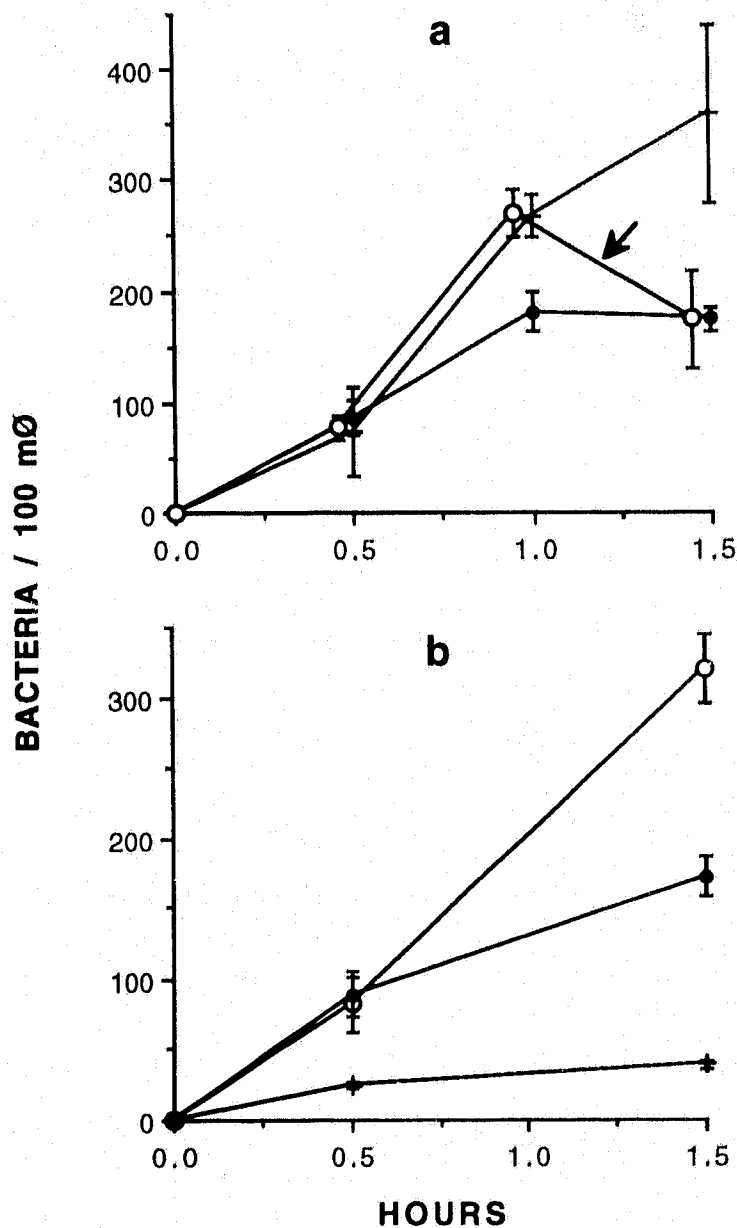
The A-layer of strains A450-10S and A450-10SR were previously shown to exhibit structural alterations (refer to chapter III). The former exhibits WD patterns and the latter discontinuous A-layer aggregates. Furthermore, A-layer aggregation on the surface of A450-10SR is known to occur only after 3 days in culture (Thornton *et al.*, 1991). Younger A450-10SR cultures produce A-layers similar to those of the parental strain A450. In reference to wild type A450 and young A450-10SR cultures, A450-10S consistently showed a reduced capacity to associate with mØs (Fig. 30a and b). However, a dramatically decreased mØ association with older A450-10SR cultures was observed, presumably related to the A-layer aggregation process (Fig. 30b).

### **Effect of hemin coatings on mØ association**

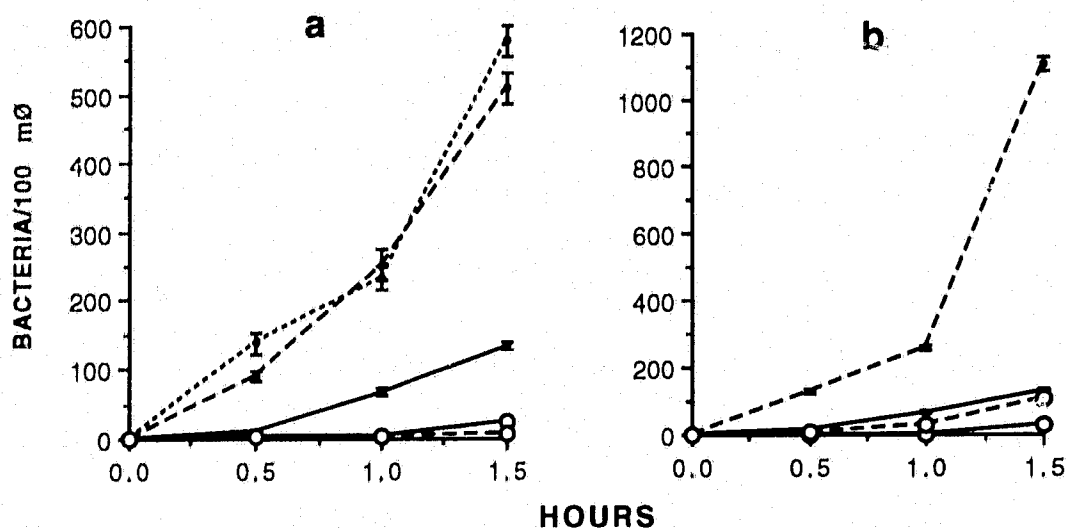
Bacterial cells, coated in liquid phase with hemin or the hemin analog Congo Red (CR) (Kay *et al.*, 1985), were used to study the effect of these coatings on their ability to associate with mØs. While A<sup>+</sup> bacteria coated with hemin associated with mØs better than uncoated cells (Fig. 31a), a CR coating, was ineffective (not shown). Furthermore, A450-3 cells exposed to either of these compounds were still unable to effectively associate with mØs (Fig. 31a). Bacteria grown on TSA/blood plates exhibited much higher levels of mØ association (Fig. 31b), as compared with uncoated or hemin coated bacteria (compare ordinants of Fig. 31). The effect of trout serum components was eliminated as the cause of enhanced adherence, by carrying out a simple association assay with bacteria opsonized in 10% fresh trout serum in incomplete L-15. None of the three bacterial strains tested (A450, A450-3, and A450-10SR) showed a significant increase in mØ association, mediated by the presence of serum alone (Fig. 32).

### **Cytopathic effects of *A. salmonicida* on trout mØs**

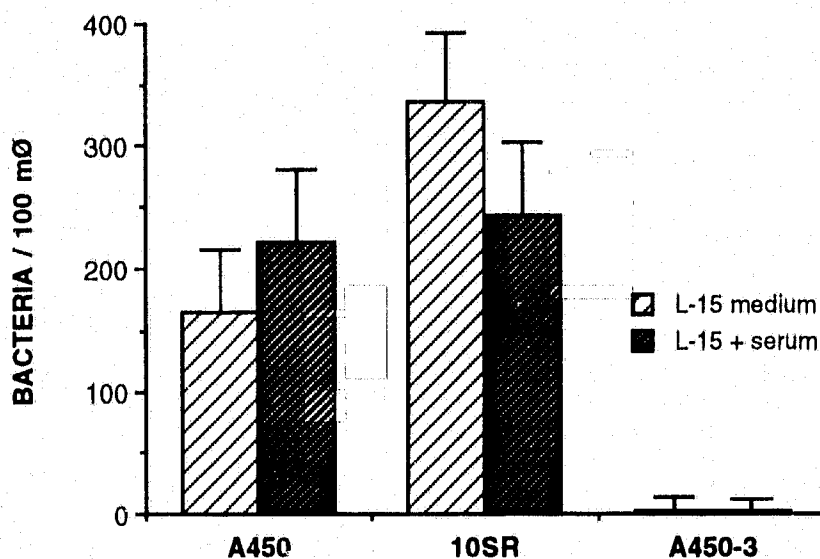
During the bacteria-mØ association assays, it was observed that an increase in the incubation time, as well as in the bacteria to mØ cell ratio (with a consequent



**FIGURE 30.** Macrophage association of two different A450 mutants with structural alterations in their A-layers. (a) Average results from two independent bacteria-mφ association experiments in which fresh TSA cultures were used. (b) Results from an experiment where two week old TSA cultures were used. A450 (o—o), A450-10S (●—●), A450-10SR (+—+). Arrow indicates an apparent decrease in the macrophage association level of A450, presumably due to cytotoxicity of this *A. salmonicida* strain to mφs.



**FIGURE 31.** Effect of different surface coatings in the mØ association levels of A<sup>+</sup>, A450 (●), and A<sup>-</sup>, A450-3 (O) bacterial cells. (a) Effect of hemin coating. Coating was carried out by mixing 1 OD<sub>650</sub> bacterial cell suspensions with hemin at room temperature for 10 min. Hemin at 1 µg/ml (- - -), and 0.2 µg/ml (.....); uncoated strains (—). (b) Effect of growth on TSA/blood plates (- - -), as compared with TSA alone (—). All experiments represented in this figure were performed using mØs from the same batch.



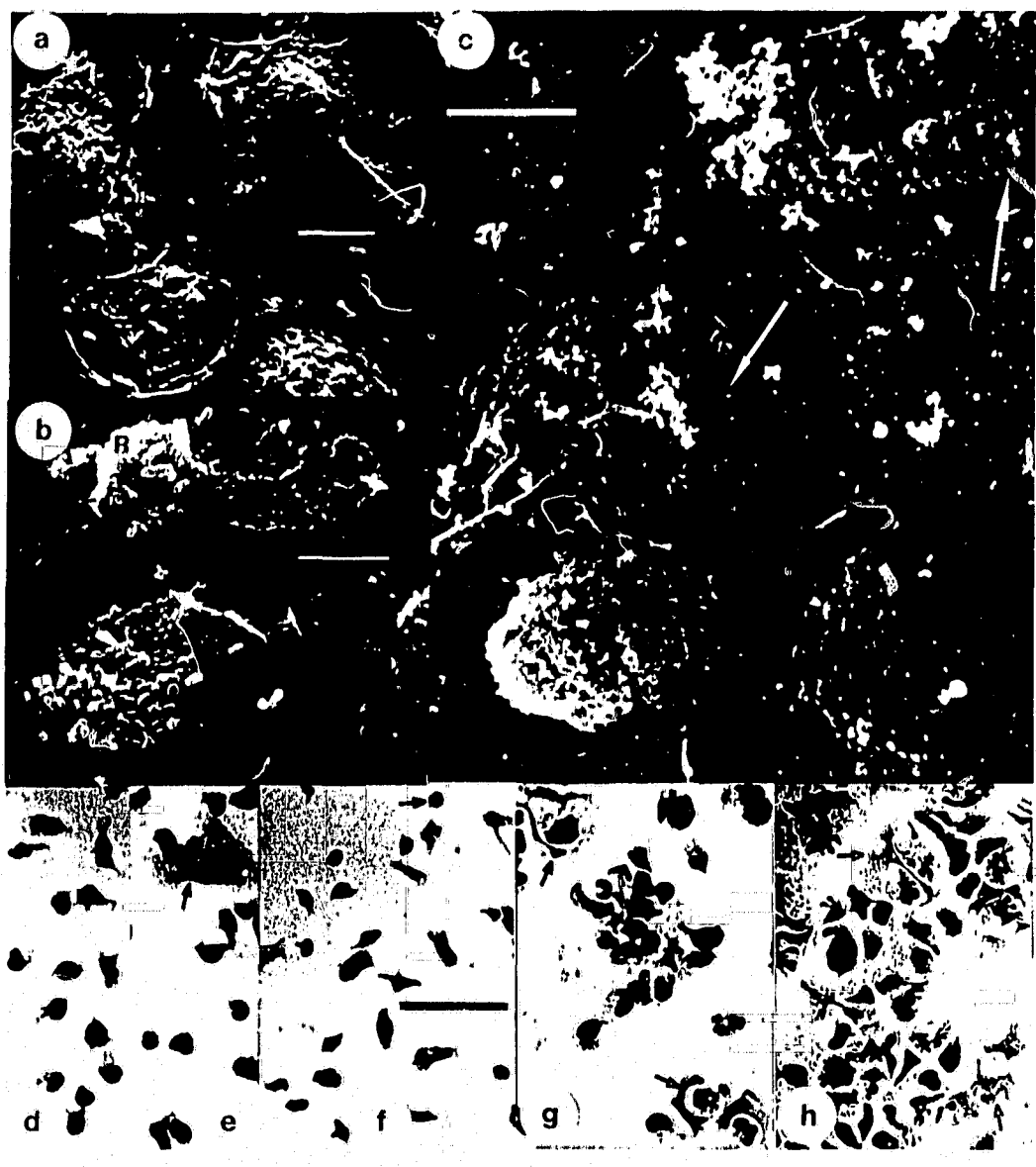
**FIGURE 32.** Effect of opsonization of *A. salmonicida* upon mØ association. Bacteria-mØ association assays, using the A450, A450-10SR, and A450-3 strains, were carried out in L-15 medium with or without 10% non-immune fresh trout serum. Levels of association were determined after 1.5 h.

increase in mØ association levels), was invariably accompanied by an increase in mØ detachment from the substratum, and mØ lysis. As observed before with murine mØs (chapter V), most of the mØs exposed to the A<sup>-</sup> strains A450-3 and A450-1 had well conserved morphological features, similar to those of control mØs not exposed to bacteria (not shown). Cytopathic effects were mostly observed in association with A<sup>+</sup> bacteria, accounting for the abrupt reduction in the mØ association index of A<sup>+</sup> strains (450, A450-10S, and reconstituted A450-3), usually seen in the last sampling time of the association assays (e.g. arrow in Fig. 30a). It is of interest to note that in spite of being an A<sup>+</sup> strain capable of associating with mØs, A450-10SR never showed this reduction in mØ association, suggesting that it was probably not severely cytopathic to trout mØs.

It was evident, from microscopic observations, that *A. salmonicida* also produced morphological changes and lysis of trout mØs (Fig. 33). It was common to observe pronounced cell rounding and compaction ("R" in micrograph b, and arrow in micrograph f). Macrophages exposed to *A. salmonicida* grown in the presence of hemin presented a particularly peculiar morphology (Fig. 33h), interpreted as massive lysis due to cell fragility. In some instances, mØ cell nuclei were also observed to lyse (arrows in micrographs g and h). Macrophages observed by SEM had a bizarre morphology, presumably as the result of lysis during EM processing, due to increased susceptibility to chemical or physical stress. The coverslips prepared for SEM were almost entirely analyzed to determine the frequency of lysis, and I found that the majority of cells exposed to A450 cells had the morphological features presented in micrographs b and c. Similarly, some mØs exposed to A450-3 presented the same features. It was interesting that lysed mØs revealed A<sup>+</sup> cells that had been internalized (arrows in Fig. 33c). Further support for bacterial cell internalization came from studies on the replication of *A. salmonicida* inside trout mØs, in the presence of the aminoglycoside gentamicin (refer to chapter VIII).

## DISCUSSION

The studies presented in chapter V demonstrated that the A-layer was involved in mediating a specific interaction between murine P388D<sub>1</sub> mØs and *A. salmonicida*. These findings have been confirmed in the more relevant salmonid



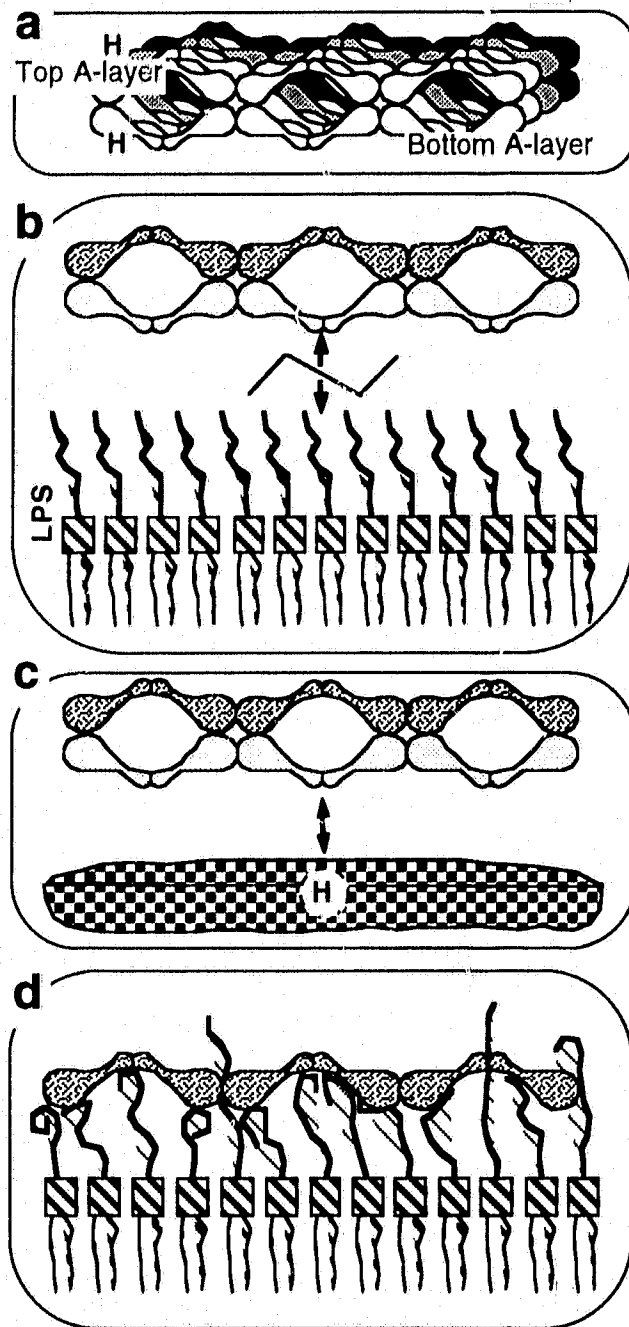
**FIGURE 33.** Effects of *A. salmonicida* upon trout mØ morphology. SEM of control, intact mØs (a) and mØs exposed to A450 for 2 h, at a bacteria to mØ cell ratio of 50:1 (b and c). R = rounded mØ covered with bacteria. Arrows point to exposed internalized bacteria. Bars represent 10 µm. Light microscopy images of control mØs in L-15 (d) and HBSS (e). A multinucleated giant cell (Secombes, 1985) is shown in the latter (arrow). Different mØ morphological changes induced by A450 are shown: pronounced mØ rounding (f), lysis and cell distention (g), and massive lysis (h). All light micrographs have the same magnification, represented by the 50 µm bar in 'f'.

(*Oncorhynchus mykiss*) mØ system, and further studies have been conducted to examine the effects of A-layer structural modifications on this mediated interaction.

The experimental system of rainbow trout mØs cultured on supported coverslips, proved to be particularly useful, and it could easily be adapted and applied to other quantitative studies in which adherent cells in culture are used.

It was confirmed that the predominant bacterial surface structure, the A-layer, acts by facilitating an enhanced interaction with mØs, whereas an intact, smooth hydrophilic LPS layer, by preventing mØ recognition. The *A. salmonicida* strain A450-1, which lacks both of these two main surface components, has a hydrophobic surface, as judged by the adherence to hydrocarbons (Rosenberg, 1984), salting out (Lindahl, 1981), and polymer partition (van Alstine *et al.*, 1986) tests (not shown). The intermediate-low level of mØ association shown by A450-1 (Fig. 29a and b), suggests that non-specific hydrophobic interactions played only a minor role in the association with mØs.

To ideally demonstrate that the A-layer has an adhesin-like role, it would be necessary to follow the so-called molecular Koch's postulates (Falkow, 1988), i.e. the re-introduction of the cloned *vapA* gene into an A<sup>-</sup> deletion mutant, with the consequent regain of adherence to host cells. This has not yet been possible since *vapA* has so far been refractory to expression in *vapA* deletion mutants (S. Chu and T.J. Trust, personal communication). As an alternate approach, I used the *in vitro* reconstitution of A-layer onto the smooth LPS surface of the deletion A<sup>-</sup> strain A450-3 (Belland and Trust, 1987), and demonstrated that the A-layer alone is capable of mediating an efficient mØ recognition. An interesting finding was that reconstitution of A450-3 with culture supernatants containing A-layer sheets from the A-layer secretory strain A450-1, was not particularly effective, as these cells did not recover the ability to associate with mØs (Fig. 29b). From a non-functional perspective, Griffiths and Lynch (1990) reported this method of reconstitution in liquid phase as effective, but the A<sup>+</sup>, O<sup>-</sup> strain that they used released into the culture supernatant what they described as floret-like material. I too, observed this kind of material in negative stained specimens of the 4X DOC A-layer preparation, but not in culture supernatants of A450-1; instead, these contained regularly arrayed A-layer sheets similar to the double layers described by Dooley *et al* (1989). The monolayers that form these double layers interact through their inner faces and thus the normal A-layer polarity is lost (Fig. 34a), accounting for the failure to achieve complete reconstitution of A450-3 cells (Fig. 34b). However, these double



**FIGURE 34.** Rationale of surface reconstitution with A-layer sheets from the A450-1 strain. (a) Simplified diagram showing the external hydrophobicity (H) and the lack of polarity of double A-layer sheets [according to Dooley *et al* (1989)]. (b and c) Schematic explanation of why the bacterial surface of A450-3, covered with a smooth hydrophilic LPS layer, was not efficiently reconstituted (b), while a hydrophobic (H) latex head surface was (c). (d) Single A-layer at the surface of a wild type strain showing its interaction with LPS O-chains. This is the putative arrangement obtained by reconstitution of A450-3 in co-culture with A450-1.

layered sheets efficiently coated hydrophobic latex beads (Fig. 34c), and mediated an efficient interaction of the coated beads with mØs (Fig. 29c, and Fig. 26a in chapter V). Coated beads therefore, displayed the correct wild type A-layer orientation on its surface (compare Fig. 34c with Fig. 34d), which seems to be critical for effective mØ recognition. The co-culturing reconstitution method proved to be functionally effective, as judged by CR binding and efficient mØ association, most likely because single A-layers, released during growth of A450-1, were adsorbed onto the A450-3 cell surface template, before they had a chance to form double layers. This also suggests that, in contrast to the surface process on plates, A-layers in liquid phase easily form double-layered sheets.

The presence of A-layer has profound effects on the characteristics of the *A. salmonicida* cell surface (Kay *et al.*, 1981; Parker and Munn, 1984 & 1985; Phipps, 1988; Trust *et al.*, 1983; van Alstine *et al.*, 1986). Therefore, it is conceivable that different A-layer arrangements are critical to the bacterial surface topology and, consequently, to mØ recognition. Both the mutant A450-10S, in which the A-layer appears disorganized, as well as A450-10SR, with aggregated A-layers, had a reduced capacity to associate with mØs. Another important structural modification in the A-layer, is that induced by growth of *A. salmonicida* in the calcium deficient medium FPM. FPM grown cells have A-layers displaying the novel BS structural pattern (refer to chapter III), which significantly increases the ability of *A. salmonicida* to associate with mØs. This will be discussed in chapter XI. Thus, it is clear that structural modifications in the A-layer had important effects on the association of *A. salmonicida* with mØs, suggesting once more, that the A-layer is indeed responsible for mediating this process.

Hemin coatings also had an important enhancing effect on adherence to mØs. Because A-protein is the hemin binding molecule on the surface of *A. salmonicida* (Kay *et al.*, 1985), it appears (in its native A-layer arrayed form) to be directly responsible for this effect. Surprisingly, growing bacterial cells on plates with whole rainbow trout blood, produced an even greater effect upon adherence to mØs (Fig. 31b). This remains as a partially unexplained effect because, although it is known that A<sup>+</sup> bacteria are capable of binding immunoglobulins (Phipps and Kay, 1988), and complement (Tomás and Kay, manuscript submitted), exposure to normal fresh trout serum alone (containing immunoglobulins and complement), did not increase adherence to mØs. Thus, the effect of whole blood is most likely attributed to i) hemin binding and ii)

bacterial cell surface modification through some as yet unidentified mechanisms. Interestingly, it has been previously reported that hemin or CR coated *Shigella flexneri* had an increased invasiveness in the HeLa cell system, when compared with non-coated bacteria (Daskaleros and Payne, 1987).

Although not a major objective of this present study, it was impossible to ignore the cytopathic effects associated with A<sup>+</sup> *A. salmonicida*. Previously observed in murine mØs (chapter V); these effects are now confirmed with trout mØs (Fig. 33). Cytotoxicity appeared not to be a direct effect of the A-layer, since the A-layer possessing strain A450-10SR did not appear as cytotoxic as A450, and A-layer coated latex beads did not induce major changes in mØ morphology. A marked effect of *A. salmonicida* upon trout mØs, not previously observed with murine mØs, was the detachment of mØs from the substratum. Similar effects on Atlantic salmon peritoneal mØs, have been reported by Olivier *et al* (1992). These authors also concluded that cytotoxicity was not directly an effect of the A-layer. The potentiating effect of hemin on the cytolytic activity of coated *A. salmonicida* remains to be explored.

In summary, I have shown that: i) adherence of the fish pathogen *A. salmonicida* to trout mØs is mediated by the A-layer, and ii) that structural alterations in the A-layer have important effects on adherence.

## CHAPTER VII

### RESPONSE OF *Aeromonas salmonicida* TO OXYGEN RADICALS. ROLE OF THE A- LAYER

#### INTRODUCTION

As professional phagocytes (in contrast to phagotrophic or non-professional phagocytes) mØs possess a diversity of oxidative and non-oxidative killing mechanisms. Oxidative killing is mediated by a multicomponent NADPH oxidase which transfers a single electron from NADPH to molecular oxygen, to produce stoichiometric amounts of superoxide anion (Morel *et al.*, 1991). Superoxide is dismutated to peroxide which then forms other reduced oxygen species (ROS) like hydroxyl radical, singlet oxygen, or, in the presence of myeloperoxidase, hypochlorate; all with potent microbicidal activities (Hurst and Barrette, 1989). Rainbow trout macrophages are quite capable of mounting, upon stimulation with live virulent or avirulent strains of *A. salmonicida*, a respiratory burst leading to production of ROS (Sharp and Secombes, 1990; Secombes *et al.*, 1988) by a NADPH oxidase-like activity (Secombes *et al.*, 1992). Therefore, *A. salmonicida*, that are able to penetrate and survive in ROS-producing macrophages, must possess protective mechanisms against oxidative killing.

A growing body of experimental evidence indicates that bacterial pathogens are constantly sensing their environment and responding to it through proper adjustments (Buchmeier and Heffron, 1990; Clark, 1990; DiRita and Mekalanos, 1989; Groisman and Saier, 1990; Lee and Falkow, 1990; Maurelli *et al.*, 1992; Miller *et al.*, 1989; Smith, 1988). Thus, a bacterial pathogen that goes from the extracellular to the intracellular environment, and ends up inside a phagolysosome, soon realizes that the surrounding environment is highly hostile. How the pathogen responds and copes with this hostile environment will determine the final outcome of the infection process. Aerobically grown *A. salmonicida* (as many other respiring, cytochrome-containing organisms) should be able to cope with oxidative stress, since ROS are usually generated as a consequence of aerobic metabolism through incomplete reduction of

molecular oxygen to water during respiration, or by other membrane-associated or cytosolic processes (Farr and Kogoma, 1991; Storz *et al.*, 1990). In the presence of oxidative stress during its interaction with mØs, *A. salmonicida* may be capable of inducing an effective response, accounting for survival inside ROS-producing mØs. A series of experiments were initiated to determine the resistance of *A. salmonicida* to superoxide, peroxide, and intracellular redox-active agents. The role of the A-layer in protecting *A. salmonicida* against ROS was especially considered.

## MATERIALS AND METHODS

Refer to chapter IV.

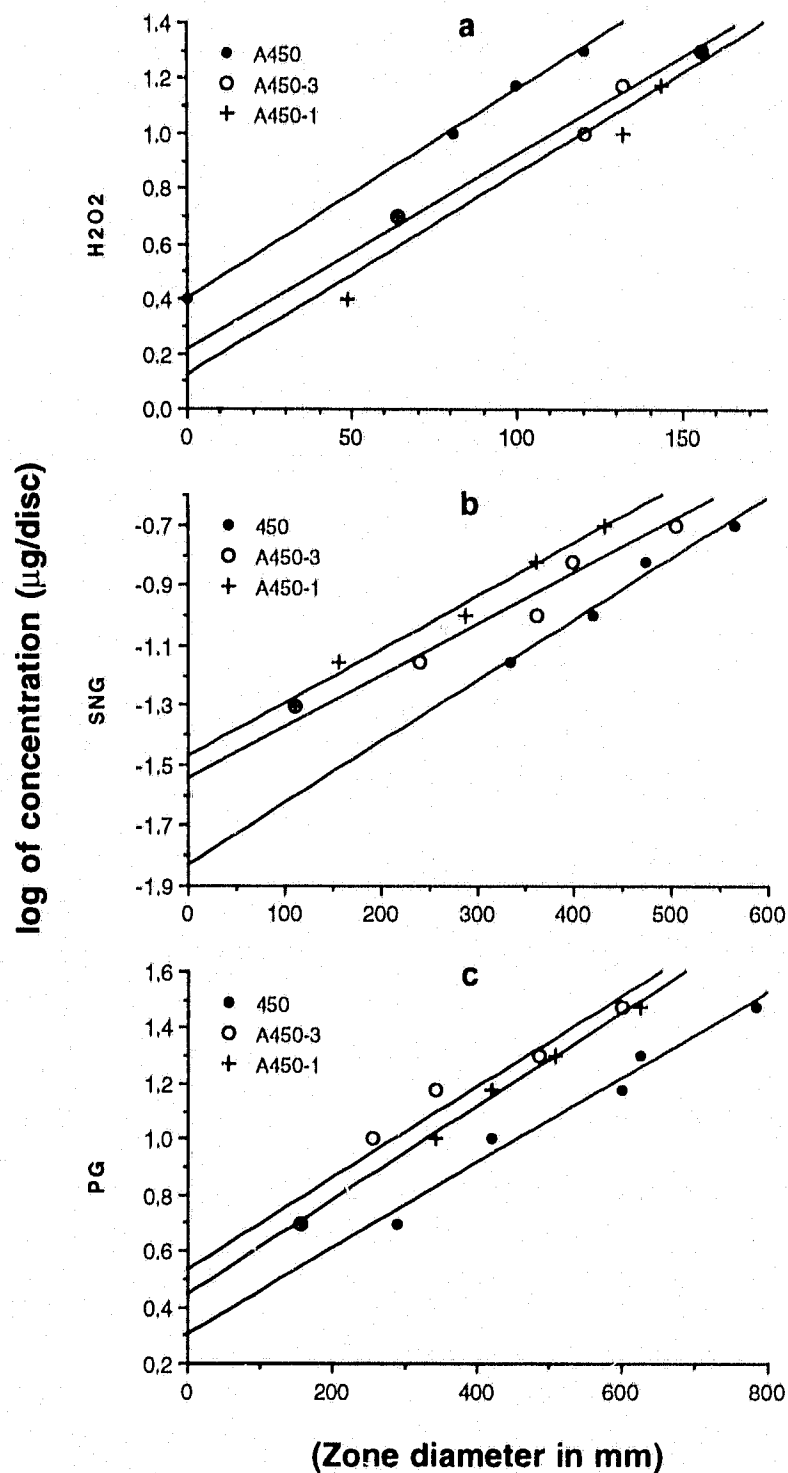
## RESULTS

### Assays on plates

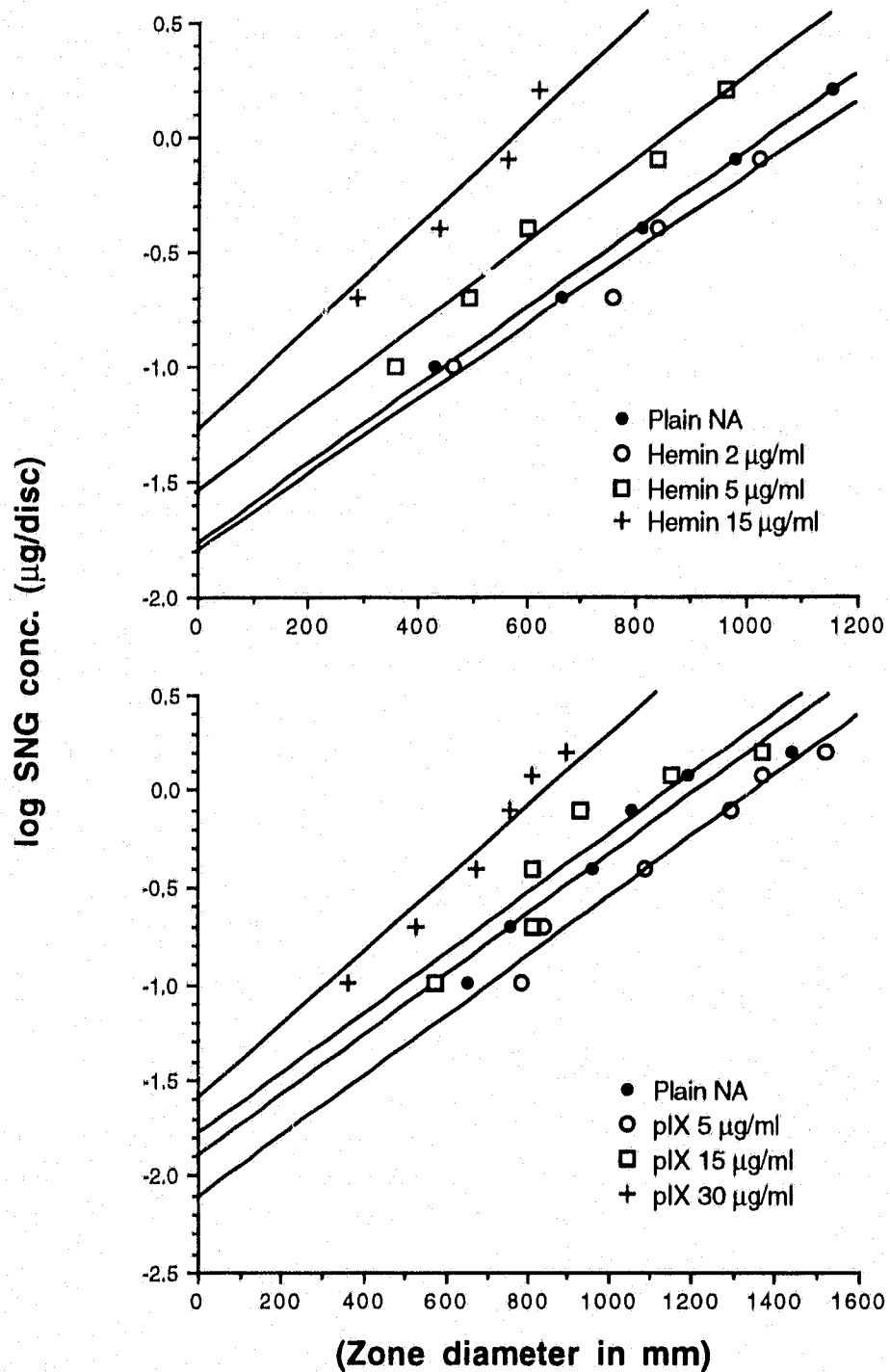
Results from disc inhibition assays on plates indicated that A<sup>+</sup> *A. salmonicida* cells were about two-fold more resistant to H<sub>2</sub>O<sub>2</sub> than A<sup>-</sup> cells (Fig. 35a). On the other hand, A<sup>+</sup> cells were more sensitive than A<sup>-</sup> to the intracellular redox-active compounds streptonigrin (SNG), and plumbagin (PG) (Fig. 35b and c). A450-1, owing to the lack of LPS O-polysaccharide chains and an anchored A-layer, behaved more similar to A450-3 than to A450.

Disc inhibition assays in nutrient agar (NA) containing different concentrations of Congo Red (CR), hemin, or protoporphyrin IX (pIX), indicated that pIX and hemin reduced SNG toxicity to the A<sup>+</sup> strain A450 in a concentration dependent manner (Fig. 36) whereas CR had virtually no effect (not shown). In contrast to A450, A450-1 (O<sup>-</sup>, phenotypic A<sup>-</sup>), and A450-3 (A<sup>-</sup>, O<sup>+</sup>) were more sensitive to SNG in the presence of hemin or pIX (Table 1). Again, CR had virtually no modulating effect upon SNG toxicity against A450-1 or A450-3.

These results clearly pointed to a relationship between the A-layer and susceptibility of *A. salmonicida* to peroxide or redox-active compounds. This was further explored in assays conducted in liquid phase.



**FIGURE 35.** Toxicity of (a) H<sub>2</sub>O<sub>2</sub>, (b) streptonigrin (SNG), or (c) plumbagin (PG), to different strains of *A. salmonicida*, as determined by the disc inhibition assay on nutrient agar. The intersection of the curve with the ordinate marks the minimum inhibitory concentration, and the slope of the curves is related to the diffusion of the toxic compound in the system used. Results from one of three independent experiments showing similar results are shown.



**FIGURE 36.** Effect of hemin, or protoporphyrin IX (pIX) on the toxicity of streptonigrin (SNG) to *A. salmonicida* A450. Toxicity was determined by the disc inhibition assay on plates of nutrient agar containing the indicated concentrations ( $\mu\text{g/ml}$ ) of the different compounds tested. Results from one of three independent experiments showing similar results are shown.

**Table 1.** Effect of hemin and protoporphyrin IX on the toxicity of streptonigrin to *A. salmonicida* strains A450-1 and A-450-3, as compared with the wild type A<sup>+</sup> strain A450

	MIC <sup>a</sup> (ng streptonigrin/disc)		
	A450-1	A450-3	A450
<b>Agent added:</b>			
<b>A. Hemin (μg/ml)</b>			
0	63	66	17
2	28	59	15.5
5	34	57	29
15	24	45	52
<b>B. Protoporphyrin IX (μg/ml)</b>			
0	56	42	13
5	22	24	8
15	15	32	17
30	14	28	26

<sup>a</sup> MIC, Minimal inhibitory concentration. Determined from the graphs presented in Fig. 36 (for A450) and similar graphs obtained for A450-1 and A450-3.

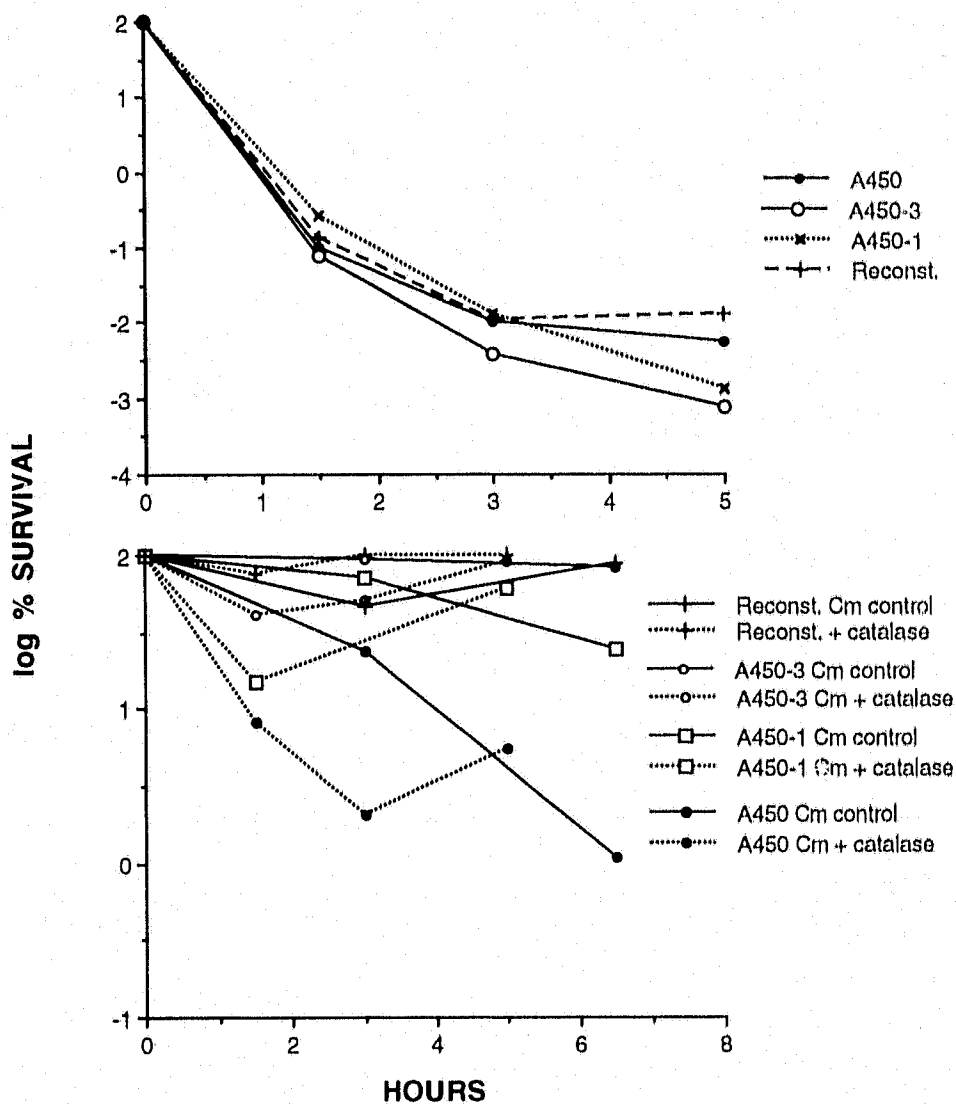
## Assays in liquid medium

### Challenges with externally added ROS.

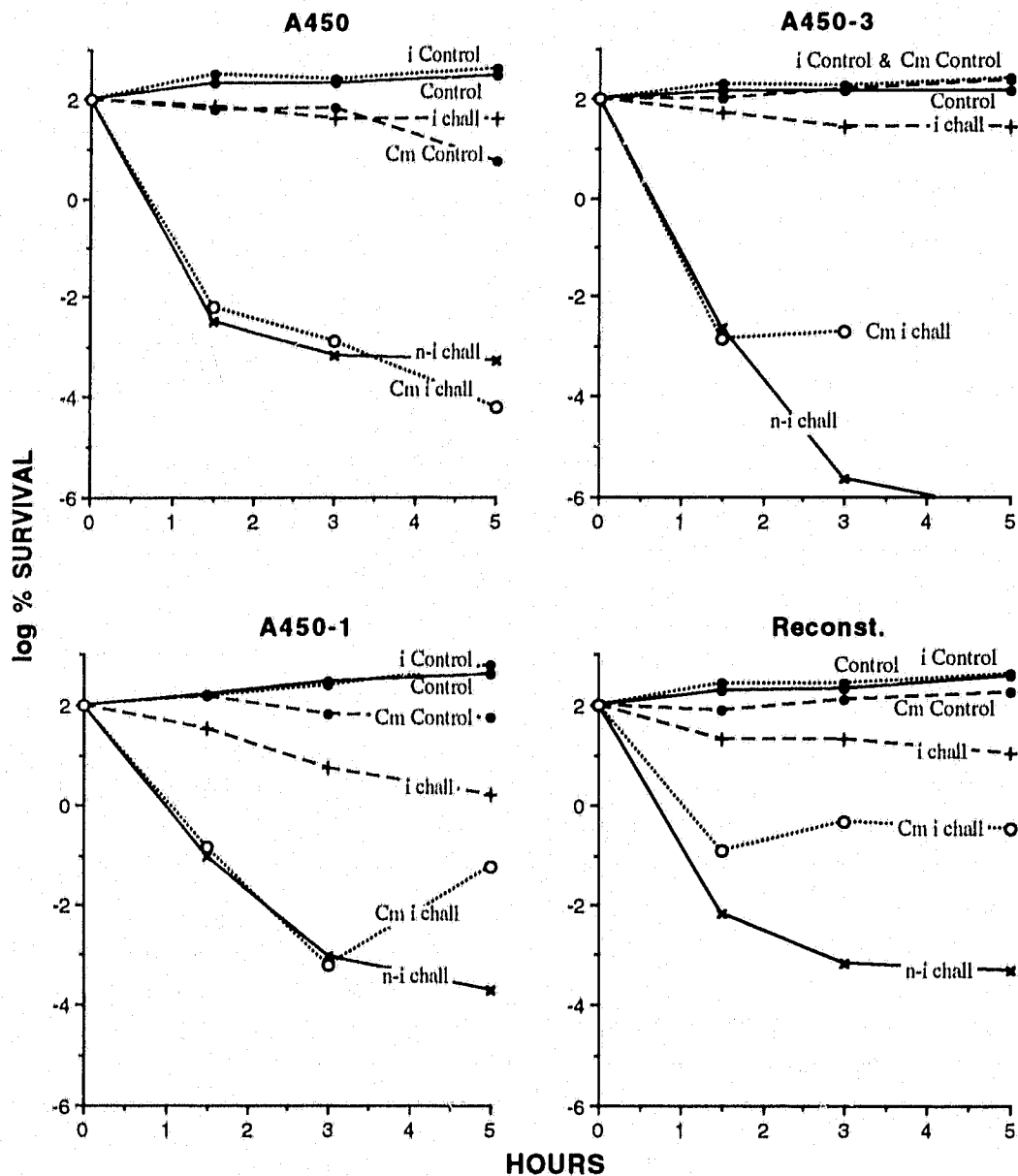
It was once more confirmed that the A-layer provided some protection against peroxide (Figs. 37 and 38). In addition, the A-layer had a significant protective effect against superoxide (Fig. 39). Again, A450-1 ( $O^-$ , phenotypic  $A^-$ ) had a sensitivity to ROS more similar to A450-3 ( $A^-$ ,  $O^+$ ) than to A450 ( $A^+$ ,  $O^+$ ). The protective effect of hemin against SNG was also confirmed (Fig. 40), suggesting that it is cell bound hemin (and not only the excess of hemin in solid media) which reduced SNG toxicity. In these challenges, it was also possible to assay the effect of A-layer reconstitution on resistance to ROS. Reconstituted cells gained resistance to either peroxide (Figs. 37a and 38) or superoxide (Fig. 39). However, increased sensitivity of reconstituted cells to SNG was not detected.

Exposure to sublethal doses of peroxide (Fig. 38), or peroxide and SNG (Fig. 39), rendered *A. salmonicida* highly resistant to subsequent lethal challenges by  $H_2O_2$  and superoxide, respectively. This inducible response was not due to the presence of the A-layer, since  $A^+$  and  $A^-$  strains acquired similar levels of resistance upon exposure to sublethal ROS doses. Cells induced in the presence of chloramphenicol were still sensitive to lethal challenges, suggesting the truly inducible nature of the response, and the requirement of *de novo* protein synthesis. The protective role of superoxide dismutase (SOD) and catalase, as well as the ROS-mediated nature of the killing process, was also demonstrated; externally added enzymes significantly reduced ROS toxicity against chloramphenicol treated *A. salmonicida* cells (Fig. 37b and Fig. 39). This inducible, A-layer independent resistance mechanism appeared to be the main protective response of *A. salmonicida* against ROS; protection obtained after induction with sublethal doses of ROS was always higher than the A-layer-mediated protection of non-induced cells.

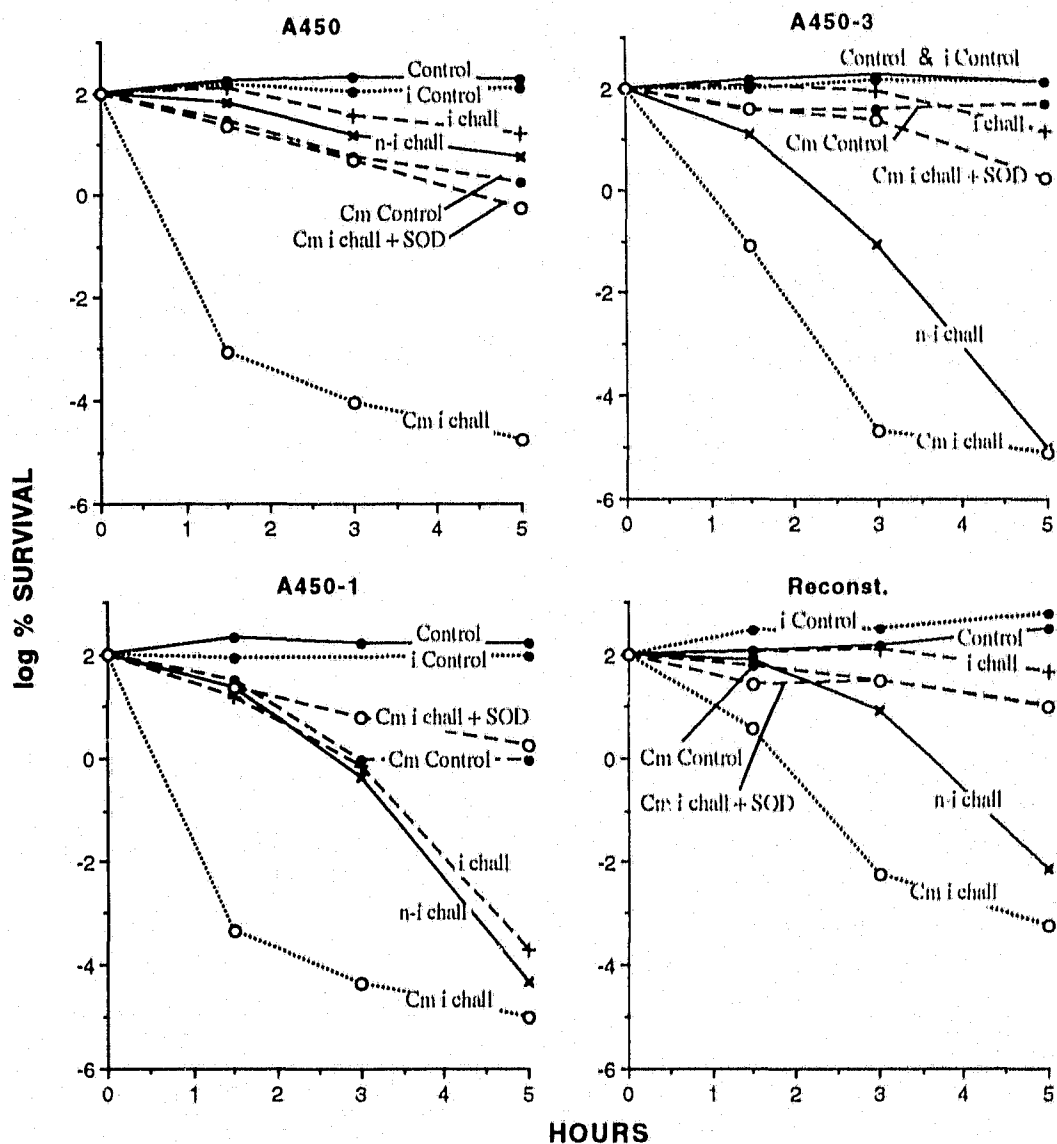
A-layer-mediated protection from ROS was not enhanced when CR, or hemin coated  $A^+$  cells were used, suggesting that the iron centers of hemin did not act as beneficial scavengers of oxygen radicals (Fig. 40). In contrast, the protective, A-layer mediated effect was impaired in hemin coated  $A^+$  cells, or  $A^+$  cells grown on TSA/Blood, perhaps due to a higher cellular iron load which has been related to increased ROS toxicity (especially  $H_2O_2$ ) (Asad and Leitão, 1991; Halliwell and Gutteridge, 1984; Sambri *et al.*, 1991). Furthermore, neither purified A-protein nor A-layer were able to inhibit the SOD-inhibitable reduction of cytochrome C by the



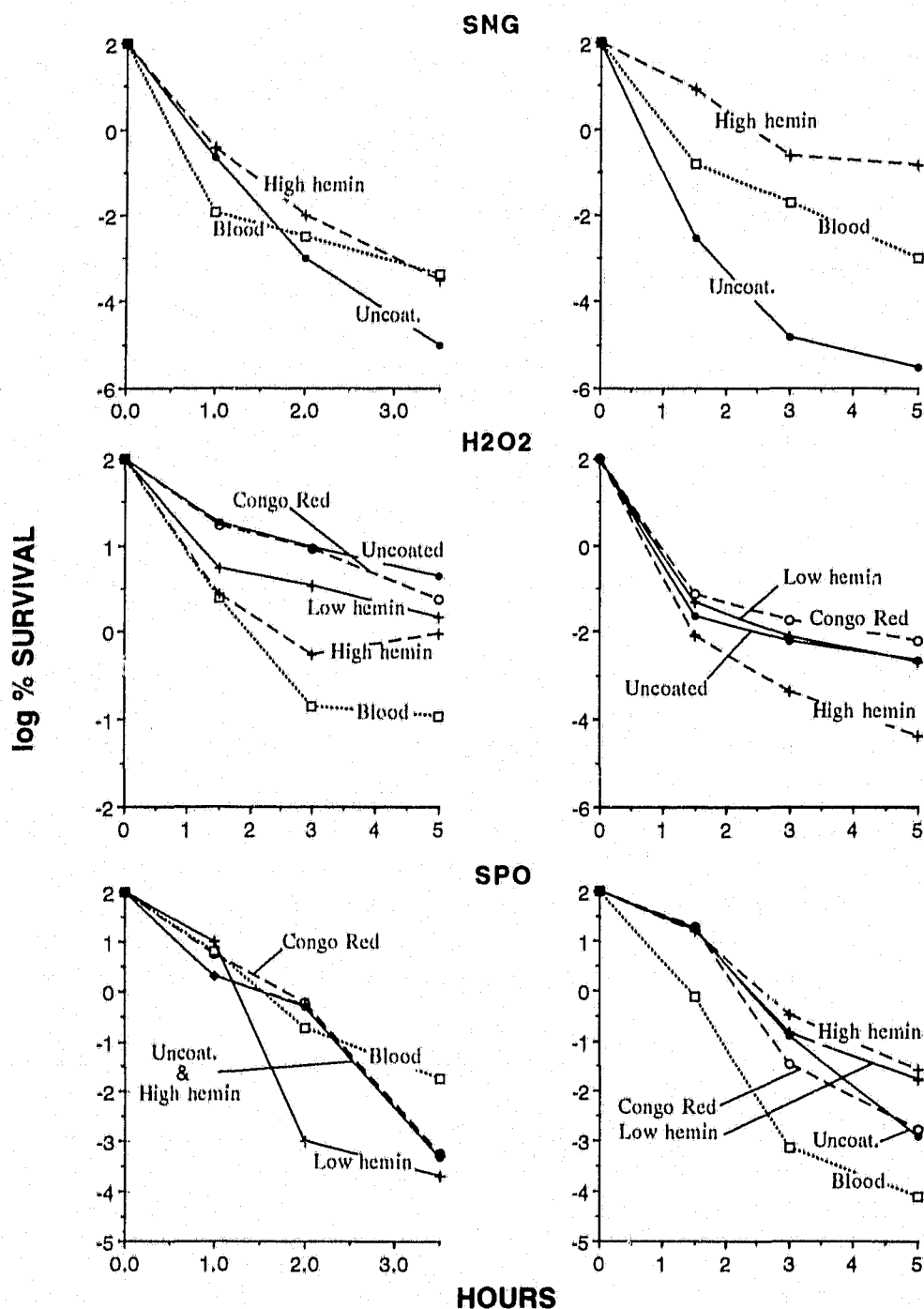
**FIGURE 37.** Toxicity of H<sub>2</sub>O<sub>2</sub> to *A. salmonicida* cells with different surface properties, as determined in liquid phase assays in nutrient broth. (a) Survival curves after a challenge with 2mM H<sub>2</sub>O<sub>2</sub>. (b) Survival curves of cloramphenicol-treated cells after a challenge with 2mM H<sub>2</sub>O<sub>2</sub> in the presence of catalase. Cm controls = Cloramphenicol-treated cells not challenged; Cm+catalase = Cloramphenicol-treated cells challenged with 2 mM in the presence of 1000 U catalase/ml. Reconst. = A-layer reconstituted A450-3 cells. Reconstitution was performed by the co-culturing method described in chapter V. Results shown are from a single experiment.



**FIGURE 38.** Induction, in *A. salmonicida*, of a protective, A-layer independent response against  $H_2O_2$ . The different strains shown were challenged with 1mM  $H_2O_2$ . Control = Bacterial cells incubated in nutrient broth (NB). i Control = Cells pre-induced with 60  $\mu$ M  $H_2O_2$  in NB and not challenged. Cm Control = Cloramphenicol-treated cells, pre-induced and not challenged. i chall = Challenged pre-induced cells. ni chall = Challenged non-induced cells. Cm i chall = Cloramphenicol treated cells, pre-induced and challenged. A-layer reconstitution of A450-3 cells (Reconst.) was performed by the co-culturing method described in chapter V. Results from a single experiment are shown.



**FIGURE 39.** Toxicity of superoxide to *A. salmonicida* cells with different surface properties, and induction of a protective, A-layer independent response, as determined in liquid phase assays in 0.07 M phosphate buffer. The different strains shown were challenged with xanthine/xanthine oxidase generated superoxide at an approximate rate of 3  $\mu$ moles/min. Control = Bacterial cells incubated in phosphate buffer. i Control = Cells pre-induced with 60  $\mu$ M  $H_2O_2$  and 0.001  $\mu$ g SNG/ml in phosphate buffer, and not challenged. Cm Control = Chloramphenicol-treated cells, pre-induced and not challenged. i chall = Challenged pre-induced cells. n-i chall = Challenged non-induced cells. Cm i chall = Chloramphenicol treated cells, pre-induced and challenged. Cm i chall + SOD = Chloramphenicol treated cells, pre-induced and challenged in the presence of the enzymes superoxide dismutase and catalase. A-layer reconstitution of A450-3 (Reconst.) was performed by the co-culturing method described in chapter V. Results from one of two independent experiments showing similar results.



**FIGURE 40.** Effect of different A-layer coatings and growth conditions on toxicity of streptonigrin (SNG),  $H_2O_2$ , or superoxide (SPO), to *A. salmonicida* strain A450. Blood = A450 grown on TSA/Blood plates. Low hemin = A450 grown on TSA plates supplemented with 1  $\mu g/ml$  hemin. High hemin = A450 grown on TSA plates supplemented with 10  $\mu g/ml$  hemin. CR = A450 grown on TSA plates supplemented with 30  $\mu g/ml$  Congo Red. Uncoat. = A450 grown on plain TSA plates. Results from two independent experiments are shown.

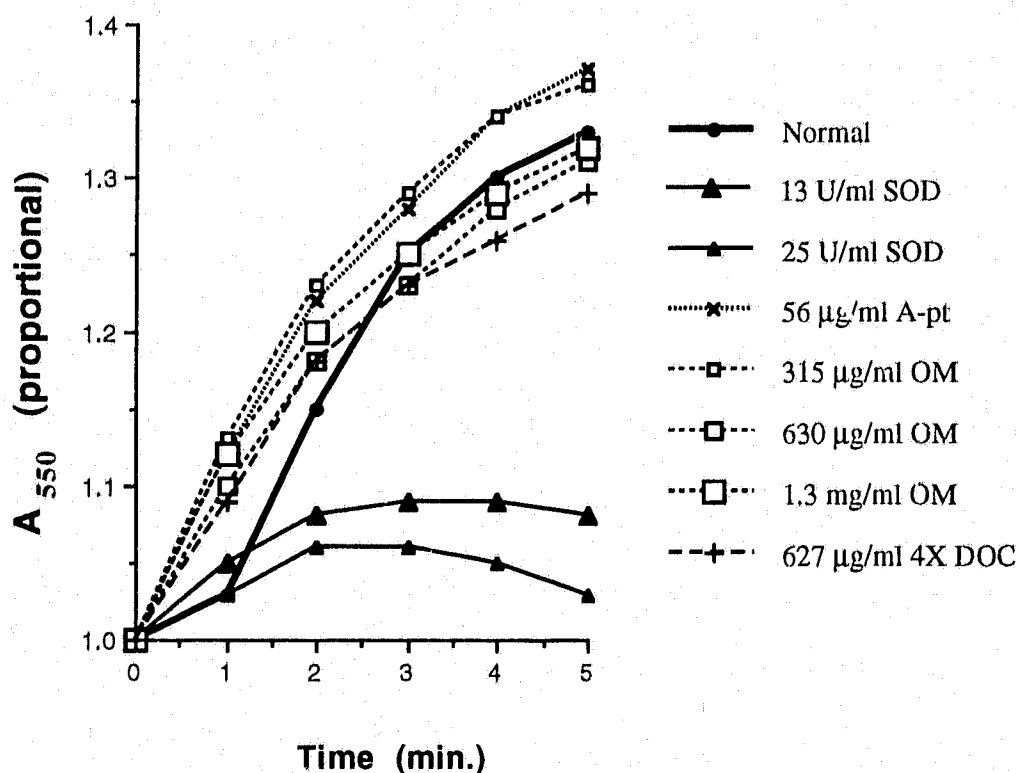
xanthine/xanthine oxidase system (Fig. 41), nor did A<sup>-</sup> cells survived better to ROS challenges conducted in the presence of A<sup>+</sup>, or coated A<sup>+</sup> cells, confirming that scavenging of radicals is not the protective mechanism of the A-layer (Fig. 42).

#### **Challenges with intracellular redox-active compounds.**

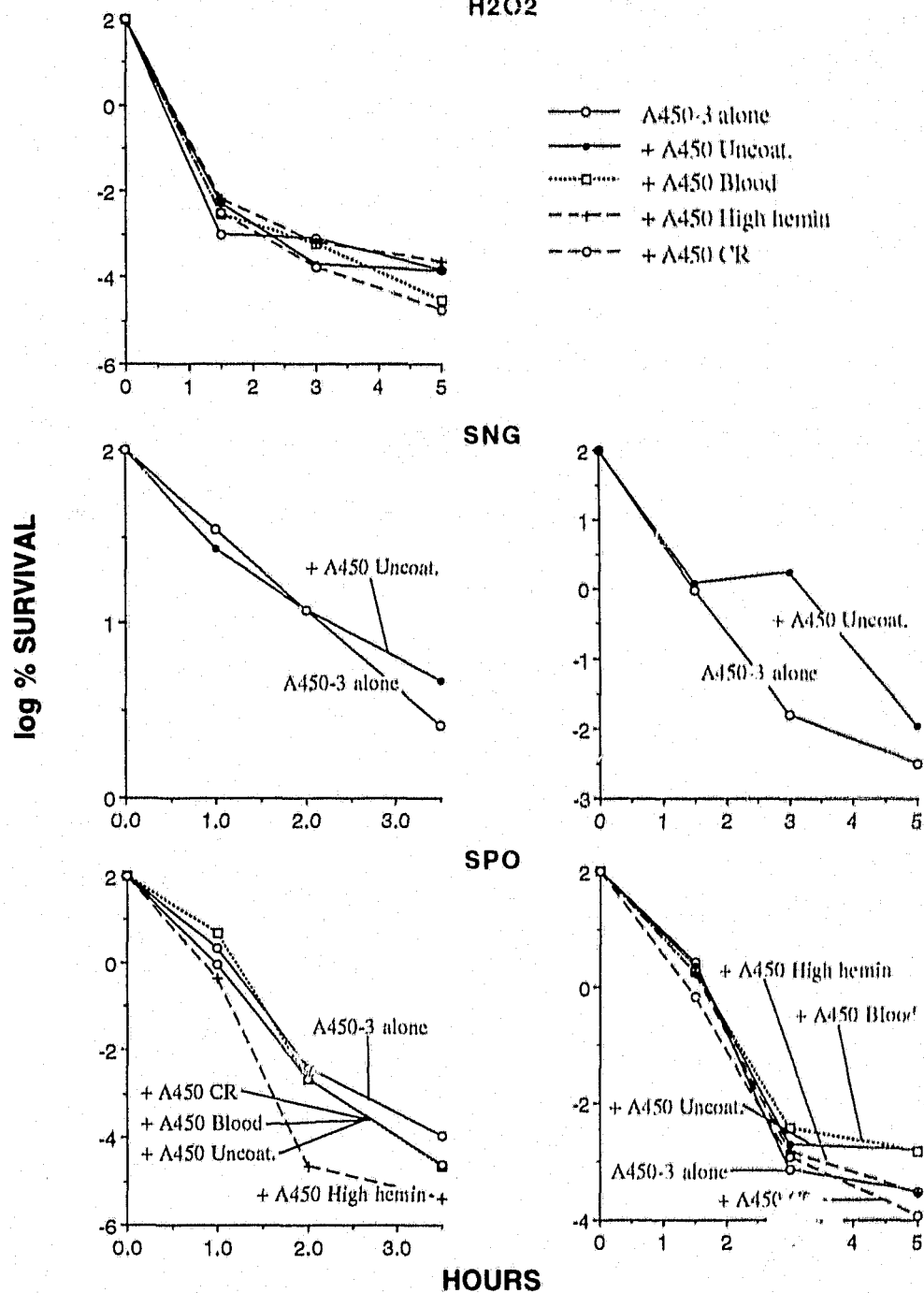
The increased sensitivity of A<sup>+</sup> cells to SNG was confirmed in the liquid phase assays. The final level of survival of A450-3 to SNG challenges was 100-fold higher than that of A450 (Fig. 43). In contrast to the results obtained with peroxide and superoxide, the pre-induction of *A. salmonicida* with sublethal doses of SNG, or the external addition of SOD, did not protect *A. salmonicida* against intracellular SNG-mediated generation of superoxide (Fig. 43). However, induction with sublethal doses of H<sub>2</sub>O<sub>2</sub> had a cross-protective effect against SNG (not shown). This suggested that: i) SNG may not be a good inducer, and ii) perhaps the intracellularly generated superoxide was not the most important effector of cell damage, but secondary H<sub>2</sub>O<sub>2</sub> (generated through the dismutation of superoxide) was. Hemin coated A<sup>+</sup> cells as well as A<sup>+</sup> cells grown on TSA/Blood plates, had a decreased sensitivity to SNG (Fig. 40), and the presence of A<sup>+</sup> cells in mixtures with A<sup>-</sup> A450-3, slightly enhanced survival of the latter to SNG (Fig. 42), both effects suggesting that SNG is indeed attracted and/or bound by A-layers, perhaps at the hemin binding site.

## **DISCUSSION**

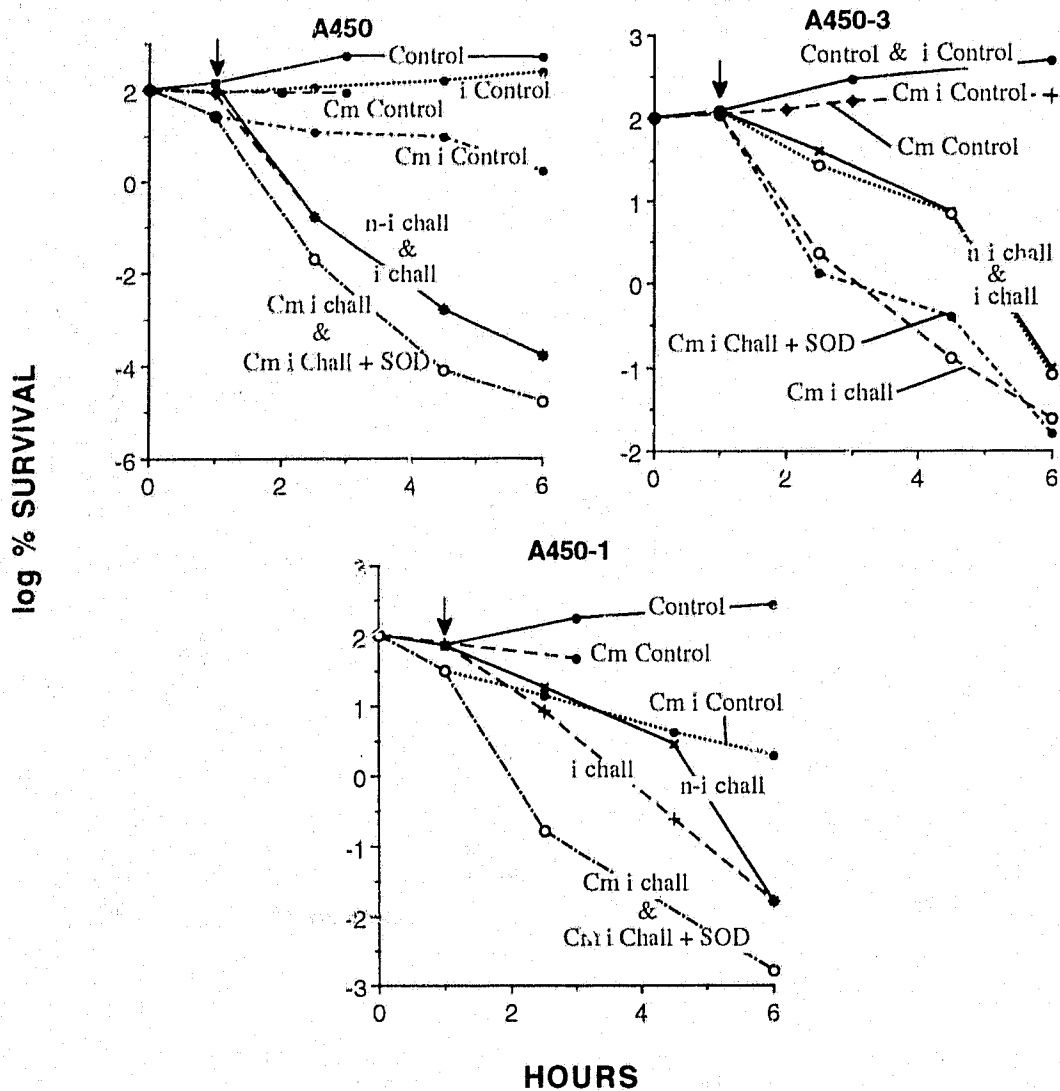
The response of *A. salmonicida* to oxidative stress has some similarities to the well studied responses of *Escherichia coli* and *Salmonella typhimurium* (Dempse, 1991; Farr and Kogoma, 1991; Tartaglia *et al.*, 1991). It was induced by similar sublethal concentrations of H<sub>2</sub>O<sub>2</sub> (but superoxide and hydrogen peroxide had different inducing properties), and required *de novo* protein synthesis, as inferred by the inhibiting effect of chloramphenicol. By analogy, this inducible response in *A. salmonicida* could also involve mechanisms similar to those in *E. coli* and *S. typhimurium*, i.e. increased synthesis of SOD, catalase and intracellular scavenging substrates like glutathione, induction of DNA repair systems, and synthesis of several stress proteins (which may overlap with those induced by other stressful stimuli). Indeed, this inducible response appeared as the main protective mechanism of *A. salmonicida* against ROS. However, it was observed that the A-layer had an interesting protective effect in non-induced A<sup>+</sup>



**FIGURE 41.** Reduction of oxidized cytochrome C by xanthine/xanthine oxidase generated superoxide. Reduction was efficiently inhibited by superoxide dismutase (SOD), but not by outer membrane preparations of  $A^+$  cells, purified A-layer (4X DOC), or purified A-protein, at different concentrations (indicated in the legend). Absorbances of the reaction mixture (measured at 550 nm) are expressed as:  $A_{550}$  at time 'x' /  $A_{550}$  at time zero ('x' = 1, 2, 3, 4, and 5 min.). Results from a single experiment are shown.



**FIGURE 42.** Effect of the presence of coated or uncoated A<sup>+</sup> A450 cells on survival of the A<sup>-</sup> strain A450-3 against H<sub>2</sub>O<sub>2</sub>, streptonigrin (SNG), or superoxide (SPO) challenges. The following mixtures were challenged: + A450 Blood = A 1:1 mixture of 450-3 and A450 grown on TSA/Blood plates. + A450 high hemin = A 1:1 mixture of 450-3 and A450 grown on TSA plates supplemented with 10 μg/ml hemin. + A450 CR = A 1:1 mixture of 450-3 and A450 grown on TSA plates supplemented with 30 μg/ml Congo Red. + A450 Uncoat. = A 1:1 mixture of 450-3 and A450 grown on plain TSA plates. Results from two independent experiments are shown.



**FIGURE 43.** Toxicity of streptonigrin to *A. salmonicida* cells with different surface properties. Toxicity was determined in liquid phase assays in nutrient broth. The different strains shown were challenged with 1  $\mu\text{g}$  SNG/ml. (arrow). Control = Bacterial cells incubated in nutrient broth. i Control = Cells pre-induced with 0.01  $\mu\text{g}$ /ml SNG in NB and not challenged. Cm Control = Chloramphenicol-treated cells, incubated in nutrient broth. Cm i Control = Chloramphenicol-treated cells, pre-induced and not challenged. i chall = Challenged pre-induced cells. ni chall = Challenged non-induced cells. Cm i chall = Chloramphenicol treated cells, pre-induced and challenged. Cm i chall + SOD = Chloramphenicol treated cells, pre-induced and challenged in the presence of the enzyme superoxide dismutase. Results are from one of two independent experiments showing similar results.

cells of *A. salmonicida*. This protective effect was significantly higher against superoxide, probably as a consequence of the fact that, unlike  $H_2O_2$ , superoxide cannot freely diffuse through biological membranes (Hasset and Cohen, 1989). Thus, possession of an A-layer could represent an initial advantage for cells facing the hostile phagolysosomal environment of mØs, before induction of the main protective response. Other investigators have observed a relationship between the presence of A-layer, survival inside mØs, and resistance to *in vitro* generated superoxide (Graham *et al.*, 1988; Karczewski *et al.*, 1991; Olivier *et al.*, 1986), but the protective mechanisms associated with the A-layer were not further investigated.

The results presented in this chapter clearly indicate that, in contrast to the protection mediated by some microbial surface exposed glycolipids (Chan *et al.*, 1989), the A-layer-mediated protection is not direct and apparently does not involve radical scavenging. Surprisingly, not even A-layer bound hemin had an enhanced beneficial effect, which was expected owing to the affinity of heme iron centers for oxygen. Since it is difficult to conceive that the A-layer would act as a physical barrier for such small radical species, explanations for the protective role of the A-layer must involve indirect mechanisms. A simplistic mechanism may be related to the propensity of  $A^+$  cells to agglutinate.  $A^+$  cells located in the centre of cell clumps may be more protected from ROS than single, dispersed  $A^-$  cells. Another possible explanation constitutes an A-layer mediated retention of outer membrane (OM) blebs. According to EM observations (not shown) and biochemical evidence (J.C. Thornton, personal communication), *A. salmonicida* is regarded as a prolific producer of OM blebs. Physically constrained blebbing in the presence of A-layer, may cause bleb retention underneath the A-layer. Alternatively, blebs could remain associated with the outer face of the A-layer (through specific A-layer-OM interactions) leading to the formation of a cloud of blebs around  $A^+$  cells. Since OM blebs have been demonstrated to have a protective effect against serum in *Neisseria gonorrhoeae* (Pettit and Judd, 1992) and *Porphyromonas gingivalis* (Grenier and Bélanger, 1991), serving as sites for complement fixation away from the cell wall and cytoplasmic membrane, it seems reasonable to hypothesize that a concentration of blebs at the cell surface could, as well, serve as a trap for ROS, reducing their probability to reach vital membrane-associated or cytosolic targets (especially DNA). Finally, the possibility that the presence of a surface anchored A-layer may modulate some OM functions (e.g. permeability) cannot be ruled out at this point. Such a mechanism could also partially explain the puzzling

susceptibility of A<sup>+</sup> cells to SNG and its modulation by hemin, the increased sensitivity of A450 to chloramphenicol (Figs. 37b, 38 and 39), and the difference in the behavior of reconstituted cells against external ROS or SNG (i.e. reconstituted cells were more resistant to peroxide or superoxide but not more sensitive to SNG).

Owing to the apparent hydrophobicity of the redox-active compounds SNG and PG, a working hypothesis was formulated postulating that the A-layer acts as a hydrophobic surface to which these compounds could be attracted. Being in close proximity to the outer membrane (and in potential agreement with the view that an outer membrane anchored A-layer modifies permeability), further partition of SNG or PG into the outer and cell membranes would lead to higher intracellular concentrations of these compounds in A<sup>+</sup> cells, accounting for the extreme sensitivity of A<sup>+</sup> cells to these compounds. In support to this view, A<sup>-</sup> cells (possessing or not a hydrophilic layer of smooth LPS) were more resistant to redox-active compounds and chloramphenicol, suggesting that it was the A-layer, but not LPS or surface hydrophobicity, which was responsible for increased toxicity. Since reduction of SNG toxicity by hemin was exclusive of A<sup>+</sup> cells, and A<sup>-</sup> cells challenged with SNG survived better in the presence of A<sup>+</sup> cells, a direct relationship between SNG, hemin and A-layer must exist. Moreover, the reduced SNG toxicity, clearly seen in the presence of hemin, was less noticeable in the presence of protoporphyrin IX, and virtually absent in the presence of the iron-less hemin analog CR, pointing to a possible involvement of iron in the process. Interestingly, a direct relationship between SNG toxicity and cell iron content exists (Yeowell and White, 1982). This relationship, which has been largely exploited in the isolation of iron uptake defective mutants owing to their resistance to SNG (Dyer *et al.*, 1987; Genco *et al.*, 1991; Holland *et al.*, 1991), may also explain why A450-3 and A450-1 were more susceptible to SNG in the presence of hemin (Table 1). Excess hemin and pIX, but not CR, may have increased the cell iron content, which, in the absence of A-layer, led to increased SNG toxicity.

Although the mechanisms involved in A-layer-mediated sensitivity to SNG are still unknown, this effect, as well as its modulation by hemin, has opened new opportunities to study the relationship between A-layer, hemin binding, iron uptake and virulence. SNG resistant (SNG<sup>r</sup>) mutants have been isolated and will be characterized as to their ability to bind hemin, produce siderophore, and infect trout. Preliminary results (not shown) have indicated that the A<sup>-</sup> or the O<sup>-</sup> phenotypes are predominant

among  $SNG^r$  mutants, and that most  $A^+$ ,  $O^+$ ,  $SNG^r$  mutants are siderophore negative. One of these mutants also had a reduced capacity to bind CR on plates.

## CHAPTER VIII

### INTRAPERITONEAL CHAMBER MODEL FOR *IN VIVO* GROWTH OF *Aeromonas* *salmonicida*

#### INTRODUCTION

While furunculosis, and its causative agent *A. salmonicida*, have been known for nearly a century (McCarthy and Roberts, 1980), a truly effective furunculosis vaccine awaits development. Numerous potential vaccines have been formulated, but there is as yet, no consistent correlation between protection from disease and anti-*A. salmonicida* agglutinin titers. Even the most recent results from immunization studies against furunculosis are either highly variable or difficult to reproduce (Hastings and Ellis, 1990; Lund *et al.*, 1991; Thornton *et al.*, 1991). Many factors may contribute to this disappointing failure in achieving consistent protection, even in the presence of a good antibody response. One possibility is that the *A. salmonicida* antigens presented in current vaccines are not relevant to protection, implying that other virulence-related determinants, perhaps only expressed *in vivo*, may constitute important protective antigens. The cell surface of *in vitro* grown *A. salmonicida* is mainly defined by the A-layer and the smooth LPS layer, which comprise O-polysaccharide chains of homogeneous length. Owing to their functional diversity and their strategic location at the cell surface, these determinants have been considered the main antigens of *A. salmonicida*. On the other hand, no virulence determinants exclusively expressed *in vivo* have been as yet identified.

Alternatively, if *A. salmonicida* is a facultatively intracellular pathogen, it may find protection from humoral defense mechanisms by readily penetrating macrophages (mØs), inside which *A. salmonicida* has been shown to survive (Graham *et al.*, 1988; Olivier *et al.*, 1986; Trust *et al.*, 1983; and chapters V to VII). In this case, successful activation of the cellular arm of the immune response (Kaufmann, 1988) would likely be necessary for protection against furunculosis. Although there are sporadic reports of protection against furunculosis by transfer of immune serum (Olivier *et al.*, 1985; Ellis

*et al.*, 1988), it is not as yet clear whether immunity to furunculosis is mediated via cellular and/or humoral responses.

To examine these possibilities more closely, an intraperitoneal chamber (IPC) model was developed, that permitted the study of the *in vivo* growth of *A. salmonicida* cells inside rainbow trout (*Oncorhynchus mykiss*). In this chapter the results of this study are reported. Of particular significance are two findings: that the peritoneal fluid of insulted rainbow trout appears to contain a potent bacteriolytic activity capable of severely impairing the *in vivo* growth of virulent *A. salmonicida*, and that *A. salmonicida* is able to replicate inside *in vitro* cultured mØs. These findings support the view that *A. salmonicida* is a facultatively intracellular pathogen that escapes humoral defense mechanisms by readily penetrating mØs in the early stages of infection.

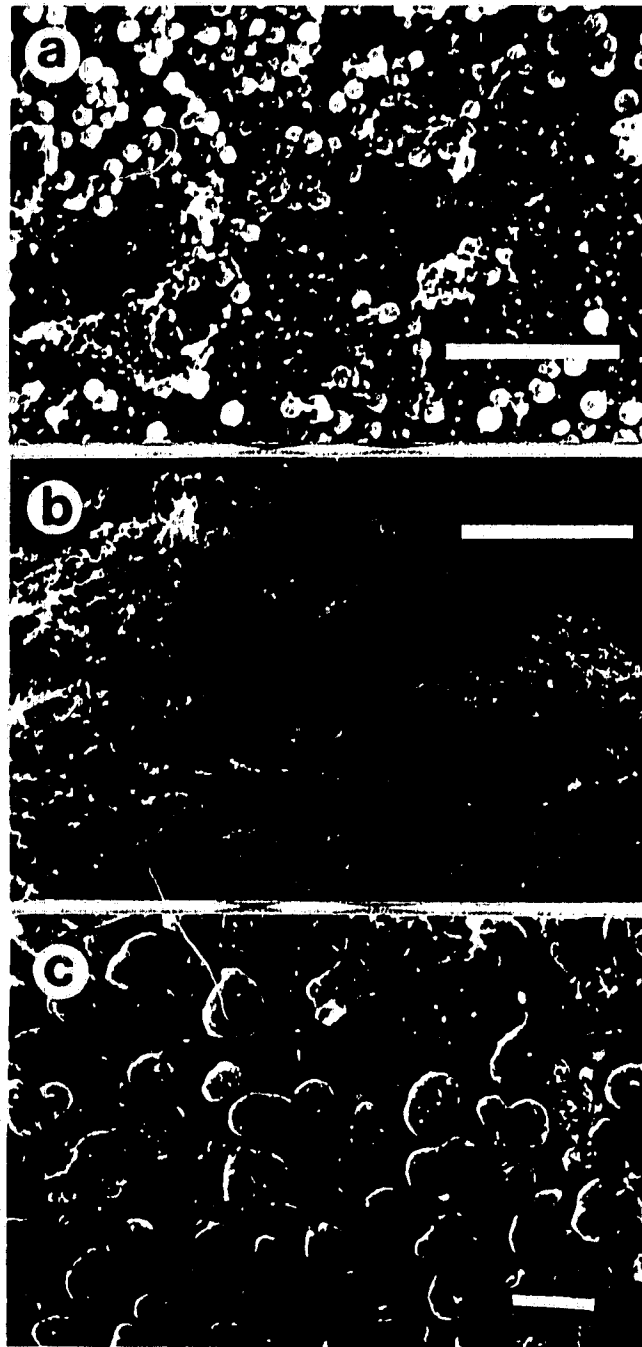
## **MATERIALS AND METHODS**

Refer to chapter IV.

## **RESULTS AND DISCUSSION**

### **Removed intraperitoneal chambers (IPCs)**

IPCs kept for up to one week were found to be partially encapsulated by a host-derived material, deposited around the chamber, particularly at the end closed by the membrane. SEM of membranes recovered from one week old IPCs, showed that the two faces of the Millipore™ membrane had quite different surface morphologies. The inner face (Fig. 44a) was covered by numerous microcolonies of bacteria, and the external face was heavily covered with a fibrous material (Fig. 44b). Some areas of the external face also had groups of adherent host cells (Fig. 44c). In two previous studies on the use of IPCs in mammals (Day *et al.*, 1980; Sutherland *et al.*, 1990) it was found that the implants also were encapsulated in a fibrinous material. This was considered to be the consequence of an inflammatory response, owing to the presence of numerous granulocytes in the material. Since I also observed a similar phenomenon it is reasonable to surmise that rainbow trout are capable of mounting an inflammatory response stimulated by the presence of the chambers and, most likely, by bacterial



**FIGURE 44.** Scanning electron micrographs showing the internal (a) and external (b & c) sides of a Millipore™ membrane recovered from an intraperitoneal chamber, after an incubation period of one week. (a) Microcolonies of *in vivo* grown *A. salmonicida* cells, (b) Detail of the host-derived fibrous material deposited on the membrane, (c) Group of host cells attached to the membrane. Bars represent 10 µm.

factors diffusing through the Millipore™ membrane. It is possible that after one week, the fibrinous coat impaired the exchange of nutrients, gases and metabolites across the membrane.

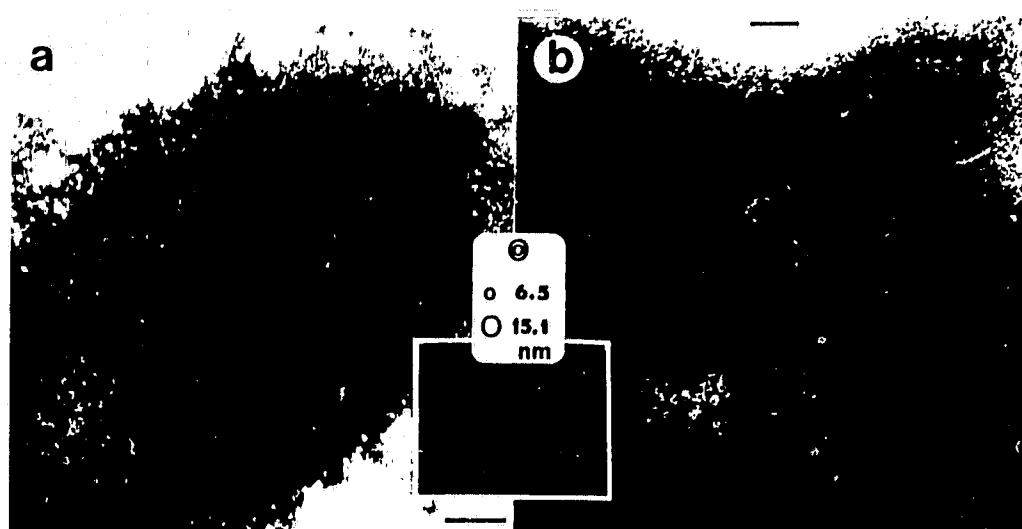
### **Survival of *A. salmonicida* inside IPCs**

Even after repeated attempts, no viable bacterial cells could be recovered from IPCs containing from  $5 \times 10^3$  to  $5 \times 10^5$  cells, after one, two or three day incubations. Negatively stained specimens prepared for electron microscopy confirmed that there were no whole cells present in the removed IPCs. Instead, many cell wall fragments were observed, displaying, upon closer inspection, numerous doughnut-shaped pores (Fig. 45). Only when the chambers were filled with  $7 \times 10^6$  cells, were survivors recovered (Fig. 46). In sharp contrast with these findings the intraperitoneal LD<sub>50</sub> for the virulent strain A450 in juvenile coho salmon, *Oncorhynchus kisutch*, was reported as  $2 \times 10^4$  cells (Kay *et al.*, 1981; Ishiguro *et al.*, 1981). Moreover, in juvenile rainbow trout, the intraperitoneal LD<sub>50</sub> for A450 was determined to be  $1.7 \times 10^2$  cells (J.C. Thornton, personal communication).

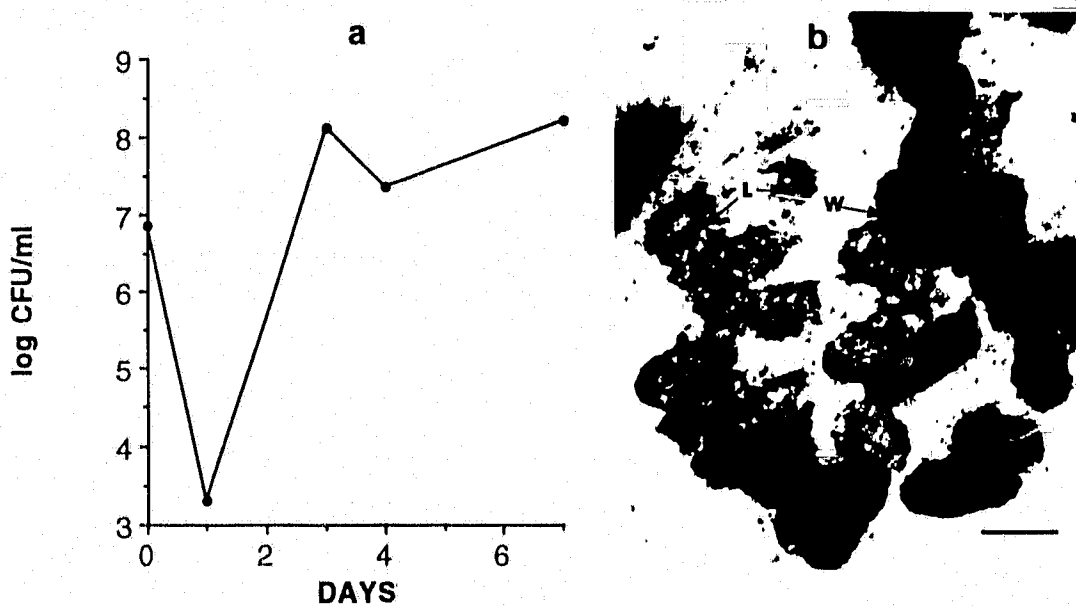
Two salient facts emerged from these studies. First, a bacteriolytic activity is present in rainbow trout peritoneal fluid, capable of diffusing across the 0.45 μm membrane. Second, there exists a 350–40,000-fold difference between the effective LD<sub>50</sub> of free intraperitoneally injected cells and the dose required to survive inside the peritoneal cavity when bacterial cells were confined inside IPCs. Although puzzling indeed, these facts suggest that the peritoneum constitutes a hostile environment and that migration from the peritoneum must be essential for bacterial survival. A closer examination of this hypothesis was conducted.

### **The bacteriolytic activity of peritoneal fluid**

The morphology of the pores associated with the envelopes of lysed cells resembled that of the membrane attack complexes formed upon fish complement fixation (Jenkins *et al.*, 1991). Therefore, a series of assays were carried out to determine if the pores were lesions associated with lysis. Normal or chloramphenicol-treated *in vitro* grown bacteria were challenged with fresh or treated peritoneal fluid. The results (Table 2) indicated that the appearance of pores was always associated with lysis and that the pores were caused by factors of host origin. The lytic activity of fresh peritoneal fluid was relatively non-specific, since a variety of bacterial species were



**FIGURE 45.** Transmission electron micrographs of negatively stained specimens showing the pores formed upon exposure to peritoneal fluid. (a) Cell wall fragment from the contents of an intraperitoneal chamber filled with a cell suspension of the A<sup>-</sup> strain A450-3 (chosen because the doughnut-shaped pores were more clearly distinguished in the absence of A-layer); inset: detail of a pore. (b) section of a membrane ghost from a horse erythrocyte challenged *in vitro* with fresh peritoneal fluid. Bars represent 0.1  $\mu\text{m}$ .



**FIGURE 46.** Growth of *Aeromonas salmonicida* A450 inside peritoneal chambers surgically implanted in rainbow trout. (a) Growth curve inside chambers containing an initial inoculum of  $7 \times 10^6$  bacterial cells/ml. Notice the sharp decrease in viability observed during the first 24 h post implantation, and the enormous recovery of the survivor cells by the third day. (b) Transmission electron micrograph of negatively stained specimens recovered from the chambers used to construct the growth curve in panel 'a'. There was always a large proportion of lysed ghost cells ("L" in the micrograph) in the midst of whole darkly stained cells ("W" in the micrograph). Bar represents 1  $\mu\text{m}$ .

**Table 2.** Bacteriolytic activity of trout peritoneal fluid and serum under different conditions.

	Peritoneal fluid			Serum		
	Fresh <sup>a</sup>	Treated <sup>b</sup>		Fresh	Treated	
		Heat	Freeze-thaw		Heat	Freeze-thaw
<b>Bacterial cells:</b>						
<b>A. Normal<sup>c</sup></b>						
Pores formed <sup>d</sup>	+	-	-	+	-	+
Killing by lysis <sup>e</sup>	+	-	-	+	-	+
<b>B. Inhibited<sup>f</sup></b>						
Pores formed	+	-	-	ND <sup>g</sup>	ND	ND
Lysis <sup>h</sup>	+	-	-	ND	ND	ND

<sup>a</sup> Used immediately after collection

<sup>b</sup> Heat treatment was achieved at 55°C for one hour. Freeze-thaw treatment consisted of a single cycle of freezing at -20°C overnight, and thawing at room temperature

<sup>c</sup> Bacterial cells used for challenges were obtained from fresh TSA cultures suspended and washed in PBS

<sup>d</sup> Determined by electron microscopy

<sup>e</sup> Determined by a reduction in viable cell counts and appearance of cell ghosts by electron microscopy

<sup>f</sup> Bacterial protein synthesis was inhibited with chloramphenicol (200 µg/ml)

<sup>g</sup> ND, not determined

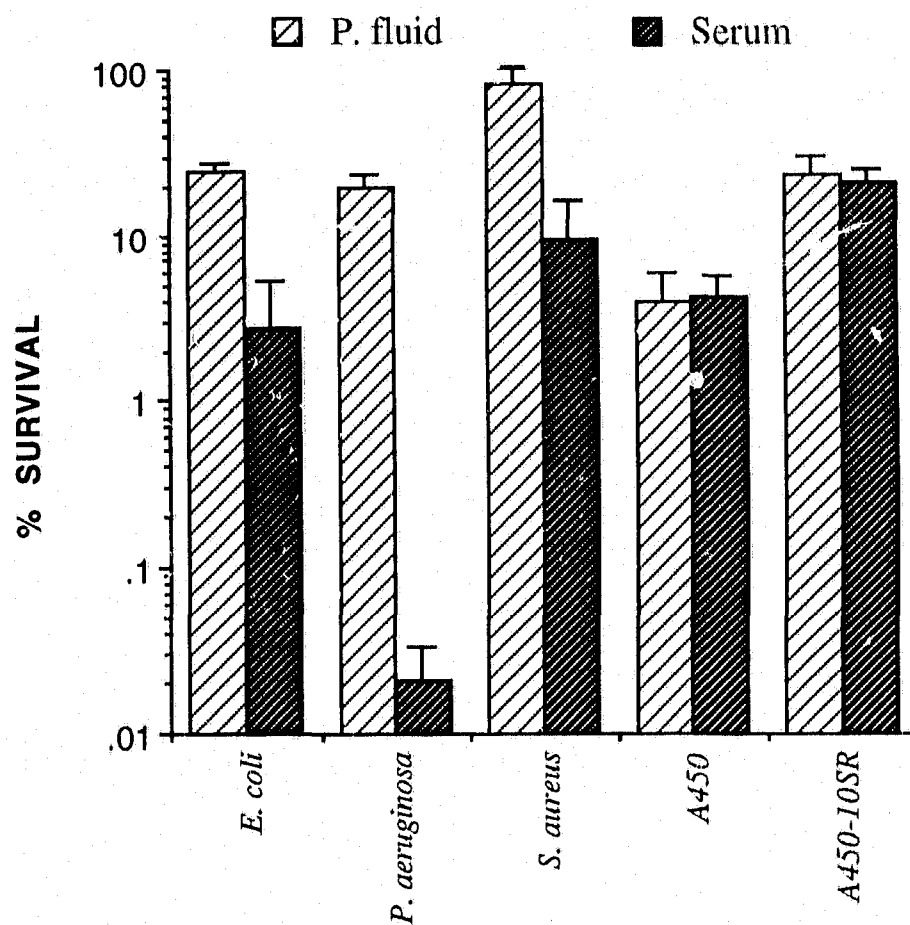
<sup>h</sup> Appearance of cell ghosts determined by electron microscopy

susceptible to lysis (Fig. 47). In general, peritoneal fluid was less bactericidal than serum, but there were important differences in the relative killing of *Pseudomonas aeruginosa* and *A. salmonicida* strains by these fluids. Moreover, bovine, sheep, horse, guinea pig, rabbit, and chicken erythrocytes were also lysed by trout peritoneal fluid, and the membrane ghosts from lysed red blood cells displayed the same morphologically distinct pores, previously observed in association with envelopes of lysed bacterial cells (Fig. 45).

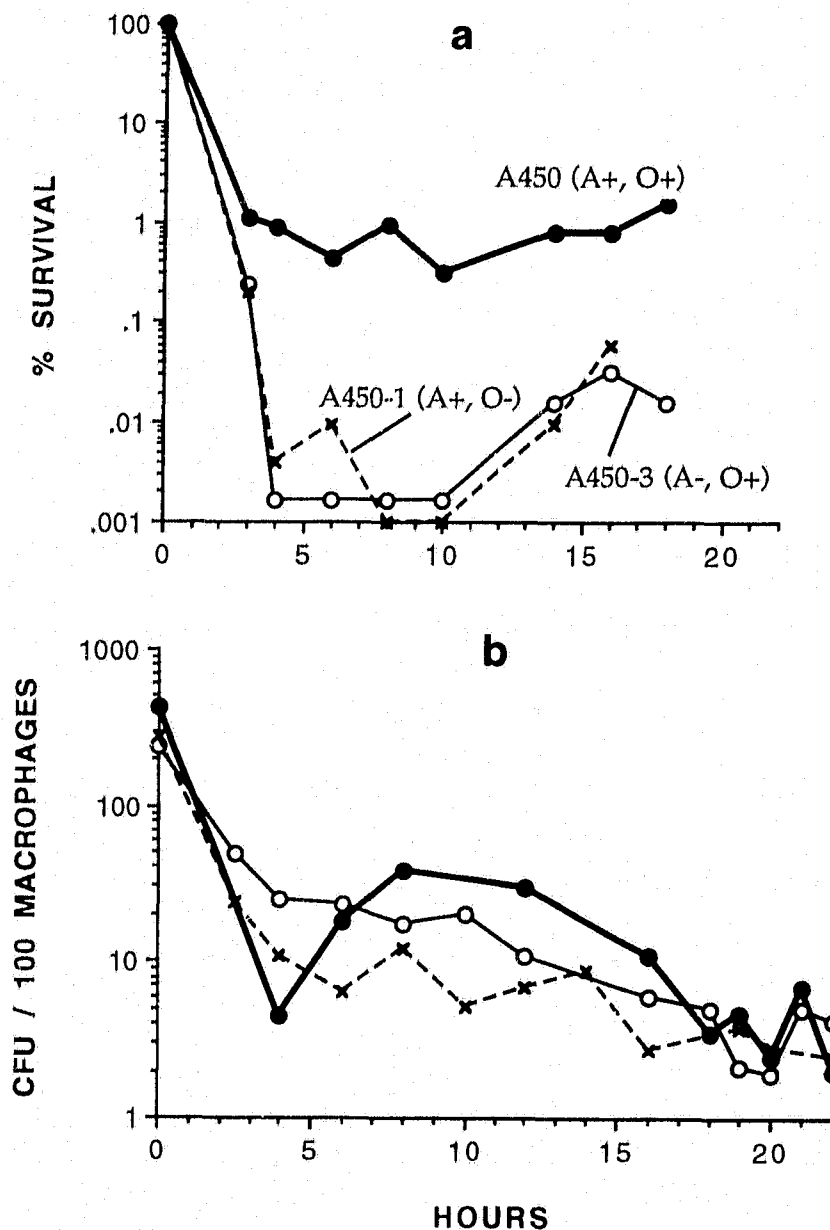
Interestingly, while the lytic activity of peritoneal fluid was inactivated by a single freeze-thaw cycle at  $-20^{\circ}\text{C}$ , the lytic activity of serum was resistant to this treatment (Table 2). Also, the kinetics of hemolysis was quite distinct between serum and peritoneal fluid; while serum-mediated hemolysis was complete after few minutes of challenge, peritoneal fluid-mediated hemolysis required an overnight incubation at  $14^{\circ}\text{C}$  to be complete. Lastly, as mentioned before, the serum sensitivity profile of a spectrum of bacterial species was different from their sensitivity profile to peritoneal fluid (Fig. 47). Therefore, it is clear that the lytic activities of serum and peritoneal fluid differ. Nonetheless, they may represent the same complement-mediated lytic activity, and the differences between serum and peritoneal fluid may be explained by i) a difference in the concentration of complement components in the two fluids (this being greater in serum), or ii) a difference in complement competence due to the environment in which it is acting (e.g. absence of some serum components in the peritoneal fluid, or vice versa).

### **Survival and replication inside mØs**

Notwithstanding its lack of motility, *A. salmonicida* is clearly capable of spreading into (as well as persisting in) a variety of host tissues (Johnson *et al.*, 1985; Kay *et al.*, 1986; Trust 1986), an ability assumed to be mediated by the mØ (Kay *et al.* 1986; Trust *et al.* 1983). Fig. 48a shows that, indeed, the A450 wild type strain had a high level of survival, whereas the two avirulent strains A450-3 and A450-1, containing cell surface defects, were more readily killed. These results are in agreement with previous reports indicating enhanced survival of virulent  $A^{+}$  *A. salmonicida* strains inside mØs (Olivier *et al.* 1986; Graham *et al.* 1988). Since *A. salmonicida* strains cause lysis (refer to chapter VI) and detachment of salmonid mØs from the substratum (Olivier *et al.*, 1992), I determined the number of mØs in the assay system, and calculated the number of bacteria per 100 mØs, as reported in Fig. 48b.



**FIGURE 47.** Survival of different bacterial species after a 3 h challenge with fresh serum or fresh peritoneal fluid, obtained from rainbow trout. Percentages of survival are referred to the initial inoculum ( $\sim 2 \times 10^8$  cells/ml = 100%). Controls kept in PBS did not show any reduction in viability during the incubation period. Error bars represent standard deviation of duplicate samples from a single experiment.



**FIGURE 48.** Survival of three *Aeromonas salmonicida* strains (A450, ●—●; A450-1, x---x; A450-3, O—O) inside cultured head kidney tissue macrophages, isolated from rainbow trout. In one experiment (a), the data is presented as “% survival”, calculated in reference to the number of colony forming units per tube at time zero. In a second experiment (b), the data is presented as “number of bacteria per hundred macrophages”. As compared with the first experiment, survival of A450-3 and A450-1 strains was apparently higher; however, the intracellular numbers of these avirulent strains steadily declined during the assay.

Interestingly, it became more apparent that, soon after an initial killing phase (presumably the combined result of mØ killing mechanisms and the addition of gentamicin), the virulent strain A450 replicates intracellularly. It is important to note that the number of viable A450 cells per 100 mØs declined in the final phase of the experiment (Fig. 48b). This may not be entirely the result of effective killing by the mØ, since upon microscopic inspection mØs looked feeble, morphologically altered, and still contained many bacterial cells. I attributed this final killing phase to the cytotoxic effects of *A. salmonicida*, leading to permeability problems at the membrane of the compromised mØ. Gentamicin may have reached the intracellular bacteria, causing the observed decrease in viability. Therefore, virulent A-layer positive cells, which readily associate and penetrate mØs in the absence of host opsonins (chapters V & VI), not only survive but also are capable of replicating inside mØs. The combination of this finding with the fact that IPC confined bacterial cells did not survive the lytic activity of peritoneal fluid, strongly suggests that *A. salmonicida* is a facultatively intracellular pathogen capable of protecting itself from humoral host defense mechanisms by rapidly gaining access to mØs and partially avoiding their killing mechanisms. Additional support to this view comes from the results presented in chapter VII, which demonstrated that A<sup>+</sup> *A. salmonicida* strains are more resistant than A<sup>-</sup> strains to oxygen radicals, especially superoxide. Since oxidative killing is an important defence mechanism of mØs, an initial resistance against oxygen radicals would be very valuable in assuring enhanced survival inside mØs.

In conclusion, using the IPC model described here, I have demonstrated that the peritoneal fluid of rainbow trout, possesses a potent bacteriolytic activity which impairs *in vivo* growth of *A. salmonicida*. I postulate that free intraperitoneal *A. salmonicida* must rapidly evade this potent bacteriolytic activity, and propose that one of the mechanisms involved is penetration of peritoneal and/or tissue mØs in the early stages of infection. The results obtained also dictated the conditions under which *A. salmonicida* would grow *in vivo*, and suggested that IPCs may be valuable as experimental tools in studying the potential *in vivo* expression of virulence factors not expressed upon *in vitro* growth.

## CHAPTER IX

### THE A-LAYER OF *Aeromonas salmonicida* GROWN IN VIVO

#### INTRODUCTION

The results presented in chapters II and III demonstrated that the A-layer possesses significant structural flexibility and plasticity. On the cell surface, the A-layer apparently exists as a monolayer with a single type of closed and deep lattice, but once detached from the cell surface, it demonstrates its flexibility by adopting more open conformations. Under calcium-depletion conditions the A-layer demonstrates its plasticity by forming two novel structural patterns, which are the result of structural rearrangements within the layer. The objective of the work presented herein was to examine if the A-layer of *in vivo* grown *A. salmonicida* experienced structural changes indicative of a role for the layer's plasticity in the interaction of *A. salmonicida* with its host. Since positive results were indeed obtained, the functional relevance of the BS pattern was also explored.

#### MATERIALS AND METHODS

Refer to chapter IV.

#### RESULTS AND DISCUSSION

##### **A-layers of *in vivo* grown *A. salmonicida***

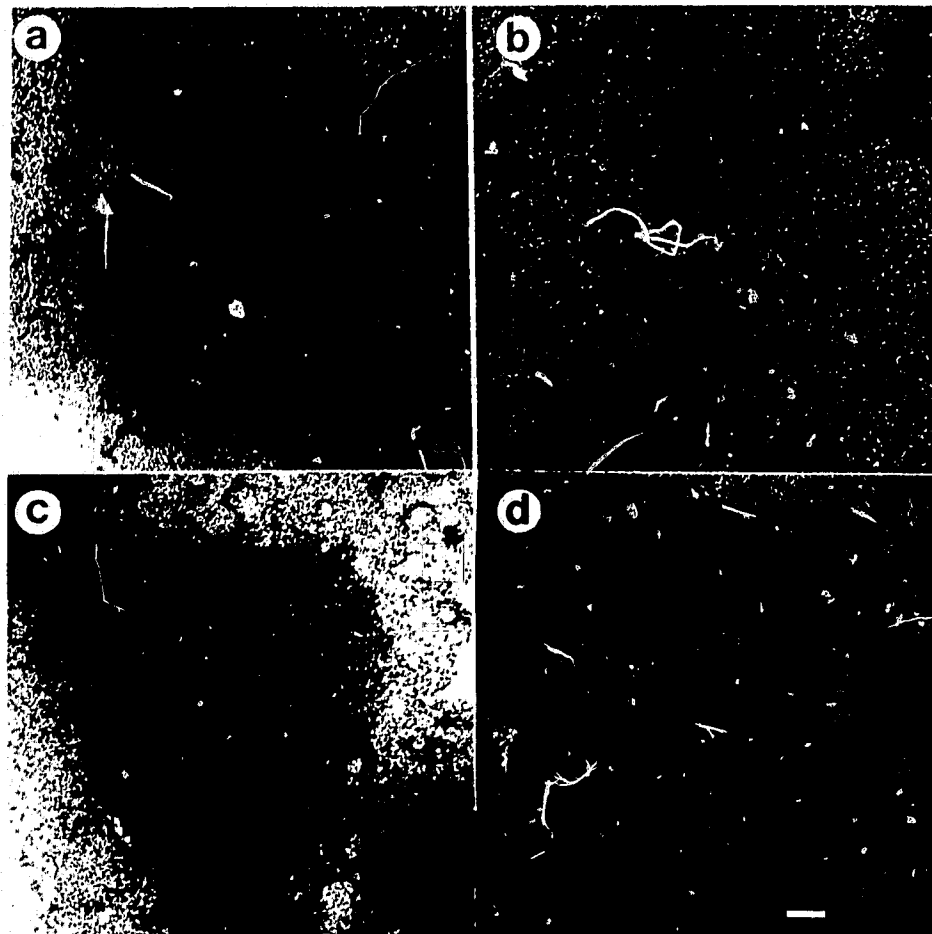
During the initial studies with *in vivo* grown cells presented in chapter VIII, it was important to determine if the A-layer could change its structure upon exposure to the intraperitoneal environment. Unfortunately, the fine structural study of the A-layers of *in vivo* grown A<sup>+</sup> *A. salmonicida* cells was not an easy task for two main reasons: First, in contrast to *in vitro* grown cells, which normally yield numerous A-layer

sloughs (refer to Fig. 4 in chapter II), *in vivo* grown cells did not easily shed their A-layers, i.e. the majority of cells had A-layers associated with the cell wall, hindering (even in lysed cells) the visualization of the fine A-layer structure. Second, the few A-layer sloughs present in negatively stained preparations were not well stained. Nonetheless, some success was obtained in studying the A-layers secreted by *in vivo* grown cells of the *A. salmonicida* mutant strain A450-1. The fluid recovered from intraperitoneal chambers, contained numerous A-layer sheets, some of which displayed the two novel WD and BS patterns (Fig. 49), suggesting that these patterns may be relevant in the biology of *A. salmonicida*. Therefore a functional characterization of the BS structural pattern (as found in FPM grown cells of the strain A450) was attempted.

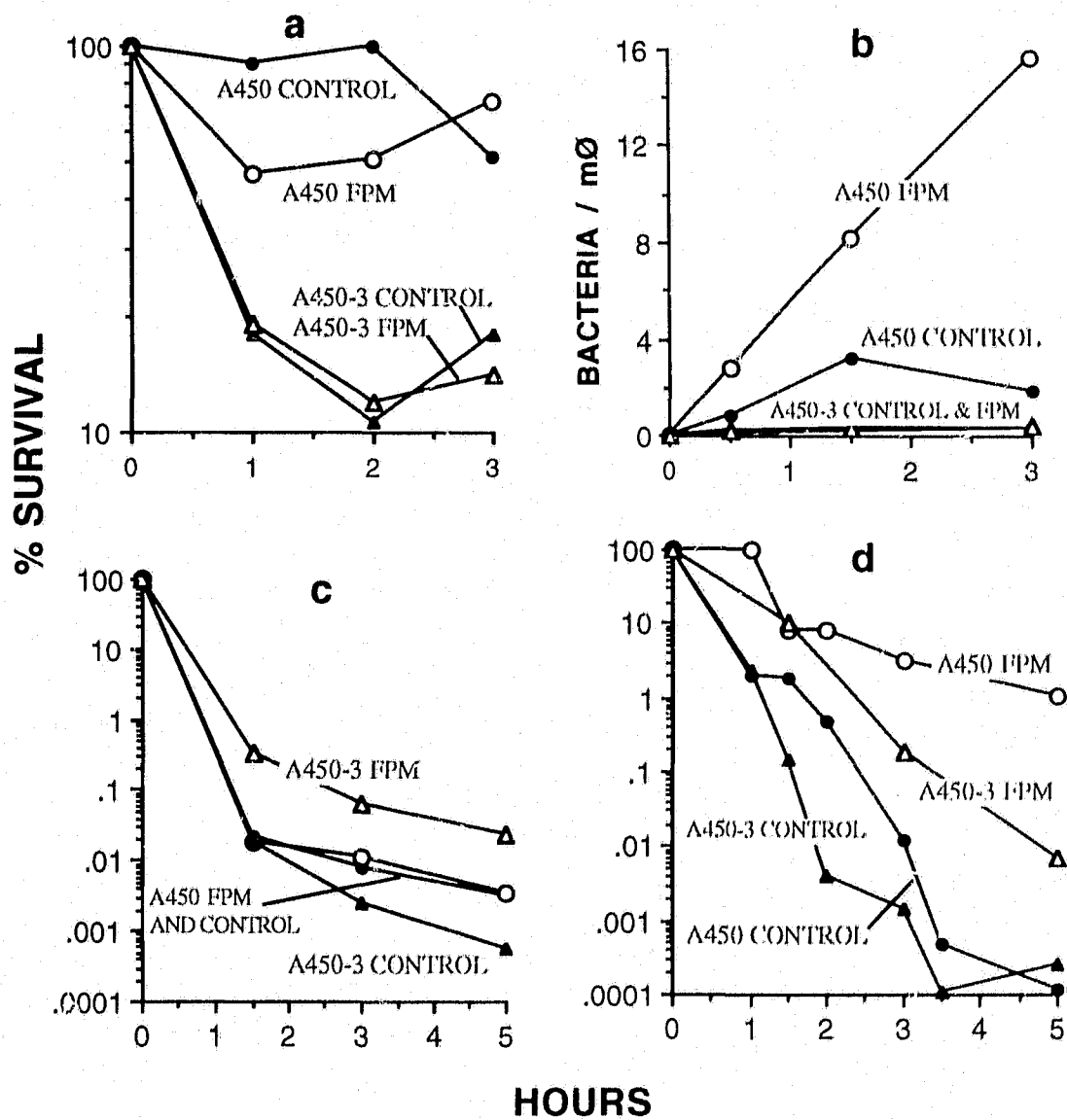
### **Functional competence of the BS pattern**

Besides possessing A-layers with prominent BS patterns, *A. salmonicida* cells grown in the calcium deficient medium, FPM, did not show any other major change in their protein or LPS profile, as detected by SDS-PAGE gels of whole cell lysates, or by Western blots using anti-*A. salmonicida* serum (gels and Western blots were run by J.C. Thornton, Department of Biochemistry and Microbiology, University of Victoria, Canada). FPM grown A450 *A. salmonicida* cells were examined for their ability to resist non-oxidative (complement-mediated), and oxidative (peroxide- or superoxide-mediated) killing, as well as in their ability to associate with mØs. Control cells were grown in the calcium replete medium trypticase soy broth (TSB). The A<sup>-</sup>, O<sup>+</sup> strain A450-3, grown in TSB or FPM, also was included as a control, to define A-layer-independent processes. The results presented in Fig. 50a indicate that the BS pattern is as effective as the normal A-layer in protecting cells from complement-mediated bacteriolysis. The lower level of serum resistance observed in A<sup>-</sup> cells grown in FPM or TSB indicated that protection against serum in A<sup>+</sup> FPM grown cells was mainly dependent on the presence of cell-associated A-layer.

It was striking that only A<sup>+</sup> cells grown in FPM had a markedly enhanced ability to associate with mØ (Fig. 50b); control TSB grown A<sup>+</sup> cells, which associate with mØs through an A-layer mediated process (chapters V and VI), appeared (in comparison to FPM grown cells) as having low levels of adherence to mØs. Indeed, the BS pattern mediated the highest levels of mØ association in the absence of opsonins or any other surface coating. Since no major detectable changes were observed in the protein and LPS profiles of FPM grown cells, and the A<sup>-</sup> strain A450-3 grown in FPM



**FIGURE 49.** Altered A-layer patterns after *in vivo* growth of *A. salmonicida*. (a) and (b) altered patterns obtained as a result of calcium limitation *in vitro*, or (c) and (d), as a result of growth *in vivo*. WD patterns are shown in (a) and (c) and BS patterns in (b) and (d). Bar represents 50 nm for all micrographs.



**FIGURE 50.** Functional characterization of A-layers displaying the BS pattern in *A. salmonicida* A450. FPM grown bacterial cells (displaying the BS A-layer pattern) were compared with control cells grown in TSB (displaying normal A-layer patterns) to compare the functional competence of the BS pattern. The A-layer negative mutant A450-3 was also included as a control to define A-layer-independent processes. (a) Results from the serum resistance assay. (b) Results from the macrophage association assay. Results from challenge assays with H<sub>2</sub>O<sub>2</sub> (c), and superoxide (d).

was still unable to associate with  $m\emptyset$ , I have ascribed the increase in  $m\emptyset$  association to the major structural change in the A-layer, from a normal to a BS altered pattern. It is possible that some protein domains, necessary for efficient adherence, became more accessible in the A-protein subunits arranged in the BS pattern.

As indicated in chapter VII, the A-layer protects *A. salmonicida* against reduced oxygen species (ROS), but the main protective mechanism against oxidative killing is inducible and A-layer independent. When FPM or TSB grown bacteria were challenged with  $H_2O_2$  (Fig. 50c) or superoxide (Fig. 50d) it was observed that the BS structural pattern, as well as the normal A-layer, are similarly protective against  $H_2O_2$ . However, FPM grown A450-3 displayed a higher level of resistance to  $H_2O_2$ . On the other hand,  $A^+$ , FPM grown cells were highly resistant to superoxide, as compared to cells grown in TSB. Again, FPM grown A450-3 displayed a high level of resistance to superoxide. The enhanced survival observed in  $A^-$  cells grown in FPM, clearly suggests a role for the inducible, A-layer independent mechanism in the resistance of FPM grown cells to ROS. The fact that FPM grown  $A^+$  cells had a significantly enhanced resistance to superoxide, but not against  $H_2O_2$ , whereas FPM grown  $A^-$  cells acquired resistance to both agents, confirmed previous observations (chapter VII): i) that the A-layer is definitely more protective against superoxide than  $H_2O_2$ , ii) that resistance to  $H_2O_2$  and superoxide is differentially induced (both having different mechanisms of action), and iii) that the A-layer appears to modify some functions of  $A^+$  cells. Interestingly, FPM and TSB grown cells were similarly sensitive to streptonigrin, and equally competent in binding the hemin analog, Congo Red (not shown).

Evidently, further experimentation is needed to evaluate the functional competence of the BS pattern, but the results obtained so far are quite encouraging. *A. salmonicida* cells possessing BS or normal A-layer patterns were similarly protected against oxidative or non-oxidative killing, but the BS pattern clearly facilitated adherence to host cells. In addition to *in vitro* calcium-depletion conditions, altered novel structural patterns were found to be present *in vivo* (Fig. 49) and also in starved cells (chapter III); observations that give rise to the question: does the A-layer possess more than one functional organizational state? Free cells in the environment, perhaps calcium- and/or energy-limited, may produce altered A-layers with enhanced adhesiveness, presumably an important advantage to efficient colonization and penetration of the host. Once in the host, changes in the levels of calcium and

availability of nutrients may modulate the expression of different structural A-layer patterns on the surface of *A. salmonicida* with consequent implications for pathogenesis. It has not yet been determined which natural environments or host compartments may contain low enough levels of calcium to induce a structural change in the A-layer, but the possibility that the A-layer may exist in more than one functional organizational state cannot be ruled out.

## CHAPTER X

### *IN VIVO* GROWN *Aeromonas salmonicida*

#### INTRODUCTION

Classically, bacterial pathogens have been isolated and studied *in vitro*, then used to infect animals to elucidate virulence factors, to define pathogenic mechanisms, and to reproduce the infectious process following Koch's postulates, in either their classical or molecular forms (Falkow, 1988 & 1990; Khavkin, 1991). However, it has been amply demonstrated that *in vitro* conditions do not accurately duplicate the *in vivo* environment. Thus, *in vitro* growth is often not conducive to the expression of some, or perhaps all, of the essential virulence determinants that bacterial pathogens express upon sensing their host during the normal course of an infection (Brown and Williams 1985; DiRita and Mekalanos, 1989; Smith, 1958 & 1990; Williams, 1988). In many cases, this response to the *in vivo* environment is coordinately regulated by intricate global regulatory networks mediated by two-component systems, and involve the participation of different cell functions (Aricò *et al.*, 1989; Bernardini *et al.*, 1990, Clark, 1990; DiRita and Mekalanos, 1989; Groisman & Saier 1990; Leimeister-Wächter *et al.*, 1992; Maurelli *et al.*, 1992; Melton and Weiss, 1989; Miller *et al.*, 1989 & 1992a).

The studies introduced in chapter VIII have been continued to study the expression of putative virulence determinants during *in vivo* growth. Besides the A-layer, other known virulence determinants of *A. salmonicida* include a layer of smooth lipopolysaccharide (LPS) of homogeneous O-polysaccharide chain length, as well as several extracellular products (Kay and Trust, 1991). Smooth LPS is necessary for anchoring the A-layer to the cell surface of *A. salmonicida* (Belland and Trust, 1985), and plays a role in serum resistance (Munn *et al.* 1982). Smooth LPS may be also responsible for the sugar-inhibitable haemagglutination, observed by Brooks and Trust (1983) using an A-layer deficient strain. Moreover, Lee and Ellis (1991) have demonstrated that purified LPS, from *A. salmonicida* and seven other bacterial pathogens, cause lysis of salmonid erythrocytes.

Extracellular products, extracted from culture supernatants of *in vitro* grown *A. salmonicida*, mainly include proteases (Price *et al.*, 1989; Gudmundsdottir *et al.*, 1990), hemolysins/cytolysins (Lee and Ellis, 1990; Nomura *et al.*, 1988), and enzymes involved in lipid metabolism (Campbell *et al.*, 1990, Lee and Ellis, 1990). These extracellular products (in particular a 70 kDa protease and the glycerophospholipid:cholesterol acyltransferase—LPS complex) have been associated with cytotoxicity, production of typical furuncular lesions, and mortality, upon injection in salmonids (Huntly *et al.*, 1992; Kawahara *et al.*, 1990; reviewed by Ellis, 1991). Lastly, in iron deficient media, *A. salmonicida* produces a siderophore (Chart and Trust 1983; Hirst *et al.*, 1991). Siderophores are important virulence factors involved in efficient uptake of iron, a scarce essential micronutrient in the *in vivo* environment (Bergeron, 1986; Payne and Lawlor, 1990). As well, the siderophore pyochelin may also be involved in tissue injury and inflammation during *Pseudomonas aeruginosa* infections (Coffman *et al.*, 1990).

Using the intraperitoneal chamber model presented in chapter VIII, it has now been demonstrated that *A. salmonicida* cells grown *in vivo* acquired total resistance to host lytic factors and phagocytosis; a phenotype associated with the presence of an undefined slime layer as the outermost structure on the surface of *in vivo* grown *A. salmonicida*. Since this putative novel virulence determinant apparently shields the A-layer and LPS O-chains, the role of these known surface determinants is also discussed. This work was conducted in collaboration with J.C. Thornton (Department of Biochemistry and Microbiology, University of Victoria, Canada), who is currently investigating the expression of other *in vivo*-induced surface antigens of *A. salmonicida*, as well as the nature and origin of the slime layer described in this chapter.

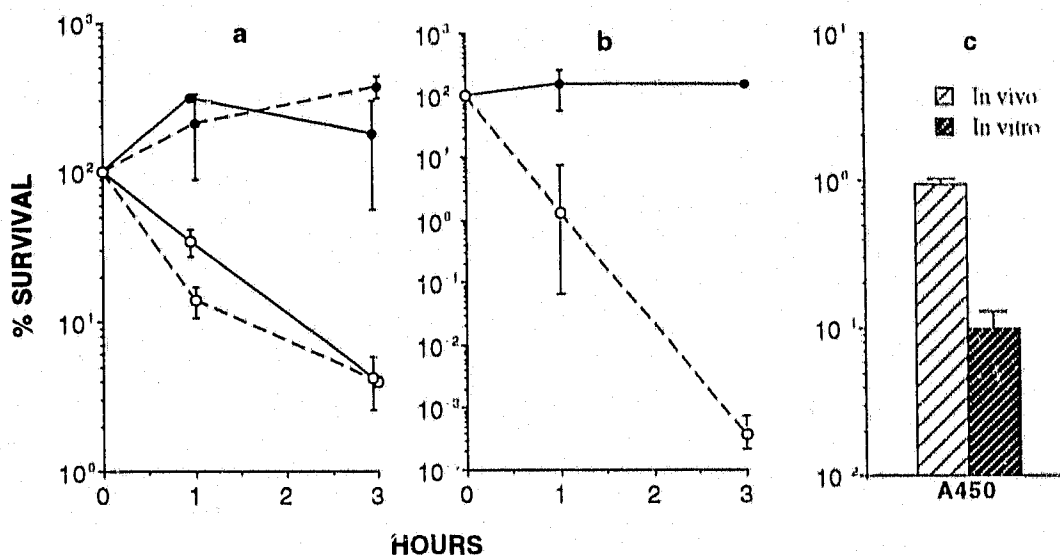
## **MATERIALS AND METHODS**

Refer to chapter IV.

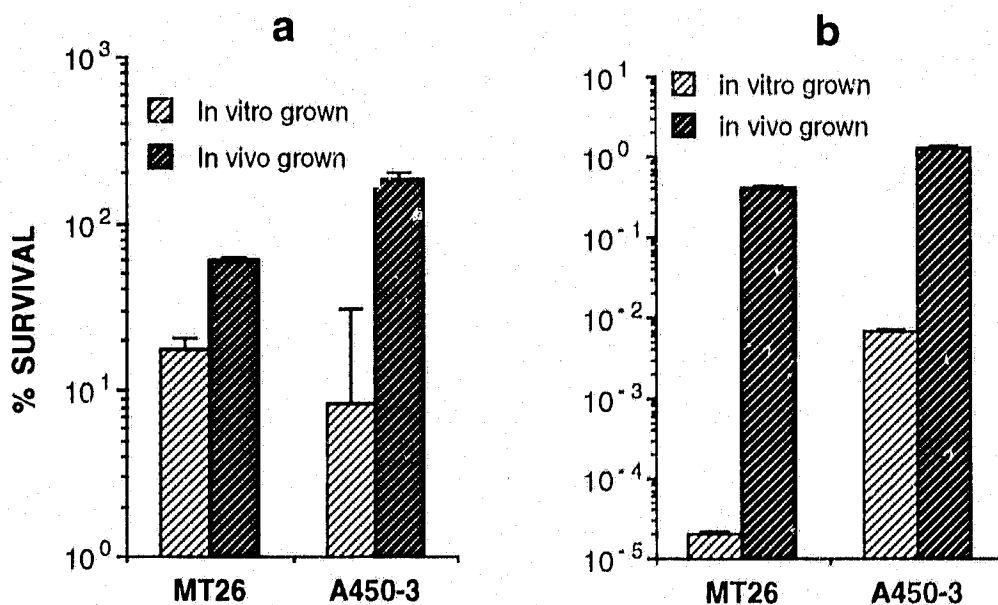
## RESULTS AND DISCUSSION

*In vitro* grown A<sup>+</sup> strains of *A. salmonicida* are somewhat resistant to complement-mediated bacteriolysis (Munn *et al.* 1982). However, even this level of resistance was markedly surpassed after *in vivo* growth, since cells of the *in vivo* grown, virulent strain A450 were completely resistant to both serum and peritoneal fluid challenges (Fig. 51a). These cells had also acquired an unusually high resistance to peroxide (Fig. 51b) and their ability to survive superoxide challenges was ten-fold higher than that of *in vitro* grown cells (Fig. 51c). Similar resistance patterns against the lytic activity of peritoneal fluid and killing by peroxide were observed for the more virulent strain MT26, and for the avirulent A<sup>-</sup> strain A450-3 (Fig. 52a and b). These resistance patterns appeared to represent truly inducible responses rather than an *in vivo* selection process of resistant variants, since resistance was lost upon a single *in vitro* subculture, as demonstrated for both MT26 and A450-3 strains. When recovered from intraperitoneal chambers and subcultured once on TSA plates, these strains regained sensitivity to peroxide and peritoneal fluid (results not shown).

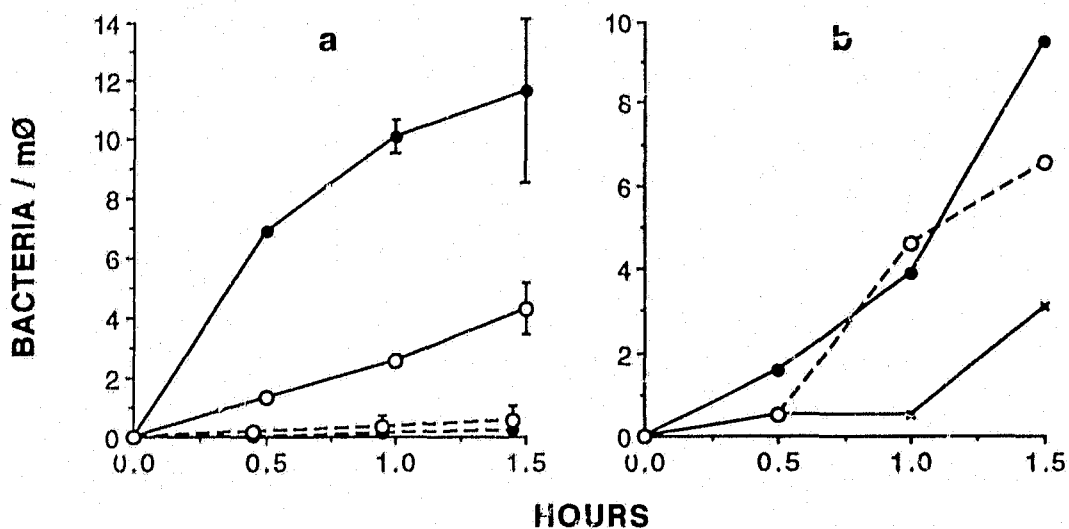
*In vivo* grown A450 cells were found to have lost the ability to associate with mØs. Moreover, *in vivo* grown cells of the A<sup>-</sup> mutant A450-3 showed an even lower capacity to adhere to cultured macrophages than *in vitro* grown cells (Fig. 53a). This was not simply the effect of coating the bacterial cell surface with host-derived molecules, since *in vitro* grown MT26 cells pre-exposed for one hour to fresh peritoneal fluid (Fig. 53b), or exposure to 10% trout serum during the mØ association assays (not shown), did not have significant effects upon mØ association. Since it was previously demonstrated that the A-layer mediates a specific interaction with the mØ cell surface, and that A<sup>-</sup> mutants are poorly adherent to mØs (refer to chapters V and VI), the fact that *in vivo* grown cells adhered to mØs at very low levels (even lower than those observed for *in vitro* grown A-layer negative mutants), suggested that either: i) the A-layer was lost upon *in vivo* culture, ii) the A-layer was structurally modified, or iii) the A-layer was shielded by an even more external structure. Electron microscopy of *in vivo* grown cells pointed to the latter possibility as the most feasible explanation. Firstly, arrayed A-layers were still present in A450 and MT26 cells (refer to chapter IX). Secondly, immunogold labeling of A450 cells with anti-A-protein antibody (refer to "Materials and methods used in functional studies" in chapter



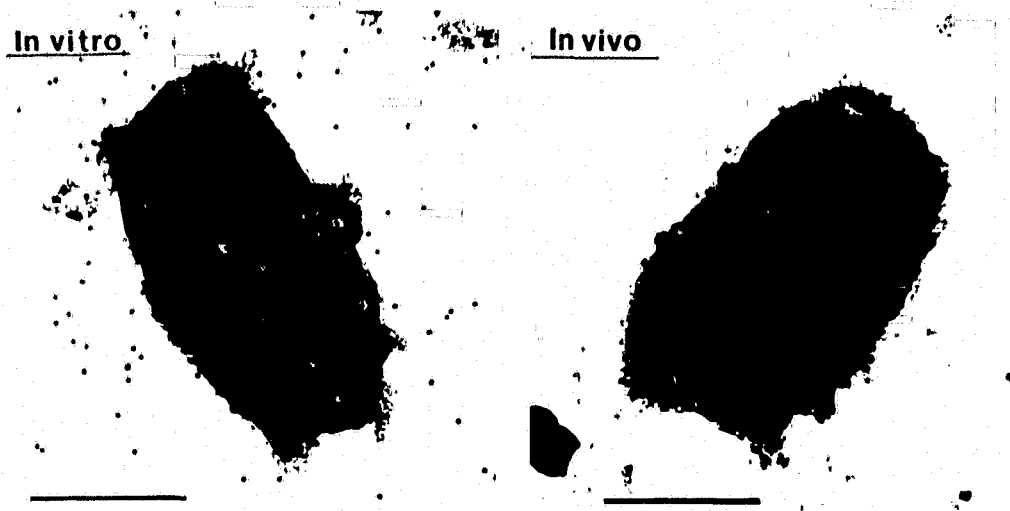
**FIGURE 51.** Resistance of *in vivo* grown *A. salmonicida* A450 to different bactericidal challenges. (a) Survival of *in vivo* (●) or *in vitro* (○) grown cells after being challenged with serum (solid lines) or fresh peritoneal fluid (broken lines). (b) Survival of *in vivo* (●---●) or *in vitro* (○---○) grown cells after a 1 mM H<sub>2</sub>O<sub>2</sub> challenge. (c) Survival to a superoxide challenge of 4 h.



**FIGURE 52.** Resistance of *in vivo* grown *A. salmonicida* strains MT26 (A<sup>+</sup>, O<sup>+</sup>) and A450-3 (A<sup>-</sup>, O<sup>+</sup>) to different bactericidal challenges. (a) Survival after a 3 h challenge with fresh peritoneal fluid, (b) survival after a 3 h challenge with 1 mM H<sub>2</sub>O<sub>2</sub>.



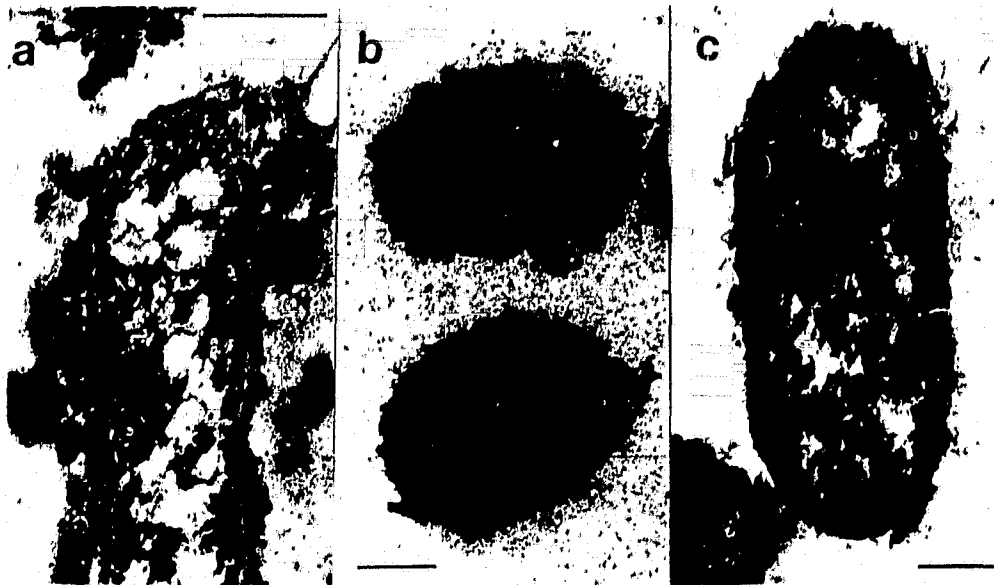
**FIGURE 53.** Effects of *in vivo* growth and pre-exposure to fresh peritoneal fluid on mØ association. (a) MØ association assays with *in vivo* (broken lines) or *in vitro* (solid lines) grown cells of the virulent strain A450 (●), and the avirulent, A-layer negative strain A450-3 (○). (b) *In vitro* grown cells of the strain MT26 were exposed to straight fresh peritoneal fluid for one hour to determine the effect of this treatment upon macrophage association. Pre-exposed cells (O---O) were compared with *in vitro* (●—●) or *in vivo* (x—x) grown cells of the same strain.



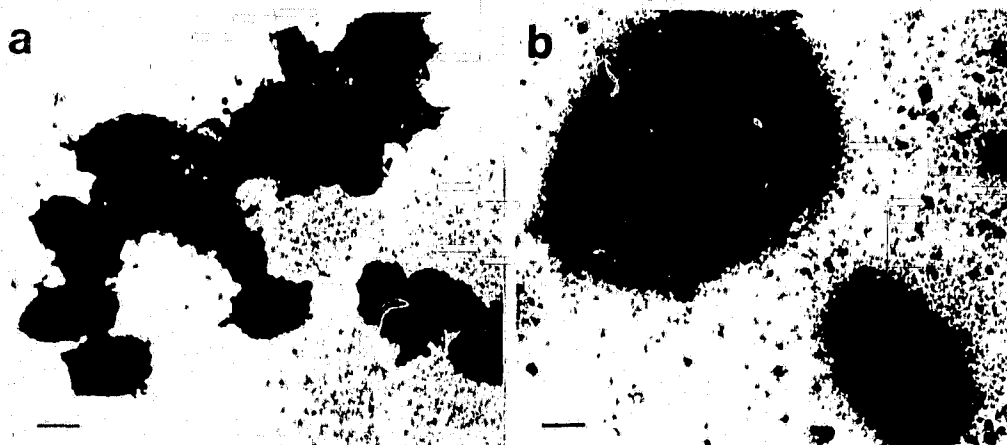
**FIGURE 54.** Immunogold labelling with anti-A-protein antibody, of *A. salmonicida* A450 grown *in vivo* or *in vitro*. *In vivo* grown cells were not labelled, whereas *in vitro* grown ones were heavily labelled. Both preparations were processed simultaneously using the same reagents. Bars represent 0.5  $\mu$ m.

IV) showed that *in vivo* grown cells were not labeled, whereas *in vitro* grown cells were heavily labeled (Fig. 54). Thirdly, a slime layer was observed in thin sections of cells fixed in Ruthenium Red (Fig. 55a) or Alcian Blue (Fig. 55b) fixatives, which enhance visualization of structures composed by polyanionic molecules, especially polysaccharides (Bayer, 1990). Control cells obtained from TSA plates and prepared similarly for electron microscopy did not possess this extra coat on their surface (Fig. 55c). Interestingly, it was noticed that in negatively stained specimens of *in vivo* grown cells it was not possible to observe any surface structure resembling the slime layer observed in thin sections. Therefore negatively stained specimens of *in vivo* grown A450-3 cells (A<sup>-</sup>, O<sup>+</sup>) previously fixed in Ruthenium Red fixative were prepared, which confirmed the presence of a diffuse, slightly electron dense structure, corresponding to the slime layer of thin sections (Fig. 56). That this structure was only visible after enhancement with Ruthenium Red stain, suggested a polyanionic and most likely, a polysaccharidic nature. These experimental observations are fully compatible with an *in vivo* expression of a polysaccharide slime layer, since slime layers and capsules have been recognized as a frequent means of avoiding phagocytosis and mediating serum resistance (Cross, 1990; Cross and Kelly, 1990; Moxon and Kroll, 1990; Horstmann 1992; Roberts *et al.*, 1989). *In vivo* grown bacterial pathogens usually develop resistance to serum or to phagocytosis (reviewed by Williams 1988), and, at least in one particular case, these effects were specifically correlated with enhanced production of exopolysaccharides (Simon *et al.*, 1982).

Alternate serum resistance mechanisms, different from glycocalix expression, should be also considered. Outer membrane blebs have been recently implicated in protection against complement-dependent lysis (Grenier and Bélanger, 1991; Pettit and Judd, 1992). Although this cannot be ruled out as a possible *in vivo* induced resistance mechanism in *A. salmonicida*, it is more likely that blebbing, and association of LPS-rich blebs with the A-layer, would be more related to serum resistance of *in vitro* grown cells. Long O-polysaccharide LPS chains have been associated with increased serum resistance in *Escherichia coli* (Porat *et al.*, 1992) and *Salmonella* (Jiménez-Lucho *et al.*, 1990). In the case of *A. salmonicida*, it would be necessary for *in vivo* grown cells to synthesize a unique type of O-polysaccharide (perhaps longer), different from the one expressed *in vitro*. This is not a very likely possibility since the A-layer-LPS interaction is O-chain dependent and quite specific. In the case of *Neisseria gonorrhoeae*, resistance to normal or immune serum (Wetzler *et al.*, 1992), as well as



**FIGURE 55.** Electron micrographs of thin sectioned *A. salmonicida* A450 cells grown *in vivo* or *in vitro*. (a) *In vivo* grown cells fixed in the presence of Ruthenium Red stain. (b) *In vivo* grown cells fixed in the presence of Alcian Blue stain. (c) *In vitro* grown control cells fixed in Alcian Blue fixative. Bars represent 0.1  $\mu\text{m}$ .

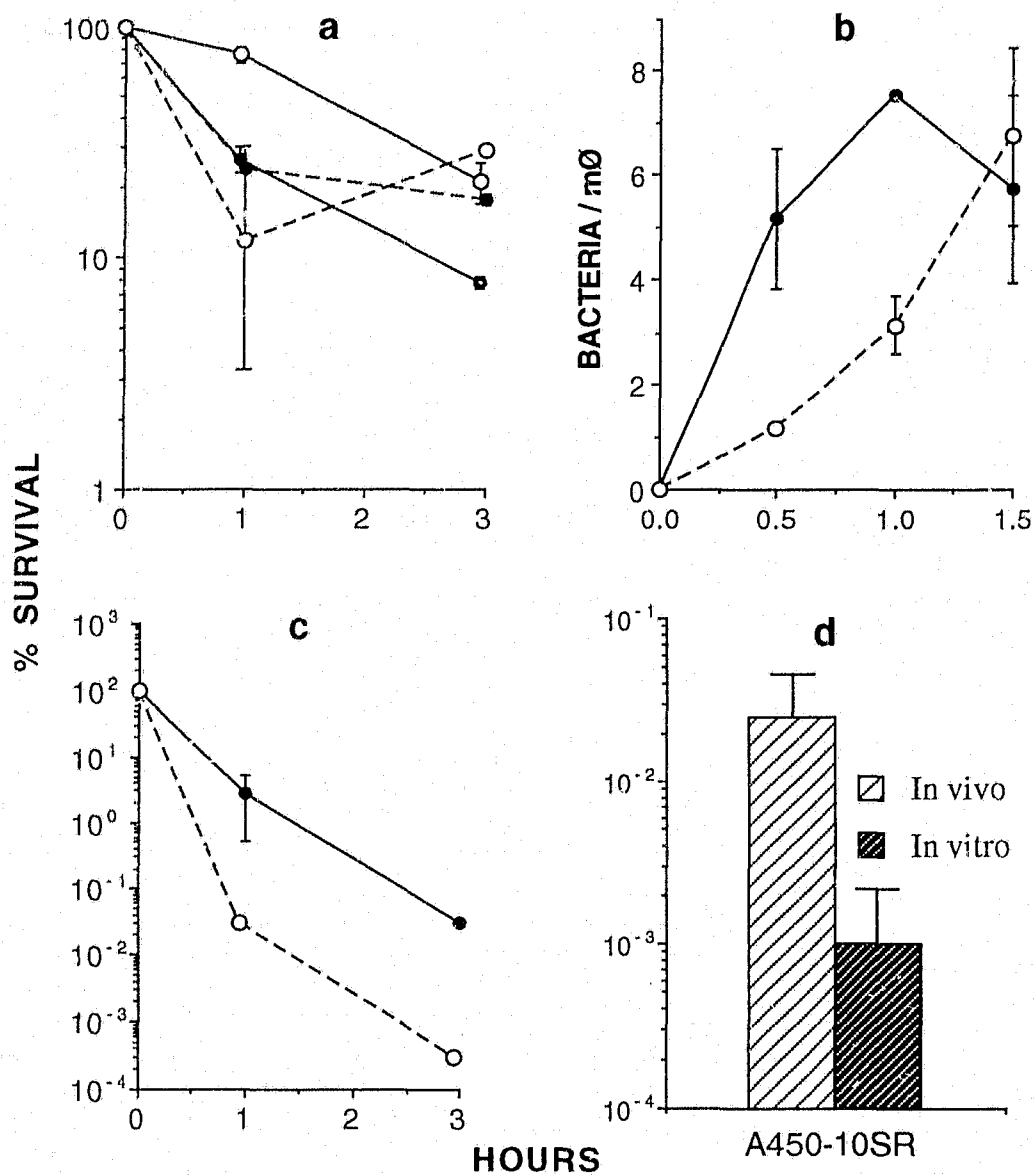


**FIGURE 56.** Transmission electron micrographs of negatively stained cells of *A. salmonicida* A450-3 grown *in vivo*. (a) Cells as recovered from intraperitoneal chambers. (b) Cells from intraperitoneal chambers fixed in the presence of Ruthenium Red. Bars represent 0.5  $\mu\text{m}$ .

resistance to phagocytosis is due to a host-mediated sialylation of bacterial lipooligosaccharides (LOS) (reviewed by Smith, 1990), with a consequent increase in LOS molecular weight, and formation of a discrete capsule which stains with Ruthenium Red (Fox *et al.*, 1991). This host-mediated mechanism may very well be in operation in the case of *A. salmonicida*. Nonetheless, the results obtained with *in vivo* grown cells of the strain A450-10SR, seem to favor production of a slime layer as the mechanism of serum and phagocytosis resistance, rather than a host-mediated modification of existing polysaccharide residues.

When cells of the multiply attenuated strain A450-10SR (harboring pleiotropic defects in cell surface organization) were grown *in vivo*, they *did not* become fully resistant to the lytic activities of serum or peritoneal fluid (Fig. 57a), and *did not* display reduced levels of mØ association. These cells adhered to mØs at levels even higher than those of *in vitro* grown cells (Fig. 57b). However, *in vivo* grown A450-10SR cells were partially resistant to peroxide (Fig. 57c) or superoxide (Fig. 57d) challenges. It was surprising to find that *in vivo* grown cells of A450-10SR *did not* display the slime layer previously observed in cells of the A450 or A450-3 strains. This constituted a fortuitous and significant observation because it provided strong evidence to further support a relationship between resistance to bacteriolysis and phagocytosis with the presence of a slime layer. Moreover, since the LPS electrophoretic profile of A450-10SR is indistinguishable from that of A450 (Thornton *et al.*, 1991), the view that the slime layer is of bacterial, and not host origin was reinforced. The fact that A450-10SR had quite different resistance profiles against lysis and oxidative killing, also suggested that these operated through different mechanisms; i.e. *in vivo*-induced resistance to oxidative killing appeared to be an A-layer and a slime layer-independent process. Most likely, this *in vivo*-induced resistance to oxidative killing is equivalent to the A-layer independent, highly protective, inducible mechanism reported in chapter VII. The fact that cells recovered from intraperitoneal chambers were resistant to reduced oxygen species (ROS), suggests that this protective mechanism has relevance for *in vivo* growth, and is presumably induced in response to granulocyte-mediated, humoral inflammatory mechanisms, that operated through the membrane of the intraperitoneal chambers.

In conclusion, *A. salmonicida* responds to the hostile intraperitoneal environment of rainbow trout, by inducing the expression of a highly protective mechanism to resist oxidative killing, as well as a slime layer. The latter appeared to be



**FIGURE 57.** Survival and macrophage association of the attenuated *A. salmonicida* strain A450-10SR grown *in vivo* or *in vitro*. (a) Survival of *in vivo* (●) or *in vitro* (○) grown cells after being challenged with serum (solid lines) or fresh peritoneal fluid (broken lines). (b) Results from the macrophage association assays with *in vivo* (●—●) or *in vitro* (○---○) grown cells. (c) Survival of *in vivo* (●—●) or *in vitro* (○---○) grown cells after a peroxide challenge. (d) Survival to a superoxide challenge of 4 h.

directly related to resistance against host lytic factors and phagocytosis. These novel virulence determinants are not observed during *in vitro* growth, and consequently represent a unique response of *A. salmonicida* to its host. The combined facts that A450-10SR is absolutely non-virulent, unable to persist in infected tissues for longer than 48 h (Thornton *et al.* 1991), and unable to produce a slime layer, strongly suggest that this outermost surface structure may be important for *in vivo* virulence. Experiments to confirm this contention are being performed by determining if *in vivo* grown cells of the otherwise avirulent strain A450-3, are virulent by intraperitoneal injection.

It was recently reported that *A. salmonicida* is capable of producing a capsule *in vitro* (apparently formed by an acidic polysaccharide and not related to the A-layer), as a response to a change in the carbon to nitrogen ratio in the culture medium (Garrote *et al.*, 1992). Besides confirming the fact that *A. salmonicida* is capable of producing a slime layer, this report also confirms that the expression of this slime layer is environmentally regulated. It is reasonable to surmise that *in vivo*, pathogens would not express all essential virulence factors simultaneously. Rather, these appear to be expressed as programmed responses following a sequential order, as has been observed during the establishment of some pathogenic (DiRita and Mekalanos, 1989; Smith, 1990) or symbiotic (Rolfe and Gresshoff, 1988) infections. In the case of *A. salmonicida* it seems that the role of the A-layer and smooth LPS layer is particularly relevant at the early stages of infection, promoting colonization, penetration of the host, and providing protection against the non-specific humoral defence mechanisms of the host. This would assure a successful initiation and establishment of the infectious process. Subsequent A-layer-mediated interaction with phagocytes would induce resistance to ROS that, in combination with the A-layer, could mediate survival inside mØs. Spreading, presumably mediated by infected mØs, would assure a continuation of the pathogenic process, by reaching other host tissues. Later, when the synthesis of the outermost slime layer is induced (or derepressed), the A and smooth LPS layers would remain covered, presumably playing a minor role in the later stages of infection. The expression of glyocalix might facilitate replication of *A. salmonicida* in different host organs, protected from the humoral response against the now covered surface. Also, since an important characteristic of some polysaccharide coats is their poor immunogenicity (Cross, 1990; Jennings, 1990; Mandrell, 1992; Moxon and Kroll, 1990), it is tempting to speculate that *A. salmonicida* cells may avoid recognition by the

host immune system. Some support for the latter comes from histopathological observations of infected fish, where foci of *A. salmonicida* microcolonies were observed in different tissues in the absence of a surrounding inflammatory response (Armstrong, 1992).

## CHAPTER XI

### IS *Aeromonas salmonicida* AN INVASIVE PATHOGEN?

#### INTRODUCTION

Since the intracellular environment represents a largely unexploited food source with extreme stability, successful adaptation to the intracellular environment may be regarded as advantageous. However, this rich, intracellular microhabitat also has an extreme selection against general colonization, implying that it is not easy to penetrate cells and establish an intracellular way of living or cytobiosis (Taylor, 1983). Indeed, intracellular bacterial pathogens have to utilize specialized mechanisms to enter and survive within their hosts (Falkow, 1991; Moulder, 1985). Entry of phagocytic hosts may be easier, owing to the very nature of the phagocyte. i.e., phagocytic hosts may acquire pathogens during their normal (feeding or defense) activities. However, the usual fate of ingested pathogens is to be destroyed and digested (for nutritional and/or defensive purposes). Based on their efficiency to carry out this latter function, phagocytes can be classified (Brubaker, 1985) as professional (good killers, like macrophages, neutrophils and eosinophils) or non-professional or phagotrophic (poor killers, like free living protozoans, some unicellular plant cells and cells of certain animal tissues, e.g. those lining the gut, or the genital mucosa). On the other hand, non-phagocytic cells are difficult to penetrate. These can be exclusively penetrated through diacytosis (forced entry without the participation of the host) or through pathogen-directed mechanisms (where the pathogen induces the host cell to efficiently internalize it through a normally inefficient mechanism; usually not used for any cellular function) (Moulder, 1985). However, contrary to phagocytes, non-phagocytic hosts display a lower capacity to destroy and digest pathogens. Thus, from the intracellular pathogen's perspective, there are two alternatives: facilitated entrance to a highly hostile intracellular environment in a phagocyte, or a difficult entrance to a less hostile environment in a non-phagocytic host.

Having demonstrated that *A. salmonicida* is a facultative intracellular pathogen capable of surviving and replicating within professional phagocytes, i.e. macrophages

(mØs) (refer to chapter VIII), it seemed important to determine if this pathogen could also penetrate non-phagocytic cells, and therefore extend its opportunities to colonize the intracellular microhabitat of the host. Previous observations during my studies with murine mØs (refer to chapter V), indicated that A<sup>+</sup> *A. salmonicida* was capable of penetrating uncompetent mØs, apparently through a pathogen-directed mechanism. Here I report that the A-layer mediates an efficient interaction with non-phagocytic fish cell lines, and that, indeed, *A. salmonicida* was able to penetrate epithelial cells.

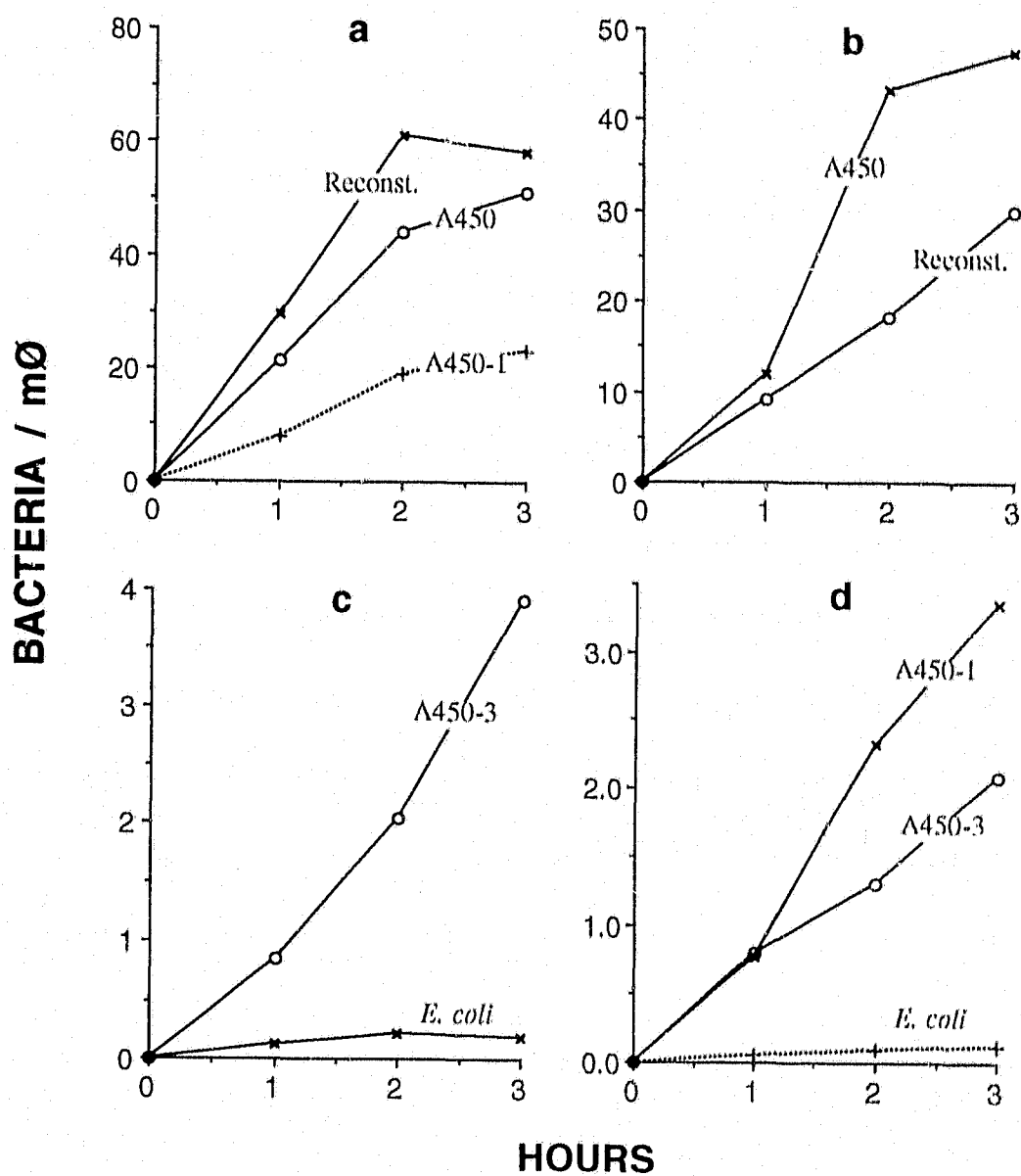
## MATERIALS AND METHODS

Refer to chapter IV.

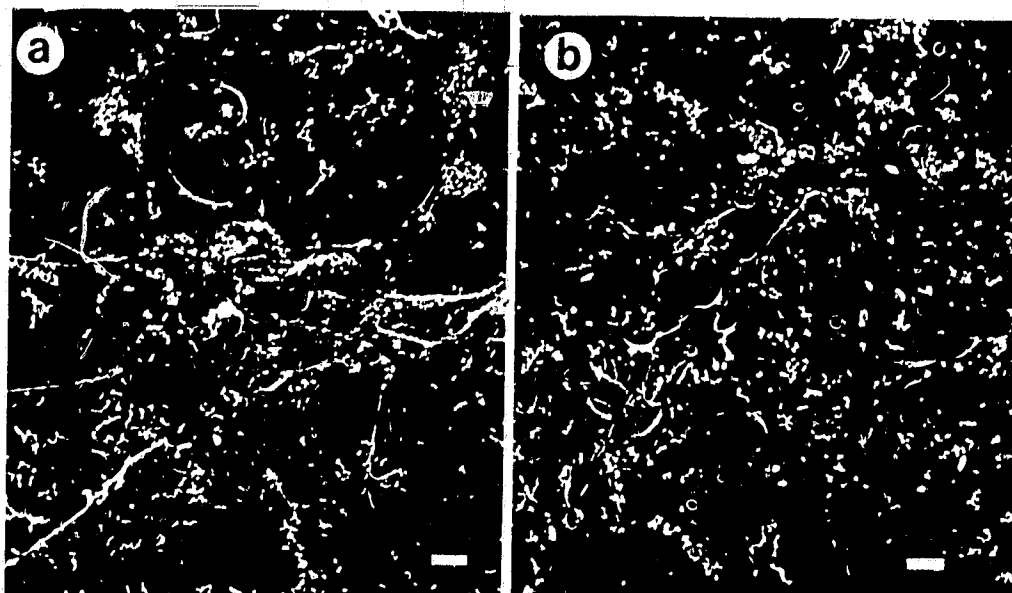
## RESULTS

Association assays of *A. salmonicida* with the fish cell lines EPC and CHSE, showed that the A-layer was capable of mediating adherence to these presumably non-phagocytic cells (Fig. 58a and b). The A<sup>-</sup> strain A450-3, was unable to efficiently associate with either of the two cell lines (Fig. 58c and d), but A-layer reconstituted A450-3 cells re-gained adherence and associated with host cells at levels even higher than those shown by the wild type *A. salmonicida* strain A450 (Fig. 58a and b). *Escherichia coli* ATCC 11775, introduced to determine background bacterial adherence to these fish cell lines, showed the lowest levels of association (Fig. 58c and d).

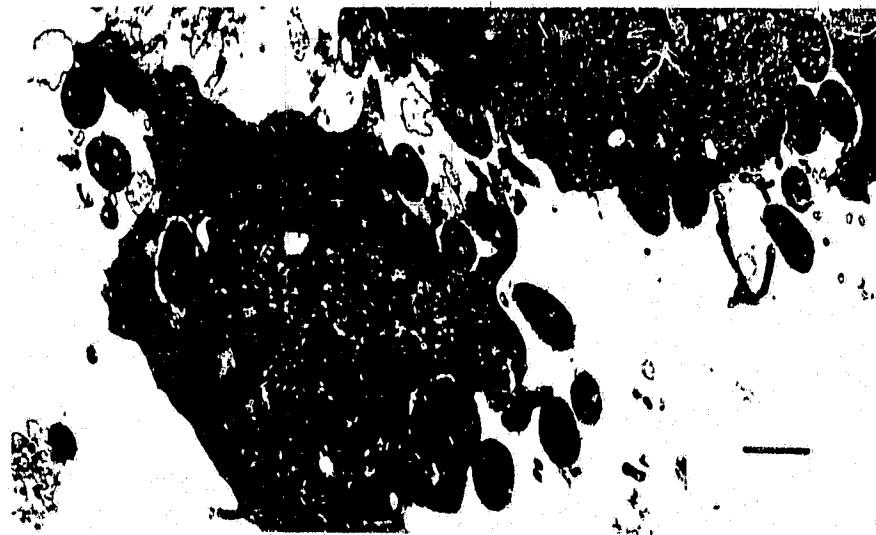
SEM revealed that only A<sup>+</sup> bacteria readily covered the surface of EPC or CHSE cells (Fig. 59), and most importantly, thin sections of infected cells showed that *A. salmonicida* was capable of penetrating epithelial (EPC) cells (Fig. 60). Interestingly, the two A<sup>-</sup> strains A450-3 and A450-1 were also internalized, although in lower numbers (not shown).



**FIGURE 58.** Association of *A. salmonicida* with the fish cell lines EPC (a & c), or CHSI (b & d). *A. salmonicida* strains with different surface characteristics were used,  $\Lambda 450$  ( $A^+$ ,  $O^+$ ),  $\Lambda 450-1$  (phenotypic  $A^-$ ,  $O^-$ ),  $\Lambda 450-3$  ( $A^-$ ,  $O^+$ ). *E. coli* was also used as a control to determine non-specific bacterial binding to these cell lines. Reconst. =  $\Lambda$ -layer reconstituted  $\Lambda 450-3$  cells. Reconstitution was carried out by the co-culturing method reported in chapter V. Results shown are from a single experiment.



**FIGURE 59.** Scanning electron micrographs of EPC (a) and CHSE (b) cells, with adhered bacterial cells of the A450 wild type strain. Only A<sup>+</sup> bacterial cells adhered readily to host cells in culture. Bars represent 10  $\mu$ m.



**FIGURE 60.** Thin section of an EPC cell showing several surface-bound and internalized bacterial cells of the A450 wild type strain. Bar represents 1  $\mu$ m.

## DISCUSSION

Although cell-entry mechanisms of intracellular pathogens are diverse, some generalizations can be made. For instance, a common property of phagocytic pathways is the dynamic cytoskeletal rearrangements that accompany penetration (Stossel, 1989; van Deurs *et al.*, 1989), which are the result of transmembrane signals involved in the activation of effector systems (Baggiolini and Dewald, 1988; Fallman *et al.*, 1989; Jesaitis *et al.*, 1989; Sarndahl *et al.*, 1989; Wick *et al.*, 1991). The fact that *A. salmonicida* could penetrate the epithelial cell line EPC, demonstrates that this bacterium is capable of producing the correct signaling required to trigger a normally non-operational particle internalization system. In order to do that, *A. salmonicida* must interact with the proper surface receptors on the surface of the host cell. The fact that A<sup>+</sup> and A<sup>-</sup> cells were also internalized, indicates that penetration is perhaps an A-layer independent process. In other words, the A-layer may act as an adhesin (as demonstrated by the fact that it significantly promotes the association of A<sup>+</sup> bacterial cells with host cells), but not as a specific invasin. As an adhesin, the A-layer may promote initial attachment to the cell surface, leading to a more efficient establishment of secondary interactions responsible of triggering internalization (Hoepelman and Tuomanen, 1992). Quantitative studies on the internalization of uncoated versus A-layer coated latex beads, and determination of the bound to internalized ratio of A<sup>+</sup> and A<sup>-</sup> bacterial cells, should provide the information necessary to determine if the A-layer has a role in both adhesion and internalization or if it only promotes adhesion. As well, the isolation of putative cell surface receptors involved in the binding and/or internalization processes, should help to elucidate the role of the A-layer (or other surface molecules) in the internalization process. Alternatively, it could be possible that the fish cell lines used were unknowingly phagocytic to some extent, as it has been demonstrated for epithelial cells lining the intestine of cod and herring larvae (Olafsen and Hansen, 1992). The normal phagocytic competence of the epithelial (EPC) and fibroblastoid (CHSE) fish cell lines should be clarified before further experimentation is conducted. In any case, as reported previously by other investigators (Parker and Munn, 1985; Sakai, 1987), A<sup>+</sup> *A. salmonicida* cells were demonstrated to have an enhanced ability to bind host cells. More importantly, a door to investigate the invasive properties of this unique and fascinating fish pathogen has been opened.

## GENERAL DISCUSSION

No S-layer has been as thoroughly studied as the A-layer of the fish pathogen *A. salmonicida*. The experimental results presented in this dissertation have significantly contributed to our understanding of the structure and function of the A-layer. Thus, the A-layer constitutes the first S-layer for which clear relationships between structure and functional competence have been established. For instance, no other S-layer has been shown to possess alternate structural conformations with potential *in vivo* functions, as it has been demonstrated for the A-layer (chapters III & IX). These novel structural conformations are not related to those previously thought to involve a change in the porosity of the A-layer (Stewart *et al.*, 1986). As demonstrated in chapter II, the putative structural conformations described by Stewart *et al.* (1986), turned out to be two different A-layer morphotypes that only exist in the context of negative staining, as artifacts of superimposition and differential staining. The functional studies reported in chapters IV to XI, also contributed to a better understanding of the architectural organization of the A-layer. For instance, they provided support for the view that the A-layer naturally exists as a monolayer, owing to the fact that superimposed or aggregated layers were not functionally competent in mediating adherence to macrophages (mØs), or in reattaching to the cell surface of A-layer negative mutants (chapter VI). The structural orientation of the layer was also important in reattachment to A450-3 cells (O<sup>+</sup>, A<sup>-</sup>), and adherence to mØs (chapter VI); clearly suggesting that the A-layer possesses two different faces which are functionally different. The inner face, defined by the plane occupied by the core mass units, interacts with the bacterial cell surface through specific interactions with the O-polysaccharide chains of LPS. The outer face, defined by the plane occupied by the linker mass units, is involved in adherence to surfaces and host cell receptors. It is tempting to speculate that the pits exposed on the outer A-layer's face, formed by the lower core mass units surrounded by the more elevated linker mass units (refer to Fig. 2 in chapter I) may constitute the preferred binding sites for host proteins. Immunoglobulins (Phipps and Kay, 1988), collagen IV (Trust *et al.*, manuscript submitted), and host receptors possessing immunoglobulin-like domains (which I have

hypothesized to be involved in mØ binding) (chapter V), bind to the A-layer in an array-dependent fashion. Perhaps these host molecules bind to a site only formed when four A-protein subunits convene at one of the two 4-fold symmetry axis of the A-layer. Alternatively, binding sites for host molecules may be present on each A-protein subunit, but they only are available when the subunits are within the array. In the latter case, binding of one large host molecule (e.g. an immunoglobulin) to one of the binding sites, may preclude binding of more host molecules through steric hindrance, accounting for the binding stoichiometry of 1 immunoglobulin molecule to 4 A-protein monomers. Lastly, the A-layer also constitutes the first S-layer for which a combined and *differential* role for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the structural integrity of the layer has been demonstrated (chapter III). This may constitute a unique and important property that allows the A-layer to exist in at least two functionally different organizational states (chapter IX).

Functionally, it was rigorously demonstrated that the A-layer possesses the properties of an adhesin (chapters V, VI & XI). Consequently, S-layers should be regarded as a new structural class of adhesin, together with fimbriae, multifunctional toxins, flagella, and other nonfimbrial adhesins. It remains to be rigorously shown that the A-layer also has a role as an invasin. However, the results presented in chapter XI regarding penetration of the epithelial fish cell line EPC, are encouraging. Mucosa invasion experiments *in vivo*, or the use of polarized fish epithelial cells, or organs in culture, should provide excellent models to study the invasive properties of *A. salmonicida*, and define the role of the A-layer in the invasion process.

An outstanding and unexpected function of the A-layer was that involving the protection of *A. salmonicida* against reduced oxygen radicals (chapter VII). The mechanism involved in this protection is ill-defined, but the available experimental evidence points to a possible physiological connection between the A-layer and the cell membrane. In this context, findings here could complement previous ones, that for many years have also suggested such a connection, and perhaps will encourage further investigation [e.g. investigation of the tight relationship that exists between incubation temperature and the synthesis of A-layer (Ishiguro, 1988; Ishiguro *et al.*, 1981)]. For example, it is known that growth of *A. salmonicida* at 30°C is only possible in the absence of an arrayed A-layer. However, some preliminary results (not shown here) have indicated that *A. salmonicida* A450 readily grows at 30°C in FPM medium without  $\text{Mg}^{2+}$ , in which A450 forms tetrameric units of A-protein that do not assemble into

arrayed layers. Also, cultures of the surface disorganized mutant A450-10SR (chapter III), which appears to have aggregated layers that do not completely cover the bacterial cell surface, possess a very high number of A<sup>-</sup> variants, as demonstrated by the large number of white colonies produced upon culture at 30°C on Congo Red (CR) plates (J.C. Thornton, personal communication). Lastly, atypical *A. salmonicida*, which can grow and form normally arrayed A-layers at 30°C, have a nutritional requirement for heme (Ishiguro *et al.*, 1986), suggesting that they have difficulty in acquiring iron. The differential sensitivity profile of A<sup>+</sup> and A<sup>-</sup> *A. salmonicida* strains to streptonigrin (SNG), and its modulation by hemin, also seems to fit as an additional piece in this puzzle. SNG uptake appears to be mediated by a wild type (but not reconstituted) A-layer (chapter VII). Previous unpublished observations by W.W. Kay, and E.E. Ishiguro (University of Victoria, Canada), also indicate that during CR binding experiments with A<sup>+</sup> cells, some dye was partitioned into the outer membrane, reaching inner cell targets. This was not observed at all during binding assays with A<sup>-</sup> cells possessing an intact smooth LPS layer, unless the smooth LPS layer was disturbed with EDTA (Ishiguro, 1985). It is very possible that A-layer-bound SNG had an enhanced access to inner cell targets, accounting for the extreme sensitivity of A<sup>+</sup> cells to this redox-active compound. Recently, it was found that A<sup>+</sup> cells grown in the presence of the iron chelator EDDA are more susceptible to SNG than LB grown cells (not reported here), which confirms the relationship between iron transport, A-layer, and SNG binding. The hemin and SNG binding kinetics of atypical strains, the SNG<sup>r</sup> mutants that have been isolated (chapter VII), as well as wild type *A. salmonicida* grown in the presence of EDDA, should provide clues for the elucidation of these puzzling results.

The *in vivo* studies, originally devised to detect structural changes of the A-layer in the host environment, have provided a tremendous insight into the pathogenic process of furunculosis, and the time frame (sequence) in which the different virulence determinants of *A. salmonicida* may be expressed. Colonization of either the external surface of fish (Cipriano *et al.*, 1992), gills, or internal mucosas (Tatner, 1987), may be mediated by the A-layer, in its adhesion role, which in addition, may provide protection against the non-specific antimicrobial activities of skin and mucosal barriers, e.g. lysozyme. It is not clear yet if subsequent penetration of the host is directly mediated by the A-layer or a consequence of the initial A-layer-mediated binding to host cells, allowing other as yet unknown mechanisms to operate. In any case, once A.

*salmonicida* has penetrated the host, the A-layer certainly provides some protection against the humoral non-immune defenses, e.g. complement and bacteriolytic activity of peritoneal fluid. Encounters with tissue mØs and granulocytes are probably frequent, either during early colonization and penetration of damaged skin (Cross and Matthews, 1991; Peleteiro and Richards, 1990), or later, when *A. salmonicida* is in contact with deeper tissues. Enhanced penetration of mØs (chapters V & VI), as well as survival within them, are two processes in which the A-layer has a definite role. Indirect effects that stem from the interaction with mØs include: i) spreading to new sites (especially the lymphoid organs), presumably mediated by infected mØs, ii) evasion of humoral defense mechanisms (chapter VIII), and iii) induction of the protective response against oxidative killing (chapter VII), which together with the A-layer, presumably constitute the means of survival inside mØs. At this point, some other virulence factors may start playing a more prominent role, while the A-layer may be relegated to a minor or secondary one. Production of aggressins (proteases, hemolysin, and cytotoxins) in different tissue locations, is most likely the cause of pathology in furunculosis. At this point the A-layer may be already shielded by the slime layer (chapter X), which in turn provides *total* resistance to serum and tissue fluids (e.g. peritoneal fluid). The presence of a slime layer, together with an apparent immunosuppression induced by *A. salmonicida* in the later stages of infection (Pourreau *et al.*, 1986 & 1987) may allow the free systemic multiplication of *A. salmonicida* and the consequent production of elevated concentrations of aggressins, accounting for the severe tissue destruction and acute mortality typically associated with furunculosis. The little success so far experienced in combating furunculosis may stem from the fact that it was totally unknown that *A. salmonicida* possessed a programmed response to the *in vivo* environment. Obviously, *in vivo* grown *A. salmonicida* shielded inside its slime layer has eluded many researchers, who considered the A and smooth LPS layers as the major targets of a protective immune response. It seems appropriate to re-direct, now, the strategy used in combating furunculosis.

In conclusion, the A-layer of *A. salmonicida* has proved to be a multifunctional structure that plays specific and important roles in the biology of this fish pathogen. Indeed, it has been rewarding to confirm that S-layers do not exist as mere decorative arrays on the surface of bacteria. It would be even more rewarding were the examination of bacterial pathogens with S-layers stimulated in light of the findings presented here. In contrast to environmental bacteria, which are usually exposed to a

fluctuating and sometimes unpredictable environment, pathogens appear to be exposed to a series of well-defined aggressions imposed by the host defence mechanisms, and therefore, they may provide researchers with some advantages in obtaining information about the biological significance and capabilities of S-layers.

As far as the A-layer, *A. salmonicida*, and furunculosis are concerned, the better understanding that we now have will certainly direct us in developing more adequate approaches to prevent infection in salmon aquaculture farms, a key step which is all the more significant, owing to the fact that the entire future of this developing industry largely depends on our ability to rear healthy fish.

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