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The Extracellular Toxins of *Aeromonas salmonicida* subsp. *salmonicida*

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INTRODUCTION

The first indication that *Aeromonas salmonicida* produced extracellular enzymes was by Griffin *et al.* in 1953 who reported beta-haemolysis and gelatin liquefaction when the bacterium was grown on rabbit blood agar and nutrient gelatin plates. In 1953, Griffin suggested that the extensive tissue destruction characteristic of furunculosis was probably due to production of protease enzymes but as haemolysis was not an obvious feature of furunculosis, the haemolysin was considered to play an unimportant role in pathogenesis. Karlsson (1962) confirmed the production of an extracellular haemolysin. However, Klontz *et al.* (1966) were the first to show *in vivo* effects by injecting saline extracts of cells which produced haemopoietic necrosis in fish. The first attempts to purify and characterize the extracellular protease were described by Dahle (1971). Fuller *et al.* (1977) purified a leucocytolytic factor which caused a transient leucopaenia after injection into fish. However, it was not until 1980 that direct evidence for potent toxin production by the bacterium was published. Extracellular products (ECP) produced by growing the bacterium on cellophane overlays were shown to have potent lethal activity as well as proteolytic, haemolytic and leucocytolytic activities (Munro *et al.*, 1980). Furthermore, upon intraperitoneal (i.p.) or intramuscular (i.m.) injection into fish the ECP was capable of inducing all the lesions associated with furunculosis (Ellis *et al.*, 1981).

Over the past 15 years a considerable amount of work has been

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performed to analyse the constituents of the ECP and to understand their role in virulence and pathogenesis. A common approach in attempts to identify extracellular virulence factors has been to compare constituents of ECP produced by strains differing in their degree of virulence and to look for correlations with the production of certain ECP components. This approach makes the basic assumption that any particular strain produces the same toxins whether it is growing *in vitro* or *in vivo*. As is described below, this assumption is clearly invalid and has led to a good deal of confused interpretation of experimental data. Essentially, the lack of a particular component in the ECP of a particular strain does not mean that that strain lacks the capacity to produce it *in vivo*. The only proof of that would be to show that the gene for that component was lacking. *In vivo* production of antigens which are known from *in vitro* studies is open for investigation using specific antibody probes on infected tissues, but the problem with investigations of virulence mechanisms is the possibility that certain factors could be produced *in vivo* but may not be produced under *in vitro* conditions by any strain. Such a possibility is difficult to investigate.

Furthermore, it is well known that *in vitro*, different enzymes are produced at different times during culture and certain enzymes, especially proteases, can degrade others; also some ECP may be extremely labile. Hence the detection of certain products may be very difficult, even though they are produced *in vitro*. It is also known that availability of certain nutrients or physicochemical characteristics of the culture medium can affect the production of certain factors, hence the use of certain growth media and conditions will favour production of some factors while suppressing others. This reflects the expectation that production of different aggressins will be stimulated by appropriate stimuli during different stages of infection and disease. Thus, the toxins appropriate for invasion of the mucous membranes are likely to be very different from those appropriate for rapid growth of bacteria within tissues or defence against host leucocytes.

A recent approach to the study of toxins is to use molecular biological techniques to produce DNA expression libraries which can be screened for potential toxins, for example proteases or cytolysins. In this way the existence of certain enzymes, which may not have been identified in culture supernatants, can be detected. Antibody probes can then be made and the production of such putative virulence factors *in vivo* can be investigated. The production of mutants lacking expression of such genes can also be assessed for virulence to provide further evidence for their importance in virulence and pathogenic mechanisms.

Thus, while we can build up a picture of the virulence factors of a pathogen based upon knowledge of factors produced *in vitro* a variety of approaches to identify them must be taken and a complete understanding may still be elusive.

EXTRACELLULAR PRODUCTS (ECP)—THE PROBLEM OF DEFINING COMPOSITION

The ECP of typical *A. salmonicida* contains a large number of extracellular proteins (Figure 9.1) (and other factors) many of which have enzyme activity (Table 9.1). Many of these components potentially have significance as virulence factors in allowing the bacterium to penetrate, survive and reproduce within the host tissues. In comparing the toxic properties of ECP from different strains, various factors need to be taken into account as the presence of many components is affected by culture parameters. For example, H-lysin is maximally produced in static broth culture (Titball & Munn, 1981). Temperature (Fyfe *et al.*, 1987), oxygen and composition of the growth medium (Fyfe *et al.*,

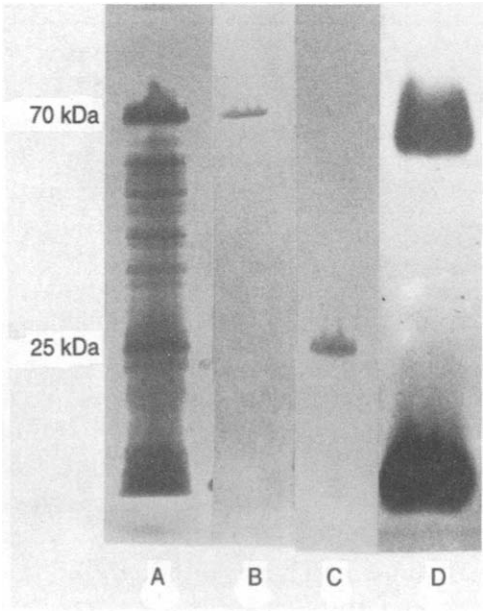

Figure 9.1 SDS-PAGE of *Aeromonas salmonicida* extracellular products (ECP). A, Protein-silver stain; B, purified 70-kDa protease, protein-silver stain; C, purified GCAT/LPS, protein-silver stain; (D) purified GCAT/LPS, LPS-silver stain.

Table 9.1 Extracellular products of *Aeromonas salmonicida* ssp. *salmonicida*

	References
Proteases	
70-kDa protease (caseinase, serine protease)	Price *et al.* (1989)
Gelatinase (metalloprotease)	Rockey *et al.* (1988)
Membrane-damaging toxins	
Leucocytolysin	Fuller *et al.* (1977)
Cytotoxic glycoprotein	Cipriano (1982a); Tajima *et al.* (1983)
T-lysin (haemolysin)	Titball & Munn (1983)
H-lysin (haemolysin)	Titball & Munn (1985)
Enterotoxin?	Jiwa (1983)
Salmolysin	Nomura *et al.* (1988)
GCAT	Buckley *et al.* (1982); Lee & Ellis (1990)
GCAT/LPS	Lee & Ellis (1990)
Other factors	
Lipopolysaccharide	MacIntyre *et al.* (1980)
Siderophore	Chart & Trust (1983); Hirst *et al.* (1991)
Brown pigment	Donlon *et al.* (1983)
Esterases	Hastings & Ellis (1988)
Amylase	Campbell *et al.* (1990)
Ribonuclease	Campbell *et al.* (1990)
Aryl-sulphatase	Campbell *et al.* (1990)
α-Glucosidase	Campbell *et al.* (1990)
α-Mannosidase	Campbell *et al.* (1990)
Alkaline phosphatase	Campbell *et al.* (1990)
Phospholipase C	Campbell *et al.* (1990)
Lysophospholipase	Campbell *et al.* (1990)
Potential toxins (identified by gene cloning)	
ASH3 (broad-range haemolysin)	Hirono & Aoki (1993)
ASH4 (haemolysin, virtually specific for fish erythrocytes)	Hirono & Aoki (1993)

1986a; Campbell *et al.*, 1990), presence or absence of A-layer (Titball & Munn, 1985) and the availability of iron (Hirst *et al.*, 1991; Neelam *et al.*, 1993) are all important factors which affect the composition of ECP. Table 9.1 includes all the factors that have been identified in ECP regardless of culture conditions.

Most investigations of the ECP components have been aimed at identifying the factors responsible for the lethal toxicity and pathology caused by the ECP and present evidence indicates that a serine protease and glycerophospholipid cholesterol acyltransferase (GCAT) complexed with lipopolysaccharide (LPS) are the most important factors responsible for these activities. The evidence for this is presented below.

EXTRACELLULAR TOXIC SERINE PROTEASE

The first reports of a lethal protease toxin were by Tajima *et al.* (1983) and Shieh (1985a), though only poor evidence for the degree of purity of the protease was presented. However, this result was substantiated by Lee and Ellis (1989) using highly purified protease. The LD_{50} of the protease in 10 g Atlantic salmon was 2.4 $\mu g\ g^{-1}$ fish.

After i.m. injection into fish, the purified protease produced haemorrhaging and muscle liquefaction but not as severe as the whole ECP containing the same protease concentration (Fyfe *et al.*, 1986b). It appeared that equivalent lesions were produced when the protease was accompanied by a haemolytic factor in the ECP (Fyfe *et al.*, 1988) and the interaction of these toxins was elucidated by Lee and Ellis (1991a) (see below).

PHYSICOCHEMISTRY OF THE TOXIC PROTEASE

The physicochemical properties of the protease are summarized in Table 9.2. The protease is a serine protease (i.e. a serine residue is present in the active site of the enzyme) as shown by its irreversible inhibition by phenyl methane sulphonyl fluoride (PMSF). For some time the molecular weight (MW) of the protease was controversial with reported values of 11 kDa (Shieh & MacLean, 1975), 43 kDa (Dahle,

Table 9.2 Physicochemical properties of the toxic protease

		Reference
MW	70 kDa	Price *et al.* (1989)
pI	5.6	Hastings & Ellis (1988)
pH optimum	9.0	Finley (1983)
Inactivation temperature	50°C	Finley (1983); Tajima *et al.* (1984)
Substrate specificity	Non-specific for high MW open structure proteins. Hydrolysis of *p*-nitroanilides indicates thrombin - specificity type	Price *et al.* (1989)
	Hydrolysis of amides indicates Factor Xa specificity type	Salte *et al.* (1992)
Low MW inhibitors	PMSF	Price *et al.* (1989)
High MW inhibitors	α_2-Macroglobulin	Ellis (1987)
	Antithrombin	Salte *et al.* (1992)

1971), 87 kDa (Mellergaard, 1983), 71 kDa (Tajima *et al.*, 1984) and 70 kDa (Fyfe *et al.*, 1986b). Most of these reports concerned the protease produced by different strains of *A. salmonicida*. However, a comparative study of the protease of these and other strains, produced under identical conditions, demonstrated the protease was in fact identical in all strains and on continuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels has an apparent MW of 70 kDa (Price *et al.*, 1989). On gradient SDS-PAGE the protease had a MW of 64 kDa (Lee & Ellis, 1990), which correlates with the predicted MW based upon its gene sequence (Whitby *et al.*, 1992).

The serine protease has only a limited degree of specificity toward protein substrates and is capable of degrading proteins with a relatively open structure, for example casein, denatured bovine serum albumin (Price *et al.*, 1990). However, proteins of a more compact structure (e.g. ovalbumin, albumin, native bovine serum albumin) were more resistant to digestion. In studies aimed to characterize the specificity of the active site a number of *p*-nitroanilides were screened as substrates (Price *et al.*, 1990). The protease hydrolysed two *p*-nitroanilides which are regarded as specific substrates for thrombin indicating the two enzymes share a specific arginine recognition site. However, the protease is much less discriminating on high MW substrates. Like thrombin, the protease markedly reduced the clotting time of trout blood but was unable to polymerize bovine fibrinogen, suggesting that activation of the clotting cascade by the serine protease was different from thrombin (Price *et al.*, 1990). This blood-clotting ability of the protease may account for the presence of microthrombi throughout the vasculature, especially in the heart in cases of clinical furunculosis and following injection of crude toxins (Ellis *et al.*, 1981, 1988b).

In pressing this line of investigation further, Salte *et al.* (1992) screened a number of chromogenic amides regarded as specific substrates for a variety of enzymes involved in the blood-clotting system and found the serine protease to have much higher activity towards substrates specifically cleaved by activated Factor X (Factor Xa) than substrates specific for thrombin or other clotting enzymes. Evidence of the similarity between the serine protease and Factor Xa was further provided by the high level of inhibition by antithrombin and chymostatin, both of which are inhibitors of Factor Xa while hirudin (an inhibitor of thrombin) and aprotinin (an inhibitor of plasmin) had little inhibitory effect (Salte *et al.*, 1992).

ROLE OF THE 70-kDa PROTEASE AS A VIRULENCE FACTOR

Evidence of a role in virulence

Questions have been raised by several workers concerning the indispensable role of the serine protease in so far as several virulent isolates of the bacterium do not produce any protease under standard culture conditions (Hackett *et al.*, 1984; Ellis *et al.*, 1988b). Furthermore, the protease-deficient ECP of these strains and the ECP of protease-producing strains which were treated with PMSF to inhibit the protease had only slightly increased lethal doses compared to ECP containing active protease (Ellis *et al.*, 1988b). Thus, neither virulence nor ECP toxicity appeared to be dependent upon the serine protease. The toxicity of protease-deficient ECP was later explained by the identification of another lethal toxin much more potent than the protease, namely GCAT/LPS (see below). Furthermore, the question concerning protease production by the protease deficient strain MT028 has been investigated under a variety of *in vitro* culture conditions. Using a specific rabbit anti-serine protease antiserum to probe Western blots of the ECP of this strain, the protease was absent when the bacteria were grown in normal culture conditions. However, in the presence of 2-2-dipyridyl, an iron chelating agent which restricts the availability of iron, a specifically staining band, typical of the serine protease was present in Western blots of the ECP (Ellis, unpublished). Thus, it appears that the availability of iron regulates serine protease production by strain MT028. As *in vivo* environments are iron-restricted it is possible that such conditions act as a signal to elicit protease production. This observation serves as an example of how the production of putative virulence factors may depend upon specific environmental stimuli and extrapolation from conventional culture conditions to the complex *in vivo* conditions must be done with caution.

Mention should be made of the existence of a second protease produced by typical *A. salmonicida* although the levels of this enzyme in ECP are usually very low. This enzyme differs from the 70-kDa serine protease in that it is a metalloprotease and lacks caseinase activity, the preferred substrates being gelatin and collagen (Sheeran & Smith, 1981). Presence of this protease has been confirmed by other workers (Rockey *et al.*, 1988; Price *et al.*, 1989), but its physicochemical properties have not yet been determined in much detail. The two proteases have been separated and partially purified and both produced lesions upon injection into trout (Sheeran *et al.*, 1984) and a mutant deficient in the serine protease only was still virulent (Drinan & Smith, 1985).

With the proviso that this mutant was not capable of producing the serine protease *in vivo*, it would appear that the metalloprotease is capable of replacing the serine protease as a virulence factor.

It is clear from the above that the evidence strongly supports the view that the 70-kDa serine protease is an important virulence factor of *A. salmonicida* and is responsible for much of the tissue liquefaction produced during disease or by injection of ECP. Many workers have also considered the protease to be the major lethal toxin (Tajima *et al.*, 1983; Shieh, 1985a). However, while the purified protease is lethal to fish, the dose required to kill is comparatively high (2.4 $\mu g\ g^{-1}$ fish; Lee & Ellis, 1989) and certainly higher than the ECP itself. Furthermore, as mentioned above, when the protease in the ECP is inhibited by PMSF, the lethal dose of the ECP is not markedly affected (Ellis *et al.*, 1988b). These findings prompted a search for another lethal toxin present in ECP and this was identified as glycerophospholipid: cholesterol acyltransferase (GCAT) much of which is complexed with lipopolysaccharide (Lee & Ellis, 1990). Furthermore, important interactions occur between the 70-kDa protease and GCAT in that the protease activates a GCAT proform and both interact in the pathogenic processes (see below).

Adaptations to activity *in vivo*

The 70-kDa serine protease is remarkably resistant to the majority of *in vivo* anti-proteases including the major anti-protease in plasma, α_1-anti-protease, which is regarded as a broad-spectrum serine protease inhibitor (Travis & Johnson, 1981). Indeed, Ellis (1987) provided evidence that the only anti-protease present in rainbow trout serum capable of inhibiting the 70-kDa protease was α_2-macroglobulin (α_2M) which accounts for less than 10% of the total trypsin-inhibiting capacity of trout serum. While Salte *et al.* (1992, 1993) showed that human anti-thrombin was capable of inhibiting the 70-kDa protease *in vitro* (in contrast to the finding of Price *et al.*, 1990), it is not known whether fish plasma anti-thrombin plays a role in inhibiting the 70-kDa protease.

Role in host protein digestion as a source of amino acids

Most bacteria can take up peptides smaller than five amino acids in length only (Gibson *et al.*, 1984) and so must be capable of digesting proteins to fragments of at least this size. As mentioned above, the 70-kDa protease has a low degree of specificity towards proteins with a

relatively open structure and is capable of degrading them to fragments small enough for absorption (Price *et al.*, 1990).

Further evidence for the nutritive role of the protease and for it being an indispensable virulence factor is from work using a protease-deficient mutant of a virulent strain (Sakai, 1985a, 1985b). This mutant was unable to grow on casein-containing medium in the absence of free amino acids. However, it grew normally when the casein was treated with the ECP of the parent strain which contained the 70-kDa protease (Sakai, 1985b). *In vivo* studies with the protease-deficient mutant indicated the importance of the protease as a virulence factor. The parent strain, which was a protease producer and also possessed the A-layer which confers protection against complement-mediated lysis (Munn *et al.*, 1982), induced clinical furunculosis upon injection into fish. An avirulent strain (lacking A-layer) did not produce disease and the bacterium survived for less than 24 h *in vivo*. However, the protease-deficient mutant, while it did not produce any lesions in the fish and did not increase in cell numbers, continued to survive within the tissues for at least 6 days (Sakai, 1985a). Thus, despite the preservation of persistence (attributed to the A-layer), the loss of virulence in the mutant may be attributed to the deficiency in production of the 70-kDa protease.

Role in iron uptake

The purified 70-kDa protease readily digests bovine transferrin rendering the iron available for bacterial growth (Hirst and Ellis, 1996). Thus, the protease may play a role *in vivo* as one of the mechanisms for obtaining iron from host transferrin.

Role in activating GCAT

The lethal cytolytic toxin, GCAT, is secreted as an inactive proform which is cleaved to a highly active cytolysin (Eggset *et al.*, 1994; see below).

Role in blood coagulation

While the role of the protease in tissue necrosis, especially in conjunction with the GCAT toxin (see below) is well recognized, a further specialized pathogenic effect of the protease may be associated with its

ability to activate the blood-clotting system through its many similarities to the major clotting enzyme, Factor Xa (Salte *et al.*, 1992).

One of the common histopathological features following injection of ECP (Ellis *et al.*, 1981) and of clinical furunculosis (Salte *et al.*, 1991) is the presence of microthrombi in small blood vessels and the heart, characteristic of disseminated intravascular coagulopathy. Price *et al.* (1990) demonstrated that the purified protease activated the clotting system in rainbow trout blood *in vitro*.

In clinical furunculosis in Atlantic salmon there is a decrease in plasma antithrombin (Salte *et al.*, 1991), and fibrinogen levels (Salte *et al.*, 1993) indicative of the activation of the clotting cascade. Injection of purified protease intravenously into Atlantic salmon induced a prolonged thrombin time and activated partial thromboplastin time, decreased anti-thrombin and fibrinogen levels and increased Factor Xa (Salte *et al.*, 1992). All these features are characteristic of consumptive coagulopathy. Salte *et al.* (1992) postulated that by virtue of its Factor Xa-mimicking activity, the protease could activate the clotting system.

The relevance of this property to virulence may be that by reducing the microcirculation through induction of microthrombi in nearby blood vessels, *A. salmonicida* may reduce the influx of leucocytes and phagocytes.

THE CYTOLYTIC TOXIN (GCAT/LPS)

As shown in Table 9.1, a number of membrane-damaging activities of ECP or partially purified ECP components have been described. Some characteristics of these toxins are summarized in Table 9.3. Although Buckley (1982) described the deacylation of phospholipids in human red cell membranes by GCAT, the erythrocytes did not lyse. Many subsequent workers therefore sought for a factor other than GCAT to identify the haemolytic factor in ECP. Some of the described toxins were not investigated for their lethality in fish (phospholipase, T-lysin, H-lysin) and while the others were injected into fish with the development of lesions, only the salmolysin and the GCAT/LPS were identified as potent haemolytic and lethal toxins. However, it is likely that all of these membrane-damaging or cytotoxic activities are properties of the single entity GCAT occurring in different monomeric or aggregated forms.

The lethal toxin was purified and shown by SDS-PAGE to contain a single protein of 25 kDa and LPS (Figure 9.1). In the native state the

Table 9.3 Properties of extracellular membrane-damaging toxins of Aeromonas salmonicida

Toxin	Physicochemical characteristics	*In vitro* activity	*In vivo* activity	Reference
Phospholipase (GCAT)	MW = 24 kDa	Effect on human erythrocyte membranes. GCAT, phospholipase A2 and lysophospholipase activities	Not determined	Buckley *et al.* (1982)
Leucocytolysin	MW = 100–300 kDa glycoprotein	Leucocytolytic	Transient leucopaenia	Fuller *et al.* (1977)
Cytotoxin	Glycoprotein	Lysed RTG-2 cells	Not determined	Cipriano *et al.* (1981)
T_1-lysin	Stable in ECP	Incomplete lysis of trout erythrocytes	Not determined	Titball & Munn (1981, 1985)
H-lysin	Unstable in ECP; heat labile; bound to cellulose filters	Non-specific haemolysin; maximum activity on horse erythrocytes	Not determined	Titball & Munn (1981, 1985)
Enterotoxin	Not determined	Fluid accumulation in rabbit ileal loop	Not determined	Jiwa (1983)
Haemolysin	MW = 56 kDa	Haemolytic only for fish erythrocytes	Enhances liquefactive lesion caused by 70-kDa protease	Fyfe *et al.* (1988)
Salmolysin	MW > 200 kDa glycoprotein; protease stable	Haemolytic for fish erythrocytes	Lethal dose 45 ng g^{-1} fish	Nomura *et al.* (1988)
GCAT	MW = 25 kDa; heat labile; pI 4.3	GCAT, phospholipase A2; incomplete lysis of fish erythrocytes	LD_{50} 340 ng g^{-1} fish; muscle necrosis	Lee & Ellis (1990)
GCAT-LPS	MW > 2000 kDa; heat stable; protease stable; heterogeneous pI	GCAT, phospholipase A2; incomplete lysis of fish erthrocytes; leucocytolysin, cytolysin (RTG-2 cells)	LD_{50} 45 ng protein g^{-1} fish; muscle necrosis; EGC degranulation	Lee & Ellis (1990)

toxin had a MW of over 2000 kDa and a variety of analytical approaches showed the toxin to be a complex between GCAT and LPS (Lee & Ellis, 1990). While much of the GCAT in the ECP is complexed with LPS, a proportion occurs as a free monomeric polypeptide with the same specific enzyme activity, as well as dimeric forms (Lee & Ellis, 1990). Monomeric, dimeric and complexes with LPS have also been confirmed to exist by Western blotting (Eggset *et al.*, 1994; see below).

The enzymatic characteristics of GCAT have been thoroughly investigated. Its activity on phospholipids is restricted to the glycerophospholipids. In the absence of an acyl receptor it acts upon phosphatidylcholine by removing a fatty acid from the 2-position to produce lysophosphatidyl choline (lysolecithin) and free fatty acid. In the presence of an acyl receptor (e.g. cholesterol), the acyl group is transferred to produce a cholesteryl ester. The enzyme can further remove the remaining 1-acyl group from lysolecithin to produce glycerophosphoryl-choline and free fatty acid. Thus, the one enzyme has phospholipase A2, acyltransferase and lysophospholipase activity (Table 9.4). Enhanced enzyme activity occurs in the presence of bovine serum albumin and human apolipoprotein A-1 (Buckley, 1982, 1983; Buckley *et al.*, 1982, 1984).

The purified GCAT/LPS complex was lethal to Atlantic salmon parr upon i.p. injection; the LD_{50} being 45 ng protein g^{-1} body weight (Lee & Ellis, 1990). The toxin possessed extremely high haemolytic activity for salmonid (but not mammalian) erythrocytes and in addition was leucocytolytic (salmonid) and cytolytic (RTG-2 cells).

GCAT MW HETEROGENEITY AND ACTIVATION BY THE 70-kDa SERINE PROTEASE

The occurrence of the different MW forms of GCAT referred to above has been further elucidated by comparative studies on a weakly haemolytic protease-negative transposon mutant (Eggset *et al.*, 1994). The ECP of the parent wild-type on fractionation by gel filtration produced three peaks with haemolytic activity, all of which were associated with cholesterol acyl transferase activity. The lowest MW form was purified and identified as 26-kDa GCAT. Using a rabbit antiserum to this GCAT to probe Western blots of wild-type ECP, three bands were stained, with MW of 26 kDa, 52 kDa and a band left in the stacking gel. These bands were considered to represent a GCAT monomer, dimer and aggregates. Using the rabbit antiserum to probe Western blots of the ECP of the protease-negative mutant, a single band with

Table 9.4 Proposed sequential reaction mechanism for the complete deacylation of phosphatidylcholine (lecithin) by glycerophospholipid: cholesterol acyltransferase (GCAT) of *Aeromonas salmonicida* (from Buckley *et al.*, 1982)

(1)	Phosphatidylcholine	—phospholipase A2———>	lysophosphatidylcholine + fatty acid
(2)	Phosphatidylcholine + cholesterol	—acyltransferase———>	lysophosphatidylcholine + cholesteryl ester
		<———phospholipase A2—	
(3)	Lysophosphatidylcholine + H_2O	—lysophospholipase———>	glycerophosphocholine + fatty acid

MW 38 kDa was stained. Upon addition to the mutant ECP of the purified 70-kDa protease from the wild-type there was a marked increase in the haemolytic activity of the ECP and on probing a Western blot of the protease-treated ECP, the 38 kDa GCAT band was converted to a 26-kDa form, identical to the GCAT of the wild-type.

Fractions from gel filtration of the wild-type ECP showed that about 50% of the haemolytic activity was associated with a free 26-kDa GCAT molecule, about 40% was associated with GCAT having a molecular mass below 70 kDa, probably representing GCAT dimers, and about 10% eluted as a high MW GCAT/LPS complex. Gel filtration of the protease-negative mutant ECP showed the presence of a small amount of high MW GCAT/LPS complex while most was present as the free 38-kDa GCAT with no evidence of dimers (Eggset *et al.*, 1994).

These data suggest that the GCAT is secreted as a 38-kDa proform of low haemolytic activity and is cleaved by the 70-kDa serine protease to produce a highly active 26-kDa GCAT which has a propensity for forming dimers and complexes with LPS.

COMPARISON OF GCAT WITH OTHER *A. SALMONICIDA* CYTOLYSINS

The biological and physicochemical characteristics of GCAT described above encompass those activities reported for other, less well-characterized cytotoxic factors (see Table 9.3). Features shared by many of these toxins are their high MW and the presence of carbohydrate moieties. Thus, the cytotoxic factor for RTG-2 cells was claimed to be a glycoprotein (Cipriano *et al.*, 1981). The leucocytolysin was characterized as a glycoprotein with a MW of over 100 kDa (Fuller *et al.*, 1977). The potent haemolytic toxin (salmolysin), with exactly the same LD_{50} as the GCAT/LPS, was reported to be a glycoprotein (with 68% carbohydrates) having a MW of over 200 kDa (Nomura *et al.*, 1988). It is tempting to speculate that the carbohydrate content of these toxins and their high MW may be explained by the aggregation of the GCAT with LPS or polysaccharides. The GCAT/LPS complex contained 65 mg carbohydrate and 2.5 mg total lipids mg^{-1} protein (Lee & Ellis, 1990) which is much higher than that reported for salmolysin, but this may be due to the different methods used in preparation of the ECP. The reports of the high MW haemolysin contrast with another claim that the haemolysin was a protein of 56-kDa MW (Fyfe *et al.*, 1987b). However, the latter is consistent with the findings that, using concentrated toxin,

the dominant band in SDS-PAGE was a dimeric form of the GCAT with MW 54 kDa (Lee & Ellis, 1990), and the dimer with MW of 52 kDa reported by Eggset *et al.* (1994).

Haemolysis of trout erythrocytes by the purified GCAT/LPS complex or free GCAT was incomplete, that is, the erythrocyte ghosts remained intact (Lee & Ellis, 1990; Eggset *et al.*, 1994), in contrast to the complete solubilization of erythrocyte membranes by the ECP (Lee & Ellis, 1990). This incomplete haemolysis is similar to the T_1 lysin activity which required the extracellular protease (present in the ECP) to cause complete lysis (Titball & Munn, 1981, 1985). When the GCAT/LPS was mixed with purified *A. salmonicida* 70-kDa protease, complete lysis of the trout erythrocytes occurred, confirming similarity of GCAT/LPS with T_1 lysin activity (Lee & Ellis, 1990).

NATURE OF GCAT HAEMOLYTIC ACTIVITY

The GCAT toxin possessed extremely high haemolytic activity for fish, but not rabbit, sheep or human erythrocytes (Lee & Ellis, 1990; Eggset *et al.*, 1994). The reason as to why the GCAT/LPS is so selectively haemolytic is probably to be found in differences in the phospholipids of the erythrocyte membranes. The optimal substrate for the GCAT has been reported to be phosphatidylcholine (PC) substituted with unsaturated fatty acids while the enzyme has no activity on sphingomyelin (Buckley, 1982). It is well established that fish tissues are much richer in polyunsaturated fatty acids than are those of mammals and, furthermore, the proportion of PC in the erythrocyte membranes of Atlantic salmon is 58.6% of total phospholipids (Lee *et al.*, 1989) compared with 29.5% in human erythrocytes (Ways & Hanahan, 1964). With over half of the fish cell-membrane phospholipids being highly susceptible to the GCAT, following exposure to the enzyme the membrane may lose its integrity resulting in cell lysis. On the other hand, the human erythrocyte membrane may remain intact because it contains only a minority of suitable phospholipid substrates. It would seem, therefore, that the enzymatic activity of the GCAT is well suited to digesting fish tissues.

Phospholipase toxins often exert their cytolytic activity by removing the charged head group, that is, phosphate, from the phospholipids in the membrane bilayer. Because the charged head group stabilizes the bilayer, removal of the phosphate destabilizes the membrane and cell lysis results. The possibility that this was the mechanism of haemolysis by

GCAT was studied by Røsjø *et al.* (1993). Analyses of Atlantic salmon erythrocyte membranes following incubation of citrated blood with GCAT/LPS showed an enzyme dose-dependent decrease in PC and increase in lysophosphatidylcholine (LPC). Haemolysis occurred as LPC levels rose to over 10% of total phospholipids. Whole salmon citrated blood also lysed when incubated with soybean LPC in a dose-dependent manner suggesting that the increased LPC of the erythrocytes following incubation with GCAT/LPS was the cause of the haemolysis.

ROLE OF THE LPS IN THE ACTIVITY OF THE GCAT/LPS TOXIN

The LPS alone has no toxic effect in the fish (Wedemeyer *et al.*, 1968) and heating the GCAT/LPS to 60°C for 30 min destroyed its toxicity (Lee & Ellis, 1990). Nevertheless, the role of the LPS in the high MW GCAT/LPS complex is considerable. As stated above, depending upon the method of culture, GCAT in ECP occurs in the form of a GCAT/LPS complex of very high MW, dimeric aggregates or as a free monomeric protein. The latter was purified and shown to have a MW of 30 kDa determined by gel filtration chromatography and a MW of 25 kDa in SDS-PAGE gels (Lee & Ellis, 1990). Experiments were performed to investigate the effect on various properties and activities of free GCAT when the latter was combined with LPS extracted from ECP (recombined GCAT-LPS). In native polyacrylamide gels, GCAT/LPS and recombined GCAT-LPS did not migrate into the gel whilst free GCAT produced a single fast-migrating protein band indicating that a high MW complex is formed when free GCAT and LPS are recombined. In isoelectric focusing gels the recombined GCAT-LPS focused heterogeneously, similar to GCAT/LPS, while free GCAT produced a single protein band focusing at an isoelectric point (pI) of 4.3 (Lee & Ellis, 1990). (This pI is disputed by Eggset *et al.* (1994) who found free GCAT to have a pI of 6.3.) Furthermore, the heat stability of GCAT was shown to be greatly enhanced when complexed or recombined with LPS. These results indicate that free GCAT and LPS can combine to form a complex with similar physicochemical properties to the GCAT/LPS in the ECP. Various other activities of free GCAT were compared with the GCAT/LPS and recombined GCAT-LPS complexes. The free GCAT was antigenically identical with the GCAT complexed with LPS since the former was stained in Western blots by rabbit anti-toxin (GCAT/LPS) antiserum. While the specific enzyme activity on egg yolk substrate or phosphatidylcholine was the same for free

GCAT, GCAT/LPS and recombined GCAT-LPS, the latter two possessed four to eight times the haemolytic and lethal activity.

The mechanism whereby LPS enhances the haemolytic and toxic activity of GCAT is not known but a likely explanation can be advanced. It is well known that LPS has an affinity for eukaryotic cell membranes and this affinity is inhibited by phospholipids and cholesterol (Kabir *et al.*, 1978). Studies with phospholipid monolayers have shown that LPS can penetrate such layers most readily when they are composed of phospholipids with unsaturated fatty acids (Kabir *et al.*, 1978). As mentioned above, such compounds are common in the cell membranes of salmonids (Lee *et al.*, 1989) and are the preferred substrates for the GCAT (Buckley, 1982). Hence, the mechanism whereby the haemolytic activity of GCAT is enhanced by complexing with LPS may be that the latter aids the enzyme to penetrate the cell membrane, delivering the GCAT precisely to where the optimal substrates for the enzyme are present. Certain other bacterial haemolysins, for example the cell-bound haemolysin of *Serratia marcescens* (Poole & Braun, 1988) and the alpha-haemolysin of *Escherichia coli* (Bohach & Snyder, 1985, 1986), or lipase of *Pseudomonas aeruginosa* (Stuer *et al.*, 1986) also exist as complexes with LPS but the role, if any, of the LPS has not been demonstrated. However, the lipase of *P. aeruginosa* is resistant to digestion by proteinase K when in the presence of LPS (Stuer *et al.*, 1986).

The haemolytic activity of the GCAT/LPS was also resistant to inactivation by proteinase K while the free GCAT was rapidly inactivated (Lee & Ellis, 1990). The effect of the *A. salmonicida* extracellular protease was similar but inactivation of free GCAT was much slower. This finding is similar to the reported protease resistance of "salmolysin", which was resistant to papain and pepsin (Nomura *et al.*, 1988). Once again, it is tempting to speculate that this property may have significance *in vivo* during inflammatory responses by protecting the toxin from inactivation by host- (e.g. leucocyte-) derived proteases and may contribute to the greater *in vivo* toxicity of the LPS-complexed GCAT. The mechanism of protection of the GCAT from proteolytic attack by the LPS may be simply by steric hinderance. Thus, the GCAT/LPS seems to be well adapted to act as a toxin in salmonid fish.

IN VIVO EFFECTS OF GCAT/LPS

While the GCAT/LPS has extremely high haemolytic activity *in vitro* there is no evidence for *in vivo* haemolysis in clinical furunculosis, or

when an LD_{50} of ECP or the purified GCAT/LPS were injected (Lee & Ellis, 1991a).

However, when larger doses of purified GCAT/LPS were injected intravenously into Atlantic salmon (causing death in about 4 h), *in vivo* haemolysis was clearly evident and red cell membranes from fish analysed 2 h following toxin injection had markedly increased levels of LPC (Røsjø *et al.*, 1993).

Interestingly, while haemolysis is not evident in moribund salmon with furunculosis, the LPC of erythrocytes was increased to about 10% of total phospholipids (Røsjø *et al.*, 1993) suggesting that the red cell membranes were destabilized (see above).

Injection of purified GCAT/LPS was also shown to induce similar signs of consumptive coagulopathy as caused by the 70-kDa protease (Salte *et al.*, 1992). The likely explanation of this is that haemolysis involves the release of thromboplastic material which causes intravascular production of fibrin (Levine, 1970).

The histopathological effects of LD_{50} doses of GCAT/LPS are not very extensive and do not seem to be able to account for death of the fish which occurs after about 20 h. Following i.m. injection of the toxin there is a coagulative necrosis of muscle fibres and restricted haemorrhaging. Both i.m. and i.p. routes induced a dramatic degranulation of eosinophilic granular cells (EGCs) in the gills (Lee & Ellis, 1991a). The gills of moribund fish were always pale and dead fish usually gaped, suggesting diminished blood flow through the gills and possibly death by respiratory failure.

Further *in vitro* studies (Lee & Ellis, 1991b) indicate a complex interaction between the GCAT/LPS and the fish's serum lipoproteins which results in enhanced phospholipase and haemolytic activities as well as a markedly increased electrophoretic mobility of the lipoproteins. Thus, it seems possible that the toxin may have some significant metabolic effects which contribute to its mechanism of *in vivo* toxicity, but this awaits investigation.

RELATIONSHIP BETWEEN 70-kDa PROTEASE AND THE GCAT/LPS TOXIN IN PATHOGENICITY

Previous workers have claimed the extracellular protease of ECP to be the major toxin (Tajima *et al.*, 1983; Shieh, 1985) or pathogenic factor (Sakai, 1985, 1985a). However, when the protease in ECP is inhibited by PMSF, while there is a prolongation of the time to death, there is only a small increase in the minimum lethal dose (Ellis *et al.*, 1988b).

Thus the protease is not a primary lethal toxin in ECP but it does hasten the time to death. Further investigations have shown that purified protease is lethal in large doses and studies with combinations of protease and GCAT/LPS complex have shown an additive relationship in lethality, with the GCAT/LPS being 55 times more lethal (ng protein g^{1} fish) than the protease (Lee & Ellis, 1989). Thus the minimum lethal dose of ECP not only depends upon the absolute concentrations of the protease and GCAT/LPS but also upon their relative concentrations in the ECP. Evidence also exists to conclude that the protease and GCAT/LPS are the major components in ECP responsible for lethal toxicity. Following the inhibition of protease activity by PMSF, the ECP was still highly toxic. However, this residual toxicity was specifically neutralized by rabbit anti-toxin (GCAT/LPS) antiserum (Lee & Ellis, 1990).

The pathogenesis of furuncle formation is also due to a combined effect of the protease and GCAT/LPS. The first evidence for this was the finding that purified protease when injected i.m. produced a much less severe lesion than ECP containing the same protease activity (Fyfe *et al.*, 1986b). However, protease fractions contaminated with haemolysin, when injected i.m., produced lesions equivalent to ECP with the same protease and haemolytic activity (Fyfe *et al.*, 1988). This finding has been confirmed using a combination of purified protease and purified GCAT/LPS (Lee & Ellis, 1991a). In this case, the GCAT/LPS alone produced coagulative necrosis of muscle fibres but with little haemorrhaging whereas a mixture of protease and GCAT/LPS produced an extensive lesion that was liquefactive and haemorrhagic, typical of that induced by ECP.

This effect is similar to the *in vitro* haemolytic effect referred to above whereby the protease is non-haemolytic (except in very high concentration), the GCAT/LPS is haemolytic but cell ghosts remain, while a combination of protease and GCAT/LPS completely solubilizes erythrocytes. Thus, it is apparent that once the GCAT/LPS has damaged the cell membrane the latter is susceptible to degradation by the protease.

OTHER CYTOLYTIC TOXINS?

H-lysin

The nature of the H-lysin is still obscure. It is active on erythrocytes from a wide range of species with highest activity towards horse

erythrocytes. Its presence in supernatants from both static (Titball & Munn, 1981) and shaken cultures (Titball & Munn, 1985) has been reported but it is maximally produced in static broth cultures. The H-lysin activity reached a peak at the end of log-phase growth and then rapidly disappeared. Other workers have failed to detect broad-range haemolytic activity in ECP (Ellis *et al.*, 1988b; Eggset *et al.*, 1994) and it is possible that certain culture conditions are necessary for its production.

Titball and Munn (1985) reported that the H-lysin was produced as an inactive precursor with MW 42 kDa which is converted to an active H-lysin with MW 29 kDa by proteolytic cleavage by the 70-kDa serine protease. While their partially purified H-lysin also contained GCAT activity these authors considered the two activities were not due to the same enzyme as membrane filtration of the preparation removed H-lysin activity but not GCAT activity (Titball & Munn, 1981).

Further work is necessary to define the nature of H-lysin but when *A. salmonicida* DNA libraries were screened for clones of T-lysin and H-lysin on trout or horse blood agars, both clones stained with a rabbit antiserum to GCAT (M. Gilpin, personal communication) suggesting that both activities were due to GCAT, possibly in different forms complexed with different factors.

ASH3 and ASH4

Although GCAT is the only haemolytic agent so far identified with certainty in *A. salmonicida* culture supernatants, gene-cloning techniques have discovered two further haemolytic peptides, ASH3 and ASH4 (Hirono & Aoki, 1993), with MW of 49 kDa and 60 kDa respectively. ASH3 lysed both mammalian and fish erythrocytes while ASH4 was more specific for fish cells. Haemolytic activity increased following exposure of both recombinant proteins to trypsin. The ASH3 gene has high homology with the aerolysin gene of *A. hydrophila* and the ASH4 gene is homologous to a gene family common to many members of the Vibrionaceae. The activities of ASH3 and ASH4 and the gene sequences are different from GCAT.

CONCLUSIONS

The data currently available indicate that with respect to the pathogenesis of furunculosis and the lethal toxicity of the exotoxins, the

GCAT/LPS and the 70-kDa serine protease are of major importance. However, *A. salmonicida* produces a range of other enzymes (Campbell *et al.*, 1990; Hirono & Aoki, 1993) and factors which, while they have not yet been implicated as having major tissue necrotizing or lethal effects, may nevertheless play an important role in virulence in terms of bacterial nutrition or as aggressins enabling the bacterium to counteract the host defence systems.

While the pathogenic and lethal effects of the protease and GCAT/LPS are more potent when the two toxins are combined, much of the effect of the protease is dependent upon an initial attack on cells by the GCAT/LPS. Furthermore, the latter is a much more potent toxin than the protease in terms of LD_{50}. However, the extent of tissue necrosis caused by a lethal dose of the GCAT/LPS does not seem sufficient to account for mortality. Injection of the GCAT/LPS into fish results in a dramatic degranulation of the EGCs in the gill arches. In fact, this is the only histopathological effect of injecting a minimal lethal dose of the toxin i.p. It is possible that the release of the EGC granules causes respiratory failure resulting in death of the fish but this is not certain. The GCAT/LPS is known to have a complex interaction with the salmonid serum lipoproteins (Lee & Ellis, 1991b) which may result in disturbances of lipid metabolism or even the activation of inflammatory mediators, for example arachidonic acid, which are known in mammals to be activated by phospholipase enzymes. A clear understanding of the *in vivo* effects of the GCAT/LPS awaits further investigations.