# Cloning, Expression, and Nucleotide Sequence of the Glycerophospholipid:cholesterol Acyltransferase gene from *Aeromonas hydrophila*.

by
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ABSTRACT

The purpose of this study was to examine the extracellular glycerophospholipid:cholesterol acyltransferase (GCAT) from A. hydrophila. The primary structure of GCAT was determined by sequencing the GCAT gene using the chain termination method of Sanger (Sanger et al. 1979). This gene, gcatA, was subsequently cloned into a wide-host-range expression vector (pMMB66; Fürst et al. 1986), and the ability of both Escherichia coli and Aeromonas salmonicida to secrete the protein was studied.

It was revealed that gcatA from A. hydrophila encoded a 31 kD enzyme capable of carrying out acyltransfer between glycerophospholipids and cholesterol. This enzyme, which is normally extracellular in cultures of A. hydrophila (MacIntyre and Buckley 1978), was secreted extracellularly by the related bacteria A. salmonicida when the A. hydrophila GCAT gene was present on a wide-host-range plasmid. In contrast, E. coli clones bearing the same recombinant plasmid only secreted GCAT to their periplasmic space, thus suggesting a lack of the necessary GCAT secretory machinery in the E. coli outer membrane.

A typical E. coli consensus-like promoter was not seen in the nucleotide sequence upstream of the GCAT gene. Although a region sharing some similarities with the CAP binding-site consensus sequence of E. coli (de Crombrugghe

et al. 1984) was identified approximately 130-150 nucleotides upstream of the start codon of the GCAT gene.

The sequence of the structural gene, gcatA, contained two regions that share sequence similarities with other lipases. These regions are believed to be the active site and the interfacial lipid-binding site of the other lipases (Maragnore and Henrickson 1986).

Polyclonal antibodies directed against GCAT from both A. hydrophila and A. salmonicida cross reacted with both proteins in ELISA and in Western immunoblots. This suggests that although the molecular weights and amino acid compositions of the two related proteins are quite different, some of the antigenic determinants of GCAT are conserved in these two proteins.

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## List of Abbreviations

Ab Antibody

Ag Antigen

Amino acids:

- Ala Alanine
- Arg Arginine
- Asn Asparagine
- Asp Aspartic acid
- Cys Cysteine
- Gln Glutamine
- Glu Glutamic Acid
- Gly Glycine
- His Histidine
- Ile Isoleucine
- Leu Leucine
- Lys Lysine
- Phe Phenylalanine
- Pro Proline
- Ser Serine
- Thr Threonine
- Trp Tryptophan
- Tyr Tyrosine
- Val Valine
- Met Methionine

AMP Adenosine monophosphate

| | |
|---|---|
| ATP | Adenosine triphosphate |
| C | Cholesterol |
| CA | Casamino acids |
| CAP | Catabolite activator protein |
| CCCP | Carbonylcyanide-m-chlorophenylhydrazone |
| CE | Cholesterol ester |
| DFP | Diisopropylphosphofluoroidate |
| DHFR | Dihydrofolate reductase |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| FA | Fatty acid |
| GCAT | Glycerophospholipid:cholesterol acyltransferase |
| HBA | Human blood agar |
| IPTG | Isopropyl-β-D-thiogalactoside |
| kb | kilobase pairs |
| kD | kilodaltons |
| LCAT | Lecithin:cholesterol acyltransferase |
| LPL | Lipoprotein lipase |
| MBP | Maltose binding protein |
| mRNA | messenger ribonucleic acid |
| MW | molecular weight |

Nucleosides

| | |
|---|---|
| A | Adenine |
| C | Cytosine |
| G | Guanine |
| T | Thymidine |

| | |
|---|---|
| OD | Optical density |
| PC | Phosphatidylcholine |
| PlA | Phospholipase A |
| PlC | Phospholipase C |
| pldA | The outer-membrane, detergent-resistant phospholipase from *E. coli* |
| PPL | Porcine pancreatic lipase |
| RLL | Rat lung lysophospholipase |
| RNA | Ribonucleic acid |
| RNAase | Ribonuclease |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SRP | Signal recognition particle |
| TCA | Tricarboxylic acid |
| TLC | Thin layer chromatography |
| TP | Translocator protein |
| TSB | Tryptic soy broth |
| X-Gal | 5-bromo-4-chloro-3-indoyl-β-D-thiogalactoside |
| YE | Yeast extract. |

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# INTRODUCTION

## Part I: Protein Secretion Across Biological Membranes

Although this discussion will mainly deal with the prokaryotic system of protein secretion, many of the useful models of protein secretion have been developed with eukaryotic systems in mind, particularly the secretion of proteins across the endoplasmic reticulum (ER) membrane to the ER lumen. However, many, if not all of these models can be applied to any system of protein secretion across a biological membrane.

### The signal hypothesis:

The first widely accepted theory of how proteins might traverse a lipid bilayer was postulated by Blobel and Dobberstein in 1975 (Blobel and Dobberstein 1975a, 1975b). Based on Blobel's earlier work on ribosomal associations with membranes (Blobel and Sabatini 1971) and on the observation that immunoglobulins are synthesized as higher molecular weight precursors (Milstein et al. 1972), "the signal hypothesis" was proposed. It was postulated that proteins secreted by eukaryotic cells contained amino terminal signal sequences that soon after their synthesis would bind to a signal recognition particle (SRP). Upon binding to the SRP, the complex of mRNA, ribosome, and nascent protein would then be directed to the target membrane where the SRP would bind a docking protein. This

membrane-associated polysome and mRNA would then synthesize the polypeptide. After docking, translation of the protein resumes and the nascent protein traverses the membrane, possibly via an aqueous pore or channel (Blobel 1980; Figure 1). This secretion of the newly-formed protein across the membrane was thought to occur cotranslationally.

This theory has been supported at the molecular level by the discovery of receptors that mediate the targeting of ribosome-mRNA-nascent protein to the membrane (Walter and Blobel 1980, 1981, 1982, 1983; Anderson et al. 1982; Ullu et al. 1982; Erickson et al. 1983). The SRP has been identified as an 11S ribonucleoprotein (Walter and Blobel 1980, 1981, 1982, 1983; Ullu et al. 1982, Andrews et al. 1985). The binding of SRP to the signal sequence of the nascent protein results in an arrest of translation (Walter and Blobel 1981), and the ribosome-mRNA-protein complex docks with the inner face of the cytoplasmic membrane via an interaction with a specific docking protein located in the inner leaflet of that membrane (Gilmore et al. 1982a, 1982b; Meyer et al. 1981, 1982).

The process of translocation is accompanied by the removal of the signal sequence by a leader peptidase enzyme located in or on the outer leaflet of the cytoplasmic membrane (Date and Wickner 1981, Wolfe et al. 1983).

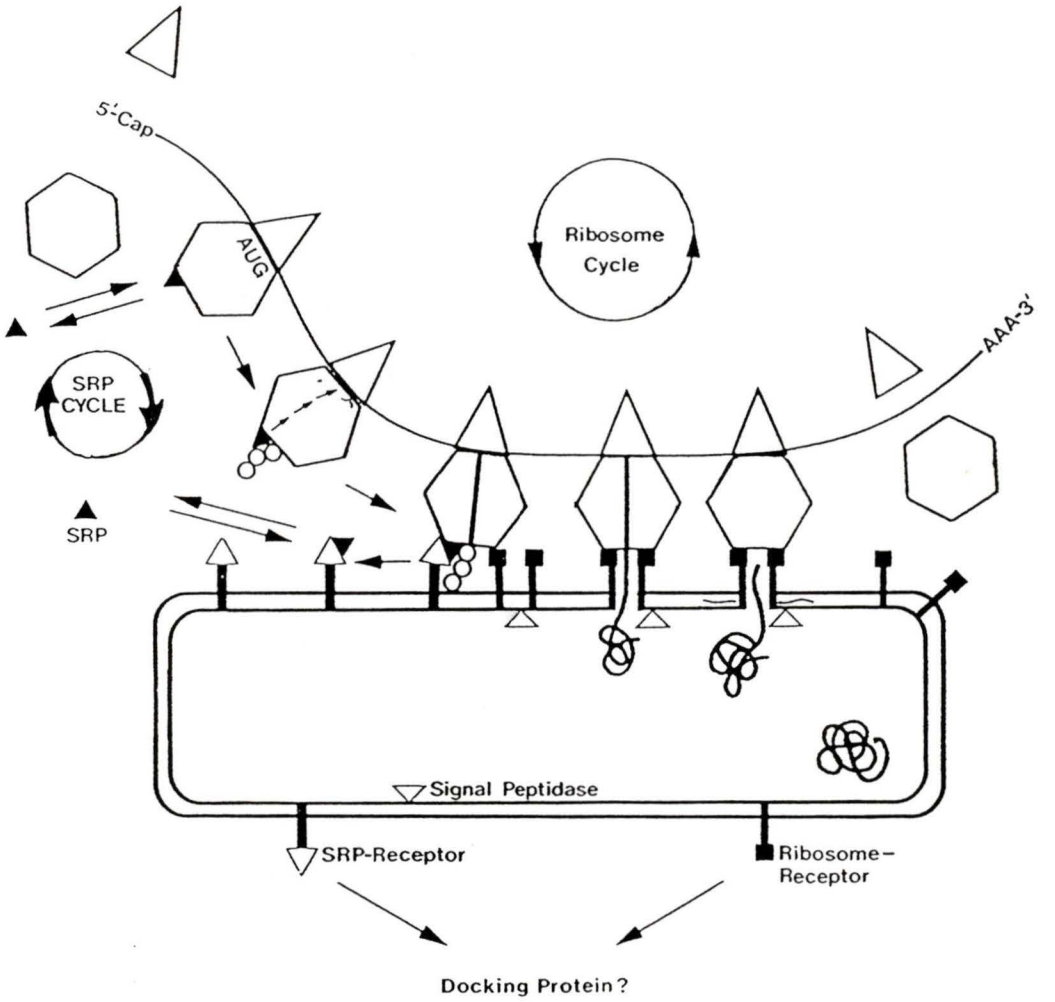



FIGURE 1. Protein secretion into the lumen of the endoplasmic reticulum according to Blobel's signal hypothesis. From Blobel., G., and B. Dobberstein 1975b.

The signal sequence:

The signal sequence is the amino terminal sequence of 15 to 25 amino acids that is present on many bacterial secretory proteins (Michaelis and Beckwith 1982). Among the signal sequences known, there is very little sequence homology but there is definite structural homology (von Heinje 1984). The sequences usually contain one or more positively charged amino acids within the first 3 to 8 amino acids. This charged region is followed by a hydrophobic core of approximately 10 to 15 amino acids. The final portion of the signal sequence is usually 5 to 6 amino acids long and is more hydrophilic than the previous portion. This final section usually contains the helix breaking residues proline and glycine. The cleavage site is usually preceded by small neutral amino acids at positions -3 and -1, and an aromatic, charged, or large polar residue must be present at position -2, (signal peptidase cleavage occurs between position -1 and +1; the (+) refers to amino acids of the mature protein and the (-) refers to the amino acids of the signal sequence).

Energy requirements for protein translocation:

Eilers and Schatz (1988) suggest that the process of protein translocation requires energy in the form of ATP, and in bacterial protein translocation across the inner membrane, a protonmotive force is also required for optimal

rates of translocation. These authors have proposed that the ATP hydrolysis is required to unfold, or somehow alter the conformation of the secreted protein to a translocationally competent form. Pulse-chase experiments with the maltose binding protein of E. coli (MBP) (Randall and Hardy 1986) have demonstrated that translocationally competent MBP is hypersensitive to protease (attributed to unfolding of tertiary structure) and that this hypersensitivity to protease is ATP dependent.

Experiments with dihydrofolate reductase (DHFR), have also demonstrated that the translocationally competent form is unfolded (Eilers and Schatz 1988). These authors observed that if DHFR is first denatured with urea or destabilized with point mutations, its import to the mitochondria is enhanced. It is important to note that the actual role of ATP in protein translocation is not known, all that is known is that ATP is required.

## Part II: Protein Secretion From Bacteria:

Although the general features of Blobel's model of protein secretion are supported by the results of research to date, some of the details of secretion, other than those already mentioned, remain unexplained. This is especially true when the signal hypothesis is applied to bacterial systems.

Secretory apparatus of bacteria

Systems analogous to the SRP have been identified genetically in bacteria. These are: the sec system (Oliver and Beckwith 1982a, 1982b; Ferro-Novick et al. 1984; Ito 1984; Wolfe et al. 1985) and the prl system (Emr et al. 1981; Bankaitis and Bassford 1985), in E. coli; and the S complex of Bacillus subtilis (Caulfield et al. 1984, 1985). Mutations of components of these systems are conditionally lethal, and the mutant bacteria accumulate many, if not all, of the normally secreted proteins in their cytoplasm. Two genes have clearly been demonstrated to be directly involved with protein secretion in E. coli. These are the secA (Oliver and Beckwith 1981) and the secY (prlA) (Ito et al. 1983; Emr et al. 1981).

Oliver and Beckwith (1981, 1982) have reported that the secA gene product is a 92 kD cytoplasmic protein that at times associates with the inner membrane and functions to maintain the preprotein (protein with signal peptide still attached) in a translocationally competent form.

Ito et al. (1983) determined that the secY gene product is a 42 kD integral inner membrane protein (Ito 1984), that functions to carry out the actual protein translocation across the inner membrane. This would make the secA protein analogous to the SRP, and the secY protein analogous to both the docking protein of the ER, and the aqueous pore for translocation. The gene product of dacA, PBP5 (Jackson et

al. 1985), located in the E. coli inner membrane; the maltose binding protein, MBP, (Oliver and Beckwith 1982) located in the E. coli periplasm; the outer membrane protein TonA that is involved in iron sequestration by E. coli (Baker et al. 1987); and the inner membrane associated leader peptidase of E. coli (Wolfe et al. 1985) have all been demonstrated to be sec dependent for secretion. In contrast there are examples of secreted proteins that are not sec dependent. Among these is the well characterized M13 coat protein. The inner membrane location of this protein is completely unaffected by mutation of the sec genes (Wolfe et al. 1985). To date, no gene product that acts solely as a docking protein for bacterial secretion has been identified.

Hengge-Aronis and Boos (1986) have reported that a truncated form of the periplasmic glycerophosphodiester phosphodiesterase (GIpQ) of S. typhimurium remains bound to the cytoplasmic membrane and interrupts the correct secretion of some, but not all periplasmic and outer membrane proteins. Ito et al. (1981), found that fusion constructs of the periplasmic maltose binding protein, malE, and the cytoplasmic β-galactosidase, also embedded into the cytoplasmic membrane causing the same type of secretion blocking of periplasmic and outer membrane proteins. These authors estimated that approximately $2 \times 10^4$ sites for secretion exist in the E. coli inner membrane.

Mode of protein translocation in bacteria

One of the central features of the signal hypothesis for eukaryotic systems is that the nascent protein is secreted through the endoplasmic reticulum, ER, co-translationally (Blobel and Dobberstein 1975a, 1975b; Rothman and Lodish 1977; Bergman and Kuehl 1979; and Miyata and Akazawa 1982). In the gram-negative bacteria, evidence exists for both co-translational and post-translational secretion. It has been demonstrated for example, that M13 coat protein can be processed and secreted both co- and post-translationally (Ito et al. 1979; Ito et al. 1980; Date and Wickner 1981; Kuhn et al. 1986). The synthesis of β-lactamase in Salmonella typhimurium, (Koshland and Bolstein 1982), was demonstrated to occur only on free polysomes in the cytoplasm, with secretion and processing occurring only post-translationally.

Many examples of co-translational secretion exist. In aerolysin secretion from Aeromonas hydrophila (Howard and Buckley 1985a; Howard, 1985), results indicate that aerolysin is only secreted co-translationally through the cytoplasmic membrane. Howard and Buckley, (1985), demonstrated that if the energy poison CCCP, which collapses the proton gradient, was added to Aeromonas hydrophila cells during a pulse labelling experiment, both with and without a chloramphenicol chase, the aerolysin signal peptide was cleaved before translation of the protein was complete.

This indicated that aerolysin is still being translated from the mRNA, while the leader peptide is available for cleavage by leader peptidase on or in the periplasmic side of the cytoplasmic membrane. Other examples of co-translationally secreted proteins are: β-lactamase of E. coli (Baty et al. 1981); alkaline phosphatase from E. coli (Smith et al. 1977); periplasmic binding proteins for maltose (Randall and Hardy 1977), and arabinose (Randall et al. 1978), also from E. coli; α-amylase from Bacillus subtilis (Smith et al. 1979);and exotoxin A of Pseudomonas aeruginosa (Lory et al. 1983).

Models for protein export from the Gram-negative bacteria

This discussion of extracellular secretion of proteins has, for the most part been concerned with protein displacement across one membrane. Gram-negative bacterial cells consist of a cytoplasmic compartment surrounded by both an inner and outer membrane. The space between the inner and outer membranes is the periplasm which comprises from 20 to 40% of the total cell volume in E. coli (Stock et al. 1977). The export of proteins from Gram-negative bacteria must then account for the translocation of proteins across two distinctly different lipid bilayers (Leive 1973; Nikaido and Nakae 1979; Lugtenberg and van Alphen 1983).

Many Gram-negative bacteria are known to be active secretors of protein. These include many bacteria of the

families Vibrionaceae and Pseudomonaceae which are secretors of many proteins such as toxins, proteases, nucleases, and phospholipases. Many early events in the translocation of proteins across the cytoplasmic membrane in Gram-negative bacteria are indistinguishable from the early events of the eukaryotic secretion models (Emr et al. 1980), one such event being the synthesis of exported proteins as higher MW precursors, (i.e., synthesized with a signal sequence; Inouye and Beckwith, 1977). The bacterial secretion models begin to differ radically when the protein bound for either the outer membrane or the extracellular milieu is confronted with the problem of traversing the periplasmic space. There are many models of how this might be accomplished, and it appears that the bacteria have developed a variety of methods for the excretion of proteins.

Emr et al. (1980), suggest that in the case of lamB (an outer membrane protein of E. coli), the transport through the periplasm is via a vesicular intermediate. Lory et al. (1983), suggest that the Pseudomanas aeruginosa exotoxin A is secreted via a region of membrane fusion very similar to the adhesion zones postulated by Bayer (Bayer 1979), thus never really entering the periplasm. These authors suggest that both leaflets of the inner membrane are continuous with the reciprocal leaflets of the outer membrane in a way that results in the formation of a functional pore. Bayer's model suggests that only the outer leaflet of

the inner membrane is continuous with the inner leaflet of the outer membrane.

Although these examples show some insight into the methods that the various bacteria employ to excrete proteins to the outside, the actual mechanism for the release of proteins from the periplasm is unknown and is therefore an topic of intense interest.

The existence of pleiotropic export mutants which accumulate secretory proteins in their periplasm supports the notion that the export of proteins is a two step process, the first being the co- or post-translational secretion through the inner membrane, the second being secretion of the protein through the outer membrane of the bacteria. Two examples of pleiotropic secretory mutants, (mutants that accumulate extracellular proteins in their periplasm), that have been identified are in (1) Erwinia chrysanthemi (Andro et al. 1984), and (2) A. hydrophila (Howard and Buckley 1983). The work of Howard and Buckley, (1983), revealed two mutants of protein export, differing in their outer membrane protein profiles. This suggests that at least two different gene products are required for the extracellular secretion of proteins from A. hydrophila. In the case of the extracellular pectinase of E. chrysanthemi, the secretion of an extracellular protease was not affected in the apparent pleiotropic export mutants. This evidence indicates that more than one mechanism exists in the Gram

negative bacteria for the secretion of extracellular proteins.

Few examples of extracellular bacterial proteins have detailed models proposed for their secretion. These include: the IgA protease of Neisseria gonorrhoeae (Pohlner et al. 1987); cholera toxin from V. cholera (Hirst and Holmgren 1987); the heat labile enterotoxin of E. coli (Hirst et al. 1984; Hofstra and Witholt 1984); colicin E2 of E. coli (Pugsley and Schwartz 1984; Luirink et al. 1986; Cavard et al. 1987); and the haemolysin of E. coli (Springer and Goebel 1980; Wagner 1983; Femlee et al. 1985; Mackman et al. 1985; Nicaud et al. 1986).

The proposed pathway for the extracellular secretion of lgA protease by N. gonorrhoeae presented by Pohlner et al. (1987), suggests that the protein is produced as a 169 kD precursor. Secretion is co- or post-translational across the inner membrane with the accompanying removal of the signal peptide by a signal peptidase. Once the entire peptide is within the periplasm, the carboxy-terminal helper domain associates with the outer membrane to form a pore that allows for the secretion of the active site domain. Pohlner et al. postulate that the protease adopts its active conformation during this secretion step, and ultimately releases itself from the outer membrane-bound helper domain by autoproteolytic cleavage.

For the secretion of the oligomeric enterotoxin from

V. cholerae, Hirst and Holmgren (1987) have shown that the subunits are synthesized by free polysomes within the cytoplasm as preproteins and that these subunits are post-translationally processed and secreted to the periplasm. Once in the periplasm the A and B subunits oligomerize to produce the active enterotoxin multimer (Hirst and Holmgren 1987). This multimer is then exported from the periplasm to the extracellular environment by an as yet undefined process. These authors do, however, suggest that V. cholerae does possess some property or component in the outer membrane that allows for the export of the active enterotoxin multimer. This is based on the following premises; a) When the toxin subunits are produced in other bacteria, no extracellular secretion is observed and the toxin remains entirely cell associated; b) E. coli produces a highly homologous oligomeric enterotoxin (LT) that remains cell associated in E. coli (periplasmic or outer membrane) (Hirst et al. 1984); c) When LT is cloned into V. cholerae, LT is processed normally and released to the culture supernatant like the wild type cholera toxin.

E. coli is known to secrete only a small number of proteins to the exterior, these include the heat labile haemolysin (Springer and Goebel 1980) and the colicins (Hardy 1975). Evidence suggests that the haemolysin, unlike most secretory proteins, is produced without an amino terminal signal sequence, (Femlee et al. 1985). It appears

that this protein is secreted actively by a process involving a specific set of secretory proteins that require only the carboxyl-terminus of the haemolysin to direct its secretion (Wagner et al. 1983; Mackman et al. 1985; Nicaud et al. 1986). There is also no evidence showing the haemolysin resides even transiently in the periplasmic space, (Mackman et al. 1986), suggesting that it is released directly from the cytoplasm via adhesion zones, protein pores, or by means of vesicles. (Bayer 1979; Nicaud et al. 1986; Mackman et al. 1986).

Colicin E2 of E. coli appears to require an outer-membrane-associated phospholipase for extracellular release (Pugsley and Schwartz 1984; Luirink et al. 1986; Cavard et al. 1987). Secretion of colicin E2 is apparently mediated by the compromising of the outer membrane of E. coli by the lipase. The lipase involved in this process is thought to be the detergent resistant, membrane-associated pldA described by Homma (Homma et al. 1984a, 1984b) and de Geus (de Geus et al. 1983, 1984). Within 2 hours of colicin E2 production, the membrane bound pldA is activated. This is followed by the subsequent release of colicin E2 and other periplasmic proteins (Pugsley and Schwartz 1984, 1985).

The fate of cloned extracellular proteins in E. coli:

The cloning of extracellular proteins from different families of secreting bacteria into E. coli almost always

results in the export of the cloned protein no further than the periplasm (Howard and Buckley 1986; Chakraborty et al. 1986; Focareta and Manning 1987; Gobius and Pemberton 1988) suggesting that the information necessary for the secretion of a protein through two membranes is not directly within the primary structure of the secreted proteins, or that the secretion machinery is species specific, and the signals within a secreted protein's primary structure are only recognized by this specific secretory apparatus.

Contrary to this evidence indicating that E. coli is unable to secrete foreign cloned proteins, some authors have reported the extracellular release of cloned proteins from E. coli. The gene products cloned from other species of bacteria that may be secreted by E. coli include the extracellular nuclease from Serratia marcescens (Ball et al. 1987), and the penicillinase, cellulase, and xylanase from alkalophylic Bacillus sp. (Kudo et al. 1983, Sashihara et al. 1984, and Honda et al. 1985), just to list a few. In the case of the penicillinase from Bacillus, extracellular release appears to be due to the compromising of the E. coli outer membrane (Kudo et al. 1983). For the other two Bacillus proteins, the authors failed to include data from the assays of culture supernatants for cytoplasmic or periplasmic enzyme activity. Nor did they assay for the release of the specific proteins at early times in the growth. These omissions raise the possibility that the release

observed could have been due to cell lysis.

The extracellular location of Serratia nuclease reported by Ball et al. (1987) appears to be an example of faithful secretion of a heterogenous protein by E. coli. The cloned nuclease was found extracellularly with no detection of cell lysis or periplasmic leakage. Other Serratia proteins have also reportedly been secreted from E. coli, including a serine protease (Yanagida et al. 1986), and two chitinases (Jones et al. 1986). The most probable explanation for this is that Serratia is an enteric bacteria closely related to E. coli, and therefore E. coli contains the necessary machinery to recognize and secrete these proteins.

Extracellular proteins from the Gram-negative bacterium Aeromonas hydrophila

As mentioned already, A. hydrophila is an active secretor of a wide variety of extracellular toxins and enzymes (Bernheimer 1970). A partial list of these proteins is given in Table 1 (from Ljungh and Wadström 1980). A number of degradative enzymes as well as a wide variety of toxins have been identified and in a few cases the protein has been well characterized.

One such protein is aerolysin, the haemolysin secreted by A. hydrophila. This protein is released from the cell as a 54 kD inactive proprotein, proaerolysin. Howard and Buckley (1985b) demonstrated that subsequent proteolytic

cleavage by a trypsin like enzyme activates proaerolysin to the 51 kD, haemolytic aerolysin. It is the most extensively characterized extracellular protein from Aeromonas spp. The protein was initially identified by Bernheimer et al. (1974). Since that time, aerolysin has proven to be a useful model for not only the study of a proteinaceous virulence factor, (Thelestam and Ljungh 1981; Ljungh and Wadström 1983; Asao et al. 1984; Rahim et al. 1984; Chakraborty et al. 1987), but also as a model for protein secretion in a Gram-negative organism, (Howard and Buckley 1983; Howard and Buckley 1985a), an example of a bacterial toxin, (haemolysin), that is activated by proteolytic cleavage, (Howard and Buckley 1985b), and also as a model for examining how a hole forming toxin might interact with the target cell membrane (Howard and Buckley 1982; Garland and Buckley 1988).

Other extracellular proteins of A. hydrophila that have been examined on a molecular basis, (although none quite as extensively as aerolysin), are: the cytotoxic enterotoxin, (Chakraborty et al. 1984); the extracellular ∝-amylase, (Gobius and Pemberton 1988); the proteases (Thune et al. 1982); and the extracellular lipase, glycerophospholipid: cholesterol acyltransferase, (GCAT), produced not only by A. hydrophila, but also by most members of the family Vibrionaceae (McIntyre and Buckley 1978; MacIntyre et al. 1979; Buckley et al. 1982; Buckley 1982; Buckley 1983).

TABLE 1. Some Extracellular Toxins and Enzymes of *A. hydrophila*

| Toxin | Age of culture | Molecular weight | Properties |
|---|---|---|---|
| Aerolysin | 24 hr | 51000 | haemolytic, heat labile |
| Leukocidin | 16 hr | - | leucocidin, hot/cold-haemolysis |
| Enterotoxin | 16-18 hr | 15000 ±3000 | affects cAMP in rabbit ileal cells |
| Protease | Stationary phase | 52000 | pH optimum 7, requires $Mg^{2+}$ |
| Proteinase A | 60-80 hr | 22100 | pH optimum 7.9 |
| Proteinase B | 60-80 hr | 43600 | pH optimum 9 |
| Staphylolytic enzyme | 8 hr | - | cleaves oligo-glycine |
| GCAT | 13-20 hr | 31300 | similar act. to GCAT from *A. salmonicida* |
| α-amylase | - | 49000 | 3 regions of homology with other α-amylases |

The enzyme GCAT has been identified in the culture supernatants of A. hydrophila (MacIntyre and Buckley 1978). These authors also demonstrated that GCAT from A. hydrophila reaches maximum levels of production during late exponential to early stationary phase (MacIntyre and Buckley 1978). Because GCAT catalyses a reaction not unlike that of the mammalian plasma enzyme LCAT (Buckley et al. 1984), this microbial enzyme along with its cellular location and biochemical activity represent some interesting areas to investigate with respect to protein secretion, lipases and lipase structure, and heterologous protein expression in bacteria.

## Part III: A general mechanism for protein translocation

In both eukaryotic and prokaryotic organisms, a great number of hydrophilic proteins synthesized within the cytoplasm are eventually transferred across one or more membranes to either a cellular organelle or to the extracellular milieu. As discussed above, secretory proteins from both types of organisms appear to interact with cellular components that partially or completely effect secretion across the cytoplasmic membrane. In eukaryotes, these components have been identified as SRP and the docking protein. In bacteria, although the research is far from definitive, a number of proteins appear to participate in secretion. Also, the presence of membrane-associated ribosomes in both eukaryotic and prokaryotic organisms suggest that the two systems are likely to share an evolutionary origin. Singer et al. (1987) found it a reasonable hypothesis that translocational events may occur by a unitary process that originated with prokaryotes, then evolved and was modified in eukaryotes. This unifying molecular model for protein translocation across a lipid bilayer includes the salient features of the signal hypothesis. This is a general model that can accommodate most situations involving protein translocation with only slight modifications for each specific example. According to this model, the protein is in contact with water at all times during the translocation process. This stipulation is consistent with the

evidence obtained by Gilmore and Blobel (1985), that the nascent polypeptide being translocated is always accessible to the aqueous media surrounding the membrane during the secretory process. Exposure to an aqueous environment is likely to be essential as other investigations of protein translocation have led to the conclusion that it is very costly energetically to remove the ionic residues of a protein from water and to then insert them into, or pass them through, the hydrophobic milieu of the interior of a phospholipid membrane (Singer 1971, Singer 1976). Singer et al. (1987), propose that it is unlikely that secretion of proteins occurs as a result of the hydrophobic signal sequence inserting spontaneously into membranes. Due to the amphipathic nature of signal sequences they tend to associate with membranes, but as is pointed out by these authors, this is only an en mass phenomenon, and several signal sequences must participate to form the aqueous pore in the target membrane. Singer et al. (1987), suggest that protein translocation is a monomolecular mechanism. The basis of their model is that there is a set of translocator proteins in every membrane capable of carrying out protein translocation. These translocator proteins, (TP), are likely to be integral membrane proteins. The TP's would be responsible for the translocation and possibly the docking of a nascent protein being synthesized. After binding the signal sequence, it is postulated that the TP then

transfers the protein either co- or post-translationally through the membrane by a series of transfers of subdomains. These subdomain units for translocation are proposed to be 10-30 amino acid residues in length (Singer et al. 1987). As peptides of this length rarely exhibit secondary structure in aqueous solution, the secondary structure required for the translocation could be conferred by the interaction of the subdomain with the TP. Starting with the signal sequence domain and ending with the translocation of the carboxyl-terminus, the entire protein would be translocated in this manner with the simultaneous utilization of energy. In bacteria the electrochemical gradient formed by the hydrolysis of ATP (Eilers and Schatz 1988) could provide this energy.

This segmental mode of transfer has been partially supported by the results of Coleman et al. (1985), showing that bacterial lipoprotein translocation is not via a threading mechanism. It was noticed that the same signal sequence was treated differently by the bacterial export apparatus depending on its position within the protein chain. Also, the existence of co-translational secretion of proteins indicates that at least these proteins are not translocated as complete units (Maher and Singer 1986; Eilers and Schatz 1986; Randall and Hardy 1986).

The TPs are proposed to be multiple subunit proteins that may function as channels, (Blobel 1980; Singer et al.

1987), similar to those of the acetylcholine receptor aggregate (Singer et al. 1987). Aggregated TP could consist of a number of subunits that are homologous but not necessarily identical. A small hydrophilic pore in the centre of the TP would allow for the charged and polar residues of the nascent protein to remain associated with an aqueous environment, and one or more domains of the TP subunits could contain an area that loosely recognizes a certain common structural feature of each subdomain in the protein that is to be translocated.

The energy for translocation could conceivably be used to cause a large quaternary rearrangement in the TP structure to allow the shifting of the "bound" nascent polypeptide to the other side of the membrane, resulting in the next subdomain for translocation entering the pore of the TP. Such large quaternary rearrangements have been detected in other proteins (Singer 1971; Unwin and Ennis 1984), but never in the context of macromolecular translocation. Singer suggests that conformational changes in the protein subdomain could be caused by phosphorylation of the TP by a kinase, resulting in subdomain displacement and eventually leading to the secretion of the entire protein across the membrane.

## Part IV: Lipases

Lipolytic enzymes are ubiquitous in the realm of living organisms. They can be involved in the biological turnover of lipids, they are required as digestive enzymes in the transfer of lipids between organisms, they are required for the deposition and mobilization of fat reserves in animals, and they are involved in intracellular lipid metabolism and the proper functioning of biological membranes (Zubay 1983). Often the function or importance of lipases is not readily apparent. Lipases are often found in the venom of poisonous snakes and insects (Joubert and Taljaard 1980), and in the extracellular milieu of a bacterial culture (Owens 1974).

Recent sequence analyses of the many characterized lipases (Komáromy and Schotz 1987; Maraganore and Heinrikson 1986) has revealed that two short stretches of amino acids show a distinct homology. These are the lipid binding domain, and the proposed active site domain.

### The classification of lipases

The classification of an enzyme as a lipase is a very general classification. There are many different lipases responsible for not only the degradation of each type of lipid, but also different lipases exist that cleave at different bonds within the lipid molecule. The lipases that hydrolyse the various bonds of a standard glycerophospholipid are divided into groups based on the bond at which

they act (see Figure 2). This discussion will concentrate on the phospholipases A and C.

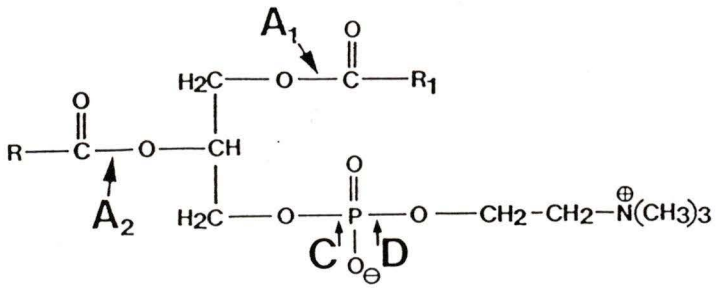
**Phospholipase C:** Phospholipase C (PlC) cleaves the phosphodiester bond of the polar head group of the lipid to release diacyl glycerol and a water soluble phosphate derivative (Figure 2).

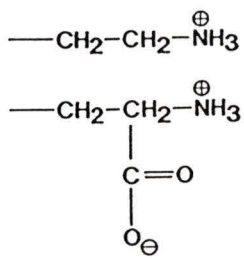
The PlC are primarily bacterial enzymes. These enzymes are known to be haemolytic, and are commonly toxins or virulence factors in pathogenic bacteria such as Clostridium perfringens (Yamakawa et al. 1976), and P. aeruginosa (Berka and Vasil 1982).

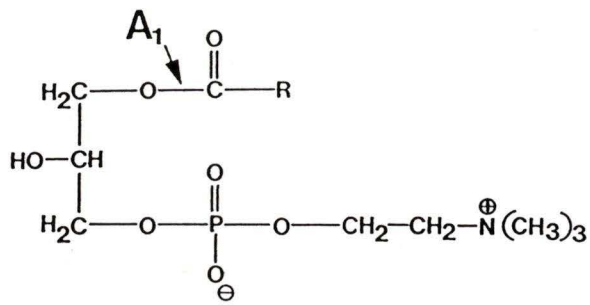
The PlC have an average MW of 30-35 kD. Of all PlC tested most show a preference for phosphatidylcholine as a substrate, although some PlC are specific for sphingomyelin or phosphatidylinositol (Watanabe et al. 1978; Taguchi and Ikezawa 1978; Ikezawa et al. 1978).

**Phospholipase A:** The phospholipases A, as depicted in Figure 2, cleave the fatty ester bonds of phospholipids. Phospholipases $A_1$ ($PlA_1$) cleave the fatty ester bond of the acyl group attached to position 1 of the glycerol moiety of the lipid, while $PlA_2$ cleave the fatty ester bond of the acyl group at position 2 of the substituted glycerol.

By far the most extensively studied of the PlA type enzymes is the $PlA_2$ from snake venoms (Maraganore and Heinrikson 1986). It has been demonstrated that $PlA_2$ is not the toxic factor that aids in the spread of the venom



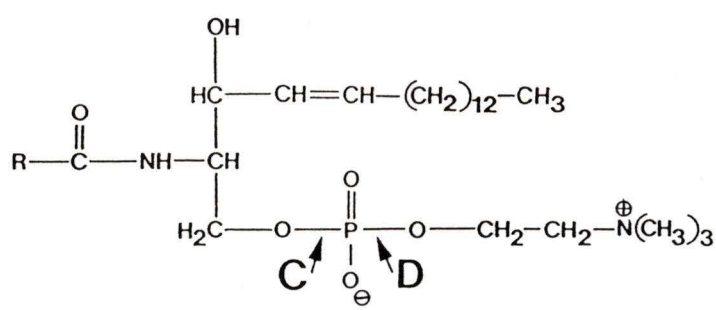




FIGURE 2. Site of action of phospholipases.

throughout the victim by degrading lipid barriers. These venom lipases have been studied both mechanistically, (Dennis 1973; Verger 1980; Wells 1972, 1974; Roberts et al. 1977, 1978; Shukla and Hanahan 1981; Barnola 1982), and structurally (Volwerk et al. 1974; Heinrikson et al. 1977; Joubert and Taljaard 1980; Dufton and Hider 1983; Maraganore and Heinrikson 1986).

The PlA are also found as digestive enzymes, that are initially produced as inactive zymogens (Brockerhoff and Jensen 1974); they can also be intracellular enzymes contained within hepatocyte microsomes (Holub 1982); they have been identified as components of the plasma membranes of human B lymphocytes (Suzuki et al. 1980), human polymorphonuclear leucocytes (Victor et al. 1981), and hepatocytes (Sakir 1981). They are also extracellular in the plasma of animals, and in the cell free growth media of certain bacteria.

The PlA enzymes are generally small, <20 kD, and are typically heat stable. Many, but not all, of the PlA require $Ca^{2+}$. It is commonly found that the enzymes requiring $Ca^{2+}$ have an optimal activity above pH 7, whereas the enzymes that do not require the divalent cation have a pH optimum of less than 7. Some of the PlA contain a histidine residue in their active sites, as p-bromophenacyl bromide will inactivate them (Volwek et al. 1974), others contain essential serine residues that are inactivated by

diethyl-p-nitro-phenyl phosphate (Paraoxon), diisopropyl-phosphofluoridate (DFP), or phenylmethyl sulfonylfluoride (PMSF). X-ray crystallographic analysis shows distinct structural similarities between the PlA enzymes (Renetseder et al. 1985).

In 1974, Wells (1974) demonstrated that the activity of a phospholipase is dependent on the form of the substrate. If a PlA is presented with a substrate above the critical micellar concentration, the specific activity of the enzyme dramatically increases. This phenomenon has been subsequently called the interfacial activation of phospholipases. There is no evidence for irreversible adsorption of the enzyme to the substrate, however, Verger (1980) has suggested that this interfacial activation is due to the lipid, in an aggregated form, being presented to the enzyme in such a manner that the site of cleavage is immediately available. The kinetics of lipolysis have been extensively reviewed by Verger (1980), and a model suggesting reversible associations between lipids and enzymes has been postulated.

Microbial Phospholipases:

In the past, much of the interest in microbial lipases has been due to the connection of these lipases with the spoilage of food, especially meats and dairy products (Cantoni et al. 1967; Castberg et al. 1975; Sutherland et

al. 1975; Papon and Talon 1988). The lipases of some microorganisms are responsible for the characteristic flavours that certain cheeses acquire with aging. More recent attention to bacterial lipases has been focused on the possible roles of lipases in protein secretion, (Luirink et al. 1986; Pugsley and Schwartz 1984) and as virulence factors or toxins (Möllby 1978; Berka and Vasil 1982; Baine 1985, 1988). Although many of the phospholipases from bacteria are cell associated, either membrane bound or cytoplasmic, only the phospholipases A and C which are the extracellular phospholipases of bacteria will be dealt with in more detail.

Extracellular microbial phospholipase C:

The phospholipases C have attracted much attention, as they have been linked to the toxigenicity of certain bacteria, including the organism responsible for gangrene, Clostridium perfringens (Möllby 1978). Baine has purified and characterized the extracellular Legionella pneumophila phospholipase C. Several roles in bacterial virulence have been postulated for this enzyme (Baine et al. 1979; Baine 1985; Baine 1988). It has been suggested, that the phospholipase could prevent phagolysosome formation by changing the lipid content of the phagosome membrane sufficiently to prevent fusion with the lysosome. This would account for the ability of Legionella sp. to survive in the phagosome (Horwitz 1983) of the infected alveolar macrophages.

Considering that PC is one of the major components of erythrocyte membranes in humans, (Mead et al. 1986), and that diacylglycerol forms unstable bilayers due to its lack of a polar headgroup, (Bernheimer 1974; Mead et al. 1986), it is not surprising to find that this enzyme acts as a haemolysin.

Another example of a phospholipase C, which is a toxin or virulence factor, is the heat labile haemolysin of P. aeruginosa (Berks and Vasil 1982). This haemolysin was shown to be a phospholipase C by Watanabe et al. (1978). It was subsequently purified and characterized by Berka and Vasil (1982). Phospholipases C have been identified in P. aureofaciens, (Sonoki and Ikezawa 1975), P. fluorescens, (Doi and Nojima 1971), and many other genera of bacteria. Although this type of phospholipase has been identified in mammalian systems, it is most commonly associated with bacterial systems (Möllby 1978).

A second phospholipase C that can be found as an extracellular toxin in bacteria is sphingomyelinase C. Some examples of sphingomyelinase C from bacteria are the β-toxin from Staphylococcus aureus (Doery 1963; Möllby 1978), and the 24 kD lipase of Bacillus cereus (Ikezawa et al. 1978). Strangely enough, both of these enzymes also possess lysophospholipase activity only on lysophosphatidylcholine, (Doery 1962; Ikezawa et al. 1978).

Extracellular microbial phospholipases A

PlA activity is associated with virtually every cell type known (Van den Bosch 1980), although in most instances the enzyme is not secreted extracellularly. In the group of bacterial extracellular phospholipases A, few have been characterized to a great extent. The list of some of these extracellular PlA includes: the $Ca^{2+}$-dependent $PlA_1$ found in cultures of Bacillus subtilis, (Kennedy and Lennarz 1979); the PlA* of Fusobacterium necrophorum (Abe et al. 1978); the $PlA_2$ of Vibrio vulnificus (Testa et al. 1984); the PlA* of V. El Tor (Chatterjee and Mitra 1962, Chatterjee and Das 1965, Magnusson and Gulasekharam 1965); the PlA* of V. parahaemolyticus (Yanagase et al. 1970); and the PlA of Aeromonas spp. (Bernheimer et al. 1975); MacIntyre and Buckley 1978). All those PlA marked with an asterisk (*) also display lysophospholipase activity (see Figure 2).

The extracellular phospholipase of the genus Aeromonas

The lipolytic activity that Bernheimer et al. (1975) detected in the cell free culture supernatants of A. hydrophila was later demonstrated to be from a single enzyme, (MacIntyre and Buckley 1978). It was also demonstrated by these authors, that aside from acting as a $PlA_2$ and a lysophospholipase, in the presence of any glycerophospholipid and cholesterol, this enzyme catalysed the transesterification between glycerophospholipids and cholesterol,

resulting in the formation of cholesterol ester, free fatty acid, and, (in the case of PC), glycerophosphorylcholine. This water soluble phosphate derivative of glycerol is the likely reason that Bernheimer et al. (1975) suggested that phospholipase C was present in A. hydrophila culture supernatants (MacIntyre and Buckley 1978). Thus this enzyme, capable of all the activities listed, was named glycerophospholipid:cholesterol acyltransferase or, GCAT.

Because it was discovered that virtually all members of the genera within the family Vibrionaceae contained similar lipolytic activities in their culture supernatants (Owens 1974), a survey of the various genera within the family, and some genera from without, was conducted (MacIntyre et al. 1979). GCAT activity was detected in culture supernatants of all members of Vibrionaceae tested with the one exception of Plesiomonas shigelloides. GCAT-like activity was detected in only one other bacterial species tested, the Gram positive Staphylococcus aureus. This research demonstrated that GCAT was a lipolytic enzyme common to the Vibrionaceae, with some minor exceptions.

The enzyme from A. salmonicida was subsequently purified and characterized (Buckley et al. 1982; Buckley 1982; Buckley 1983; Buckley et al. 1984). It was found that GCAT is a 23.6 kD protein that represents 0.6% of the total extracellular protein of A. salmonicida. It was also shown that the enzyme requires no divalent cations for activity,

and the activity is stimulated by both serum albumin and human apolipoprotein A-1. These properties, and the 2 positional specificity for acyltransfer activity, suggested strong similarities with mammalian LCAT (Glomset et al. 1962; Glomset 1962). Further studies on substrate specificities (Buckley 1982), and the mechanism of action of GCAT (Buckley 1983) revealed that the acyl donor can be any of the major glycerophospholipids, with slight differences in reaction rate occurring dependent on the nature of the substrate (micelles or liposomes). It was demonstrated that the acyl chain preference is for short chain saturated (10:0 to 12:0), or for longer unsaturated fatty acids (16:1 to 18:1). It was also noticed that a variety of alcohols can act as acyl acceptors, but that none are as effective as cholesterol. Work using other steroid acyl acceptors demonstrated that the important features of cholesterol that make it the choice acyl acceptor are the planar trans-A/B ring system, and the 3 position β-hydroxyl group (Buckley 1982, 1983). These observations, as Buckley suggests, imply that this enzyme is not involved with bacterial metabolism, but rather it is likely involved with degradation of target membranes. This is because the most effective acyl acceptor, cholesterol, is normally not a component of the membranes of bacteria, except in the case of Mycobacterium. Degradation of target membranes could be involved in virulence as in the function proposed for Legionella PlC or it could be part of

a general nutrient scavenging system. Neither of these possibilities has been confirmed and the actual role of GCAT remains a mystery.

The substrate specificities of mammalian LCAT are, as mentioned above, quite similar to those for GCAT. The acyl donor for the LCAT catalyzed reaction is reportedly restricted to PC (Assmann et al. 1978). For acyl acceptors, LCAT appears to require both the β-hydroxyl, and trans configuration of the A/B rings in sterols, and also like the microbial GCAT, most straight chain alcohols and water are able to act as acceptors for the acyl transfer, although none of these are as effective as cholesterol (Kitabatake et al. 1979; Buckley 1982).

## Part V: GCAT, the Purpose of This Thesis

Previous studies of GCAT (MacIntyre and Buckley 1978; MacIntyre et al. 1979; Buckley et al. 1982; Buckley 1982, 1983; Buckley et al. 1984) have been primarily concerned with the GCAT molecule from *A. salmonicida*. In this study, the molecule of interest is the enzyme released by *A. hydrophila*. Other than GCAT's presence at various times during the growth of *A. hydrophila*, very little is known about this GCAT. The questions addressed will be:

1) Is GCAT actively secreted by *Aeromonas*, or is its extracellular location due to cellular or outer membrane lysis?

2) Is the sequence of GCAT homologous to other lipase sequences, especially that of LCAT?

3) Is GCAT expression stringently controlled or is the gene constitutively expressed in Aeromonas?

4) Is the secretion of GCAT genus or species specific?

In order to attempt answers to these questions it was necessary to first, sequence the GCAT gene, then to examine the expression and cellular localization of GCAT in E. coli, A. salmonicida, and A. hydrophila.

The work presented in this manuscript will deal with the cloning and expression of the GCAT from A. hydrophila in E. coli and A. salmonicida. The primary structure of the protein, as derived from the nucleotide sequence of the gene, will be presented and compared to known phospholipase sequences.

# MATERIALS AND METHODS

## Materials

Egg yolk lecithin (phosphatidylcholine), cholesterol, bovine serum albumin (essentially fatty acid free), Triton X-100, ribonuclease (RNAase), trypsin, lysozyme, 5-bromo-4-chloro-3-indoyl-β-D-thiogalactoside (XGal), isopropyl-β-D-thiogalactoside (IPTG), ampicillin (Ap), tetracycline (Tc), kanamycin (Km), chloramphenicol (Cam), rifampicin (Rif), polyadenylic acid and sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Co. Acrylamide, Temed, bis(N,N-methylenebisacrylamide) and agarose were obtained from Biorad Laboratories. 7-(thienyl-2-acetamido)-3(2(4-N,N-dimethyl-aminophenylazo)-pyridinium methyl)-3-cephem-4-carboxylic acid (PADAC) was purchased from Calbiochem-Behring Co. [8-$^{3}$H]polyadenylate (500 mCi/mmol)([$^{3}$H]poly A), [4-$^{14}$C]cholesterol (53mCi/mmol), deoxyadenosine 5'-[$\alpha$,$^{32}$P]-triphosphate (>400 Ci/mmol) and deoxyadenosine 5'-[$\gamma$,$^{32}$P]-triphosphate (>2000 Ci/mmol) were obtained from Amersham Inc. Restriction endonucleases were obtained from Pharmacia Inc., New England Biolabs Inc., Bethesda Research Laboratories, or Boeringer Mannheim GmbH. DNA polymerase I (Klenow fragment), $T_4$ DNA ligase, and all deoxy- and dideoxynucleoside triphosphates (dNTP's and ddNTP's) were obtained from Pharmacia Inc. Protein and DNA molecular weight standards were purchased from Biorad Laboratories. Outdated human blood was obtained from the Royal Jubilee

Hospital, Victoria, B.C. The bacteriological growth media were supplied by Gibco. Egg yolk emulsion was purchased from Oxoid. Polyclonal sera from rabbits against A. hydrophila-GCAT and A. salmonicida-GCAT were from this laboratory. Horseradish peroxidase-conjugated goat-anti-mouse immunoglobulin was purchased from Tago Inc. All other chemicals and reagents were the purest commercially available.

## Bacterial Strains and Plasmids

All bacterial strains and plasmids used in this study and their sources are listed in Table 2.

## Culture Media

The standard medium used for E. coli HB101 was Luria-Bertani (LB) medium with appropriate antibiotics where necessary. E. coli strains used in the induction experiments were grown in LB + 0.2% glucose and appropriate antibiotic. The standard broth for A. salmonicida-As440 liquid cultures was M9 salts-yeast extract-casamino acids (M9-YE-CA) prepared as described by Bernheimer and Avigad (1974). M9-YE-CA media contained 4% Difco yeast extract, 2% Difco casamino acids, 0.0033% thiamine, 0.00012% nicotinic acid, M9 salts (Miller 1972), 1mM $MgSO_4$, and 0.1mM $CaCl_2$ at pH 7.2. When As440 was used in induction experiments the liquid media used was LB + 0.2% glucose and appropriate

TABLE 2 Bacterial Strains and Plasmids

| Strain | Genotype or description | Source |
|---|---|---|
| A. hydrophila Ah65 | Wild-type | This lab. |
| A. hydrophila Ah65-rif | Rifampicin resistant mutant of Ah65 | This lab. |
| A. salmonicida As440 | ATCC 14174 | ATCC |
| A. salmonicida As440-rif-1 | Rifampicin resistant mutant of As440 | This lab. |
| E. coli HB101 | recA13, hsdS20, ara14, proA2, lacY1, galK2, leuB6, rps120, xy15, mtl1, supE44 | E.E. Ishiguro |
| E. coli JM105 | pro-lac, thi, rpsL, hsdR44, endA, sbcB, F'traD36, $proA^{+}B^{+}$, lacIq, lacZ M15 | Pharmacia |
| pVK102 | $Km^{r}$, $Tc^{r}$, cos | E.W. Nester |
| pRK2013 | $Km^{r}$, conjugative helper plasmid | E.W. Nester |
| pBR322 | $Ap^{r}$, $Tc^{r}$ | E.E. Ishiguro |
| pMMB66EH | $Ap^{r}$, tacP | J.B. Fürste |
| pHEc1 | See the text | S.P. Howard |
| pHEc2.2 | See the text | This study |
| pJT22 | See the text | This study |
| pJTP6 | See the text | This study |
| PJTPA7 | See the text | This study |
| M13 mp18/mp19 | M13 sequencing vectors | Pharmacia |

antibiotic(s). Induction of the tac promoter was accomplished by adding IPTG to 1mM. A. salmonicida cultures were grown at 27 ± 2°C. A. hydrophila were grown under the same conditions as A. salmonicida except the temperature was 37°C.

For broth cultures, the bacteria were grown in Erlenmeyer flasks in a gyrorotary shaker at 27±2°C or 37°C and 250 rpm. Routine plate cultures were grown on LB + 1.5% agar, tryptic soy agar (Difco TSA), or HBA (TSA with 4% human blood, v/v) with the various antibiotics at the appropriate temperatures. E. coli JM105 was maintained on M9 salts media with 0.2% glucose, 0.00005% thiamine, and 1.5% agar.

McLung and Toabe Egg Yolk Agar (Owens 1974), contained 0.5% NaCl, 4% peptone, 0.001% $MgSO_4$, 0.2% glucose and 1.5% agar, pH 7.4. After autoclaving, sterile egg yolk emulsion (Oxoid) was added to 10%.

All media were sterilized by autoclaving at 121°C at 15 $lb/in^2$ for 20 min.

Plate cultures were maintained for 2-3 weeks at 4°C prior to restreaking. Long term storage of stock bacterial cultures was at -80°C in 15% glycerol.

Antibiotics, where required, were added to either solid or liquid media at the concentrations listed in Table 3.

Growth of the liquid cultures was monitored by measuring culture absorbance at 600 nm., or by plating

TABLE 3. Antibiotic Concentrations

| Antibiotic | Abbreviation | Working Concentration |
|---|---|---|
| Ampicillin | Ap | 100 $\mu g\ ml^{-1}$ |
| Chloram-phenicol | Cam | 16 $\mu g\ ml^{-1}$ |
| Kanamycin Sulphate | Km | 40 $\mu g\ ml^{-1}$ |
| Rifampicin | Rif | 40 $\mu g\ ml^{-1}$ |
| Tetracycline | Tc | 20 $\mu g\ ml^{-1}$ |

serial dilutions to determine the number of viable cells.

## Bacterial Mating

Bacterial matings were carried out essentially as described by Harayama et al. (1980). Donor strains, (pVK102- or pMMB66-based recombinant plasmids in HB101), the helper strain HB101-pRK 2013, and rifampicin resistant recipient strains of As440 (As440-rif-1) or Ah65 (Ah65-rif) were all grown in LB, (containing the appropriate antibiotics), to early log phase (optical density at 600mm = approx. 0.5). These cultures were then filtered through sterile 0.45 μm Millipore filters in volume ratios of 1:1:2 (donor: helper:recipient). Filters were then incubated on HBA plates at 30°C for 3 to 6 hours without antibiotics. After incubation, the filters were washed with 2 ml of sterile 10mM $MgSO_4$ and 0.1 ml aliquots of appropriate dilutions of the bacterial suspension were plated onto selective HBA, and the resulting transconjugants were grown overnight.

## Detection of Cells Producing GCAT

A) Plate assays:

GCAT production was detected qualitatively on solid growth media using HBA or McClung and Toabe Egg Yolk Agar. On egg yolk media, Aeromonas strains producing GCAT exhibit zones of opacity around and under the colonies which are

visible after at least 24 hrs. growth. E. coli recombinant clones producing GCAT do not produce these zones of opacity.

On HBA, GCAT-producing E. coli clones cause weak alpha-haemolysis, visible only after 36 hours. All Aeromonas species used in this study were haemolytic on plates. The haemolysis caused by A. hydrophila has been demonstrated to be due to aerolysin (Buckley et al. 1981). Haemolysis due to A. salmonicida is thought to be due to the production of other haemolysins (Titball and Munn 1981). Thus the HBA method was not used to screen strains of this genus containing the GCAT recombinant plasmids. Instead, plasmid conferred antibiotic resistance in conjunction with the ability of a recombinant Aeromonas clone's plasmid repertoire to transform HB101 to the same drug resistance as well as to GCAT positive, was used to confirm that transconjugants contained the recombinant GCAT plasmids.

B) Enzyme assay:

The quantitative assay for GCAT was essentially as described by Buckley et al. (1981). The standard GCAT assay consisted of inverted micelles (0.5 µmol of egg yolk lecithin, 0.5 µmol of cholesterol, and approx. $2.0 \times 10^4$ cpm (approx. 0.01 µCi) of [4,$^{14}$C]Cholesterol) in a Tris/KCl buffer in a final volume of 0.5 ml of 0.5% Triton X-100, 30mM KCl, 20 mM Tris-HCl, pH 7.4). Reactions were stopped after 15 minutes at 37°C by the addition of 5 volumes of $CHCl_3/CH_3OH$ (2:1,v/v). After centrifugation, 1.0 ml samples

of the lower phase were taken, dried under nitrogen, and the lipid was dissolved in 150 µl $CHCl_3/CH_3OH$ (4:1,v/v). Lipids were separated by thin layer chromatography (TLC) on plastic backed Silica-60 plates (Merck), in petroleum ether/ether/acetic acid (70/30/1). Spots corresponding to cholesterol (C) and cholesterol ester (CE), (identified by comparison with known standards), were located by brief exposure to iodine vapor and cut from the plates. Each spot was added to 5 ml of scintillation fluid (Dupont Formula 963) and counted in a Beckman LS8100 Liquid Scintillation Counter. GCAT activity is expressed as nmol of CE formed per minute.

**Aerolysin Assay**

Aerolysin activity was measured as described previously (Howard and Buckley 1985a). Haemolytic activity was expressed as the $\log_2$ of the largest dilution which caused 100% lysis, and is at times expressed as haemolytic units (HU).

**Other Enzyme Assays**

Glutamate dehydrogenase activity was measured by the reductive amination of α-ketoglutaric acid as described by Halpern and Lupo (1965). Units are expressed as umol NADPH oxidized per minute at 20°C. RNAase, a periplasmic marker, was assayed by measuring the release of acid soluble [$^3$H]adenylate from [$^3$H]polyadenylic acid at 37°C as described

by Lopes et al. (1972). Units are defined as nmol adenylate released per minute. β-lactamase activity was measured using the chromogenic substrate PADAC as described earlier (Howard and Buckley 1986). Units are arbitrarily defined as a relative amount of PADAC hydrolysed per minute.

## Cell Fractionation

Crude samples for the initial detection of GCAT in *E. coli* clones were prepared by first rupturing the cells in a French pressure cell, or by freeze-thaw lysis (see below).

Cell free culture supernatants were obtained by centrifuging bacterial broth cultures. Large samples were centrifuged at 10000 x g and 4°C for 15 minutes; small samples (<1.5 ml) were centrifuged in 1.5 ml eppendorf tubes in a microfuge at 4°C for 3 minutes.

Periplasmic fractions were obtained by the sucrose-EDTA osmotic shock method of Willis et al. (1974). Crude cytoplasmic fractions were isolated by either passing the shocked cells through a French pressure cell (1100 $kg/cm^2$), or by repeated freezing and thawing in 20 mM Tris-HCl, pH 7.4, with subsequent centrifugation (15 min. at 20000 x g, 4°C) to remove whole cells and subcellular debris.

Unless otherwise stated, the culture volumes used for the osmotic shock were 10 ml for large scale fractionation experiments, or 1 ml for standard fractionation experiments. Periplasmic fractions were in a final volume of 2 ml or 0.2

ml respectively.

## Protein Chemistry

When necessary, culture supernatants were concentrated prior to electrophoresis by ammonium sulphate precipitation, or by TCA precipitation. Ammonium sulphate precipitation was done by the addition of ammonium sulphate to 85% saturation at 0° with gentle stirring. The salt solution was then left overnight at 4°C and centrifuged at 15000 x g for 30 minutes.

TCA precipitation was performed by adding an equal volume of cold 20% TCA to samples on ice. This was followed by centrifugation and a -20°C acetone wash. The resulting pellets were resuspended in appropriate volumes of SDS-PAGE sample buffer.

Protein concentration was measured by the modified Lowry method of Markwell et al. (1978). The amino-terminal amino acid sequences of As440-GCAT and Ah65-GCAT were kindly determined by Sandy Keilland using an Applied Biosystems 470A gas-phase sequenator (Aplied Biosystems Co.).

## Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% acrylamide slabs according to the method of Neville (Neville 1971). Samples for SDS-PAGE were mixed with sample buffer and heated to

100°C for 3 to 5 minutes prior to electrophoresis. Gels were stained with Coomassie blue.

Standard separation of DNA was by agarose gel electrophoresis in 40 mM Tris-acetate, 1mM EDTA, pH 8.0 as previously described (Maniatis et al. 1982). Samples of DNA for the agarose gels were mixed with 6 x sample buffer prior to electrophoresis. After electrophoresis gels were stained with 2 $\mu g\ ml^{-1}$ ethidium bromide for 30 minutes, destained with water for 5 minutes and photographed under UV light. MW estimations were interpolated from standard plots of $\log(R_f)$ vs. MW.

**Western Immunoblots**

After SDS-PAGE proteins were transferred to nitrocellulose paper as described by Towbin et al. (1979). Following blocking, the blots were incubated with 2000 fold dilutions of the appropriate polyclonal rabbit sera. After extensive washing, these blots were then incubated with 1 $\mu g\ ml^{-1}$ horseradish peroxidase conjugated goat anti-rabbit antibodies (Tago). All immunoblots were blocked with 2% BSA. All incubations and washes were performed in 1.5 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4 (NET buffer), containing 0.05% NP40 (Sigma Chemical Co.). Blots were developed using the chromogenic peroxidase substrate N,N-dimethoxy benzidine (Eastman-Kodak) at 12.5 $\mu g\ ml^{-1}$ and 0.01% hydrogen peroxide

in 10 mM Tris-HCl, pH 7.4.

## Nucleic Acid Purifications

Plasmid DNA, when used for cloning or restriction mapping, was isolated by the cleared lysate method of Godson and Vapnek (1973) and purified by centrifugation through a cesium chloride-ethidium bromide density gradient as described by Maniatis et al. (1982). Plasmid minipreparations, used for the rapid screening of insert size in cloning experiments, were obtained according to the method of Birnboim and Doly (1979).

## Restriction Endonuclease Digestions

Digestions with restriction endonucleases were carried out as described by Maniatis et al. (1982). Standard digestion reactions for restriction mapping contained 0.25 to 1.0 µg DNA and 10 units of enzyme. The temperature was 37°C unless otherwise specified, and the buffer systems used were either those recommended by Maniatis et al. (1982), or those buffers specified by the enzyme suppliers. The standard reaction time was 1 hour. Upon completion, 0.2 volumes of sample buffer (40% w/v sucrose, 60 mM EDTA, 0.25% bromophenol blue) were added to the samples and the samples were applied directly to the agarose gel for electrophoresis.

In preparation for cloning, 200 to 300 ng of vector DNA, and a three-fold molar excess of target insert were used. Precloning digestions were terminated with 10 mM EDTA

and heating at 65°C for 10 min. Samples were extracted successively with phenol, phenol:chloroform (1:1), and chloroform, then precipitated and resuspended as previously described (Maniatis et al. 1982).

## Cloning Methods

The vectors used in cloning were pBR322 (Bolivar et al. 1977), pMMB66EH (Fürste et al. 1986), or the M13 sequencing vectors mp18 and mp19 (Norrander et al. 1983; Table 2, p. 51). The primary source of target DNA was the recombinant cosmid pHEc1 constructed by S.P. Howard (Howard and Buckley 1986). pHEc1 is a recombinant clone of the cosmid pVK102 (Knauf and Nester 1982) that contains an 18 kb insert in the SalI site of the tetracycline resistance gene.

Samples of purified vectors digested with appropriate restriction enzymes were ligated to similarly restricted fragments of target DNA, in a vector:target ratio of 1:3, overnight at 12°C (Maniatis et al. 1982). Aliquots (1 µl) were used to transform E. coli HB101 by the $CaCl_2$ shock method (Cohen et al. 1973). Transformed cells were then plated on HBA-Ap, or HBA-Tc, grown at 37°C for 20-36 hr, and then left at 4°C for 12-24 hr to allow the weak ∝-haemolysis associated with clones expressing GCAT to develop.

For the isolation of M13 bacteriophage containing various restriction fragments for DNA sequencing, restriction digestions and ligations were carried out as

above. $CaCl_2$-treated E. coli JM105 were transfected with ligation mixtures and plated with an indicator lawn of JM105 in the presence of IPTG and X-Gal as described by Messing (1983).

## DNA Sequencing Procedures

Restriction fragments of the inserts in pHEc1 or pHEc2.2 (see results) were cloned into the M13 sequencing vectors mp18 and mp19 (Messing 1983) as described above. These recombinant bacteriophage DNA derivatives were then sequenced by the dideoxy method of Sanger et al. (1979), with modifications outlined below.

In order to obtain templates for sequencing, white plaques (resulting from the interruption of lacZ in the recombinant vectors) were picked from the transfection plates and used to infect 2 ml logarithmic cultures of JM105. After growth with vigorous shaking for 2.5 hours at 37°C, 0.1 ml samples of these cultures were used to infect 10 ml cultures of the same bacteria. Following 5-7 hours of incubation the recombinant phage particles were isolated by the polyethylene glycol (PEG) precipitation method of Messing (1983).

Protein was removed from the template DNA by successive extractions with phenol, phenol:chloroform (1:1), and chloroform. The single stranded sequencing template was then precipitated with ethanol and resuspended in 10mM Tris,

0.1 mM EDTA, pH 7.5 at approximately 1 μg $ml^{-1}$.

Minipreparations of the double stranded replicative form of the M13 templates were isolated from the cell pellets of the transfectants to confirm the presence of the desired inserts by restriction enzyme digestion and agarose gel electrophoresis (see Nucleic acid purifications above).

In sequencing reactions, 2 μg of template DNA were annealed to 1 pmol of primer DNA (see below) in 10 μl of 6.6 mM Tris, 6.6 mM MgCl, 50 mM NaCl and 1 mM DTT (pH 7.4). After the addition of 40 μCi of deoxyadenosine 5'-[$\alpha$,$^{32}$P]-triphosphate, chain terminating primer extension reactions were carried out using 1.25 U DNA polymerase I (Klenow fragment) and the appropriate deoxy/dideoxyribonucleoside triphosphates (dNTP/ddNTP). The reaction concentrations of these nucleosides were previously determined by Howard (1985) to be optimal for dideoxy sequencing at the following concentrations. In the ddATP mix: 30 μM ddATP, 33 μM dCTP, 33 μM dGTP, and 33 μM dTTP; the ddCTP mix: 33 μM ddCTP, 1.6 μM dCTP, 33 μM dGTP, and 33 μM dTTP; the ddGTP mix: 50 μM ddGTP, 33 μM dCTP, 1.66 μM dGTP and 33 μM dTTP; and the ddTTP mix: 117 μM ddTTP, 33 μM dCTP, 33 μM dGTP, and 1.66 μM dTTP. The chase solution contained all four dNTP's at 2 μM in 10 mM Tris, pH 7.5.

Sequence data of the 3' end of the various inserts were derived using the 17-base, single-stranded universal primer (New England Biolabs Co.), which hybridizes to the 3' end of

the inserts M13 vectors. Internal or 5' regions were sequenced, according to the strategy depicted in Figure 5, using 18-mer oligonucleotide primers, which were synthesized using a Sam One DNA synthesizer (Biosearch Inc.), by P.J. Romaniuk. Synthetic primers were purified from contaminants by means of 7 M urea-20% polyacrylamide gel electrophoresis and reverse phase chromatography as described by Atkinson and Smith (1985).

The primer extension reaction mixtures were separated by electrophoresis on 7 M urea-6% or 8% polyacrylamide gels as described by Messing (1983), and the resulting sequences read from autoradiographs produced on Kodak XK-1 film. A computer program (SEQUENCE) from Delaney Software Ltd. was used for analysis of the sequence data.

## Radiolabelling of DNA

Single stranded oligonucleotide probes were labelled using polynucleotide kinase (PNK) and [$\gamma$,$^{32}$P]dATP (2000 Ci/mmol). Labelling reaction mixtures contained 20 pmol of 18-mer oligonucleotide (120 ng), 50 µCi [$\gamma$,$^{32}$P]dATP, and 1 unit of PNK in a total of 20 µl of 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT, and 10 mM Tris, pH 7.5. Incubation was at 37°C for 10 minutes.

It was found to be unnecessary to remove unincorporated [$^{32}$P]dATP from the labelled probes prior to hybridization experiments.

## Southern Blot Analysis

Southern dot blot assays were carried out on Gene Screen Plus membranes essentially as described in the Gene Screen Plus Manual (New England Nuclear). The prehybridization and hybridization steps were performed in 1% SDS, 1 M NaCl and 10% PEG 8000 at 40°C. Prehybridization was for 20 min., and hybridization was overnight (16-20 hours). Both steps were carried out in commercially available sealing bags. In the hybridization step, a radiolabelled 18-mer oligonucleotide was used as a probe, and yeast tRNA was used at 5 $\mu g\ ml^{-1}$ as a carrier instead of salmon sperm DNA. After hybridization, two low stringency washings were done in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M citrate pH 7.4) at room temperature. Two high stringency washings were then performed in 2 x SSC at 55°C.

Autoradiograms, using XAR-5 film exposed for 2.5 hours at -70°C in a Kodak XRay cassette with enhancing screens, were used to monitor the blot results after both wash steps.

## Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA were performed essentially as described by T.W. Pearson (personal communication). GCAT was dried onto the ELISA plates overnight at 37°C at 0.5 µg per well. After washing with PBS-Tween-20 (0.05%) (PBS is 0.01 M $NaH_2PO_4$, 0.85% NaCl, pH 7.4), the wells of the ELISA plate were blocked with 3% BSA in PBS-Tween-20 at 37°C for 2 hours.

First antibody was then added in the presence of 0.5% BSA and 0.05% Tween-20, and incubated for 2 hours at 37°C. After extensive washing, second antibody was added, (see below), and incubated with the same conditions as first antibody. Following several washing steps, colour was developed as outlined below.

First antibodies were serial dilutions of rabbit antisera beginning with a $500^{-1}$ dilution. Second antibody was horseradish peroxidase conjugated goat anti-rabbit Ig (Tago) used at 0.5 µg per well. Colour development was from the reaction of peroxidase with 2,2'-azino-bis(3-ethylbenz-thiazoline)-sulfonic acid (ABTS). ABTS was kindly supplied by T.W. Pearson. The developed ELISA's were read on an EIA autoreader EL310 (Biotek).

# RESULTS

## Cloning of GCAT

The A. hydrophila cosmid library in lambda phage was used to infect E. coli HB101 and resulting transfectants were plated on HBA-Km. A small number of the transfectants had haemolytic phenotypes. Some of these displayed definite beta haemolysis, while others displayed a weak alpha haemolytic phenotype. The beta haemolytic clones have previously been demonstrated to produce aerolysin, the A. hydrophila haemolysin (Howard and Buckley 1986). One weakly alpha haemolytic clone was subsequently tested for acyltransferase and aerolysin activity. This clone, pHEc1, (Figure 3), which displayed GCAT activity (but not aerolysin activity) in disrupted cells after overnight growth, was selected for further study.

The presence of an enzyme capable of acyl transfer suggested that pHEc1 did code for GCAT, and not for aerolysin or some other cytolytic protein. As E. coli does not normally produce an enzyme capable of acyltransfer, it was concluded that any such enzyme produced must be of both plasmid and of A. hydrophila origin.

The SalI insert of pHEc1 was determined to be 18 kb by agarose gel electrophoresis of various restriction enzyme digests. A large amount (approx. 1.5 mg) of pHEc1 was subsequently purified for further restriction mapping and subcloning (Figure 3).

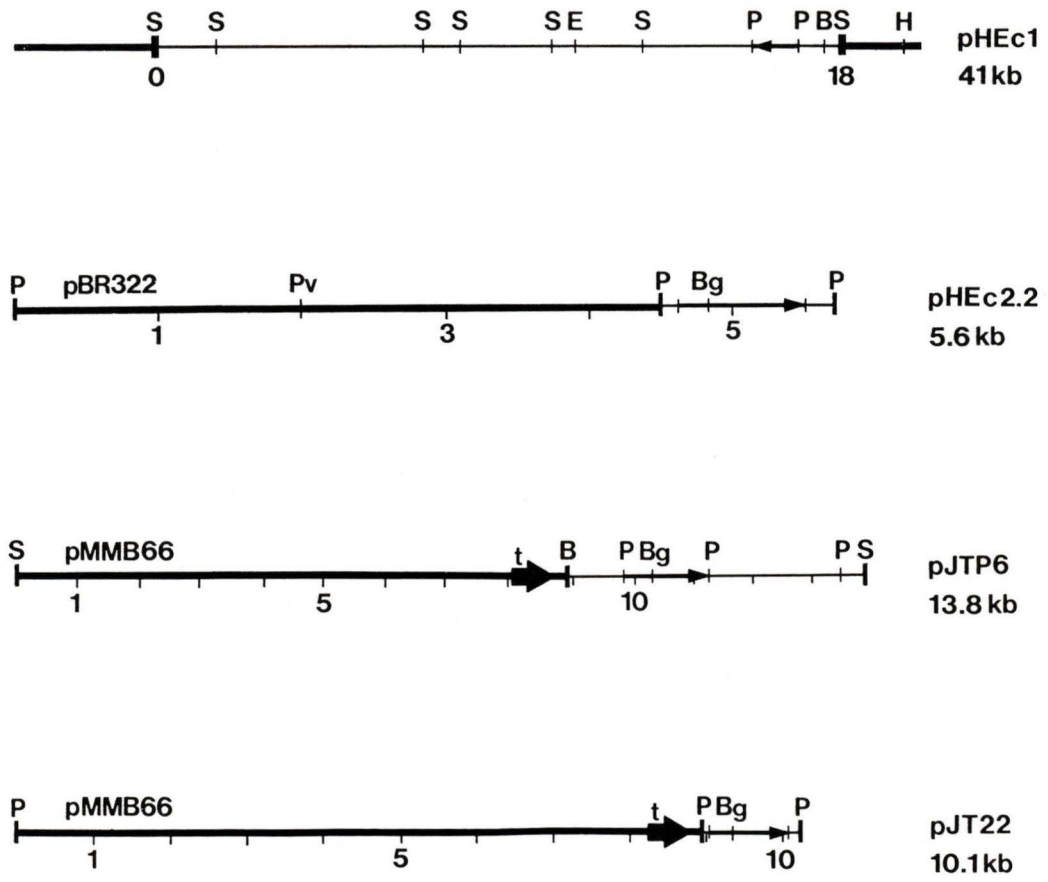



FIGURE 3. Clones containing the GCAT gene. The thick lines represent vector DNA, the thin lines represent A. hydrophila DNA. B, BamHI; Bg, BglII; E, EcoRI P, PstI; Pv, PvuII; S, SalI; t, tacP, (large arrows); small arrows represent gcatA and its orientation. Individual scales in kb are present on each plasmid map.

To sequence the GCAT gene and study the expression of GCAT in heterologous species of bacteria, it was necessary to subclone the gene into various other plasmid vectors. Such subcloning would likely eliminate other unnecessary genes that flank the GCAT gene in pHEc1. The subcloning vectors chosen were: (a) pBR322 for standard localization experiments in E. coli and for further DNA manipulations such as restriction mapping; and (b) the wide host-range tacP expression vector pMMB66 in order to study expression of GCAT in heterologous bacterial species; (see table 2 for descriptions of these vectors and their sources).

Restriction enzyme mapping of the SalI insert of pHEc1 revealed the various sites shown in Figure 3. The enzymes BamHI, PstI, and SalI were all used for subsequent subcloning of the GCAT gene into pBR322. One of the PstI subclones, created by the ligation of PstI fragments of pHEc1 to pBR322, was the only recombinant to display an alpha haemolytic phenotype.

This pBR322-based PstI subclone, pHEc2.2, contained a 1.2 kb PstI restriction fragment inserted into the PstI site of the pBR322 bla gene (Figure 3). Disrupted cells from an overnight culture of E. coli HB101-pHEc2.2 contained considerable GCAT activity. This plasmid was the primary source of restricted DNA fragments that were sequenced.

The GCAT gene, gcatA, was also subcloned into the wide host range tacP expression vector, pMMB66EH (Fürste et al.

1986), both with and without extra upstream DNA (see below). This gave rise to the plasmids pJTP6, and pJT22, (Figure 3). The plasmid pJTP6 contained a 5 kb SalI-BamHI insert cloned directly from the cosmid pHEcI. The 1.2 kb PstI fragment that could be excised from pHEc2.2 is located approximately 0.75 kb downstream from the BamHI site of the pMMB66EH multiple cloning site, as determined by restriction mapping. The orientation of this insert was also determined by restriction enzyme analysis (Figure 3). This plasmid (pJTP6) was constructed in order to determine if a GCAT promoter was located immediately upstream, or if GCAT was a distal gene within an operon. In the plasmid pJT22, the insert is the same 1.2 kb PstI fragment as in pHEc2.2, except that the DNA was obtained from the replicative form of one of the sequencing templates, (pJT1/mp19, Figure 5). This fragment was cloned into the pMMB66EH multiple cloning site using the enzymes EcoRI and HindIII of the M13-mp19 multiple cloning site, (not shown in Fig. 3), that flank the PstI sites. This insured that the orientation of gcatA was as shown in Figure 3. This plasmid was constructed in an attempt to gain complete control of GCAT expression by the tac promoter of the vector. In both pJTP6 and pJT22 the inserts were in the orientation required for transcription from the upstream tac promoter of pMMB66EH.

A hybrid plasmid containing the 750 bp BamHI/PstI fragment of pJTP6 (this is the region immediately upstream

of the GCAT functional gene), cloned immediately upstream of the functional gene for aerolysin, aerA, (flanked by NsiI and SalI restriction sites) from pKW200, (J.T. Buckley, unpublished), was constructed in pMMB66EH. This resulted in the formation of pJTPA7, (Figure 4).

**Nucleotide Sequence Analysis of the GCAT Gene**

Using the sequencing strategy shown in Figure 5, the nucleotide sequence of the gene coding for A. hydrophila GCAT, gcatA, was determined. The results are presented in Figure 6. The DNA sequence of 1368 bp was determined and the correct reading frame for gcatA was identified. Confirmation of this reading frame was achieved by first comparing the newly derived amino acid sequence with the known amino-terminal amino acid sequences of GCAT from both A. hydrophila and A. salmonicida. These two proteins differ by only one amino acid within the first 18 amino acids of their amino terminus (Figure 7). Further confirmation that the proposed reading frame was correct came from a comparison of the codon usage frequencies for the GCAT gene (Table 4) with the codon usage frequencies for the previously sequenced Aeromonas hydrophila ∝-amylase (Gobius and Pemberton 1988), and aerolysin (Howard et al. 1987). These frequencies were virtually identical to the codon frequencies for the GCAT gene and all codon frequencies correspond to those reported for E. coli nonregulatory genes (Ikemura and Ozeki 1982).

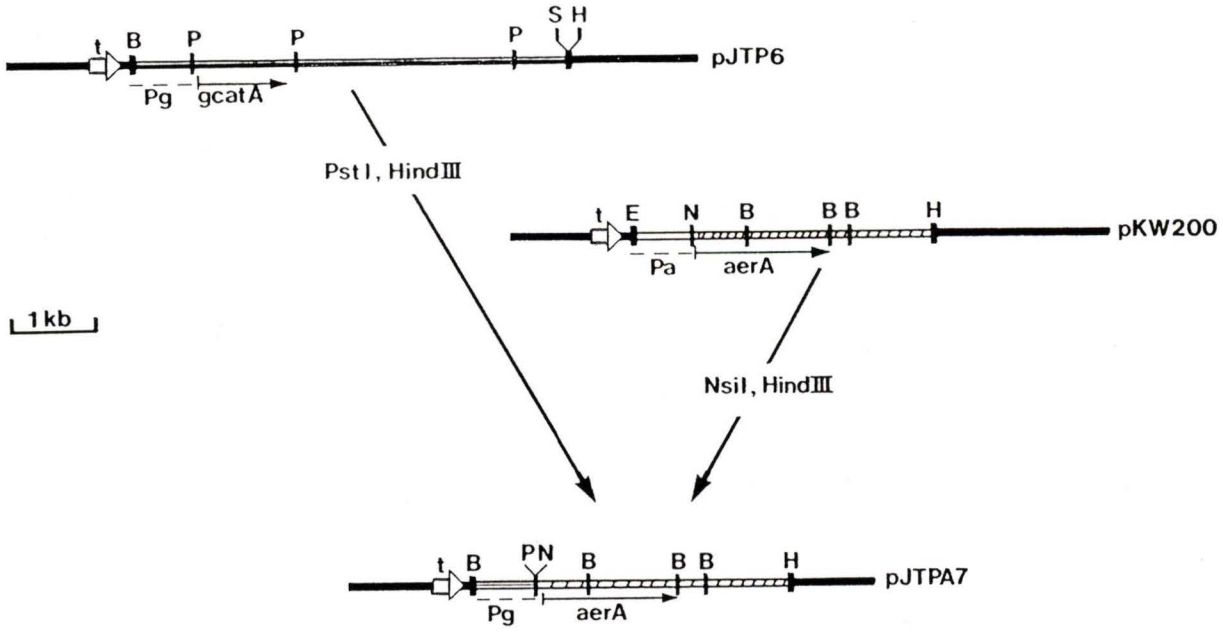



FIGURE 4. The Construction of pJTPA7.

The fusion of the aerolysin gene downstream of the proposed GCAT promoter to form pJTPA7. Pg represents the GCAT promoter region; gcatA represents the structural GCAT gene; Pa represents the aerolysin promoter region; and aerA represents the structural aerolysin gene. B, BamHI; E, EcoRI; H, HindIII; N, NsiI; P, PstI; t, tacP. pKW200 is from J.T. Buckley, unpublished data. Arrows represent orientations of the various elements.

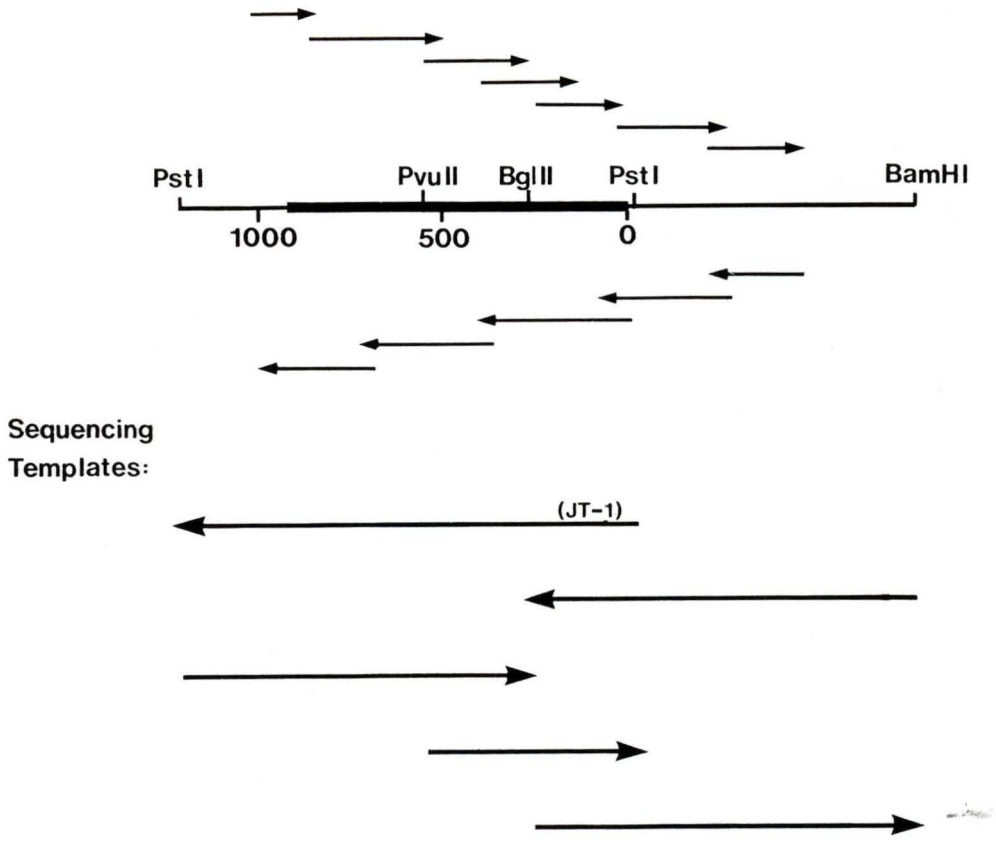



FIGURE 5. Strategy for sequencing the GCAT gene.

Small arrows represent sequences obtained using the various templates depicted (large arrows) in conjunction with synthetic oligonucleotide primers. Scale is in bp.

FIGURE 6. DNA sequence of the GCAT gene. The nucleotide residues are numbered in the 5' to 3' direction. The coding region is translated above the DNA sequence. Numbers below the sequence refer to nucleotide positions, numbers above to the amino acid positions. The sequence from -18 to -1 is the probable signal sequence. The sequence corresponding to the amino acid sequence of the enzyme from A. salmonicida is underlined (see text). Several restriction sites are identified for references to Figure 3.

```
CAT GCT CCT GTC ATT GGT ACG GGA GCA GTG TGT GCA AGC CGG GCG TGA AAC AAA CGT GAA GCC TCC
              12                  24                  36                  48                  60
CCA TCT GGG TGA AGG GGG TGT GAC CGT AAT CGA TTT AAT CAG CAC CGC CTG CTG CGA AAT TTA ATT
              78                  90                 102                 114                 126
CGG CTG GAT ACC CTG CAA AAT GGC AAA ACA GCT TCC CAG CAC CTT TGC CTA TCG AGG AAA AGC CCT
             144                 156                 168                 180                 192

Pst I                                                                 Met Lys Lys Trp Phe Val Cys Leu Leu
GCA GGC ACC ACC TGC TTT CAA AGC AAC GAG AAC AAC AAG ATG AAA AAA TGG TTT GTG TGT TTA TTG
             210                 222                 234                 246                 258
                                                 1
Gly Leu Val Ala Leu Thr Val Gln Ala Ala Asp Ser Arg Pro Ala Phe Ser Arg Ile Val Met Phe
GGA TTG GTC GCG CTG ACA GTT CAG GCA GCC GAC AGC CGT CCC GCC TTC TCC CGG ATC GTG ATG TTT
             276                 288                 300                 312                 324
                                                    25
Gly Asp Ser Leu Ser Asp Thr Gly Lys Met Tyr Ser Lys Met Arg Gly Tyr Leu Pro Ser Ser Pro
GGC GAC AGC CTC TCC GAT ACC GGC AAG ATG TAC AGC AAG ATG CGC GGT TAC CTC CCC TCC AGC CCC
             342                 354                 366                 378                 390
                                                                    50
Pro Tyr Tyr Glu Gly Arg Phe Ser Asn Gly Pro Val Trp Leu Glu Gln Leu Thr Asn Glu Phe Pro
CCC TAC TAT GAG GGC CGC TTC TCC AAC GGG CCC GTC TGG CTG GAG CAG CTG ACC AAC GAG TTC CCG
             408                 420                 432                 444                 456
                                                                                75   Bgl II
Gly Leu Thr Ile Ala Asn Glu Ala Glu Gly Gly Pro Thr Ala Val Ala Tyr Asn Lys Ile Ser Trp
GGC CTG ACC ATA GCC AAC GAG GCG GAA GGC GGA CCG ACC GCC GTG GCT TAC AAC AAG ATC TCC TGG
             474                 486                 498                 510                 522
                                                                                           100
Asn Pro Lys Tyr Gln Val Ile Asn Asn Leu Asp Tyr Glu Val Thr Gln Phe Leu Gln Lys Asp Ser
AAT CCC AAG TAT CAG GTC ATC AAC AAC CTG GAC TAC GAG GTC ACC CAG TTC CTG CAA AAA GAC AGC
             540                 552                 564                 576                 588

Phe Lys Pro Asp Asp Leu Val Ile Leu Trp Val Gly Ala Asn Asp Tyr Leu Ala Tyr Gly Trp Asn
TTC AAG CCG GAC GAT CTG GTG ATC CTC TGG GTC GGC GCC AAC GAC TAT CTG GCC TAT GGC TGG AAC
             606                 618                 630                 642                 654
    125
Thr Glu Gln Asp Ala Lys Arg Val Arg Asp Ala Ile Ser Asp Ala Ala Asn Arg Met Val Leu Asn
ACA GAG CAG GAT GCC AAG CGG GTG CGC GAC GCC ATC AGC GAT GCG GCC AAC CGC ATG GTG CTG AAC
             672                 684                 696                 708                 720
                    150
Gly Ala Lys Glu Ile Leu Leu Phe Asn Leu Pro Asp Leu Gly Gln Asn Pro Ser Ala Arg Ser Gln
GGC GCC AAG GAG ATA CTG CTG TTC AAC CTG CCG GAT CTG GGC CAG AAC CCC TCG GCC CGC AGC CAG
             738                 750                 762                 774                 786
                                175
Lys Val Val Glu Ala Ala Ser His Val Ser Ala Tyr His Asn Gln Leu Leu Leu Asn Leu Ala Arg
AAG GTG GTC GAG GCG GCC AGC CAT GTC TCC GCC TAC CAC AAC CAG CTG CTG CTG AAC CTG GCA CGC
             804                 816                 828                 840                 852
                                                200
Gln Leu Ala Pro Thr Gly Met Val Lys Leu Phe Glu Ile Asp Lys Gln Phe Ala Glu Met Leu Arg
CAG CTG GCT CCC ACC GGC ATG GTG AAG CTG TTC GAG ATC GAC AAG CAG TTT GCC GAG ATG CTG CGT
             870                 882                 894                 906                 918
                                                                225
Asp Pro Gln Asn Phe Gly Leu Ser Asp Thr Glu Asn Ala Cys Tyr Gly Gly Ser Tyr Val Trp Lys
GAT CCG CAG AAC TTC GGC CTG AGC GAC ACG GAG AAC GCC TGC TAC GGT GGC AGC TAT GTA TGG AAG
             936                 948                 960                 972                 984
                                                                         250
Pro Phe Ala Ser Arg Ser Ala Ser Thr Asp Ser Gln Leu Ser Ala Phe Lys Pro Ala Gly Ala Pro
CCG TTT GCC TCC CGC AGC GCC AGC ACC GAC AGC CAG CTC TCC GCC TTC AAA CCC GCA GGA GCG CCT
            1002                1014                1026                1038                1050
                                                                                       275
Arg His Arg Arg Gln Pro Ala Ala Gly Pro Gly Arg Arg Gln Pro His Gly Cys Pro Gln Arg Gln
CGC CAT CGC CGG CAA CCC GCT GCT GGC CCA GGC CGT CGC CAG CCC CAT GGC TGC CCG CAG CGC CAG
            1068                1080                1092                1104                1116

His Pro Gln Leu
CAC CCT CAA CTG TGA GGG CCA AGA TGT TCT GGG ATC AGG TCC ACC CCA CCA CTG TCG TGC ACG CCG
            1134                1146                1158                1170                1182

CCC TGA GCG AGC CCG CCG CCA CCT TCA TCG AGA GCC AGT ACG AGT TCC TCG CCC ACT GAT GAC AAG
            1200                1212                1224                1236                1248

CCG ACA CTC GGT CGG CCC CTC TTT TGC CTC GTC ACG ACA CCA TCA ATC AGC GAT AGC GCT TCA CTA
            1266                1278                1290                1302                1314
                                                                    Pst I
TGC CGT CAT AGA TAC GTT CTG CCG GAT GGT GGC CAG CGC CTC CTG CAG
            1332                1344                1356                1368
```

FIGURE 7. Amino-terminal Sequence of GCAT from *A. hydrophila* and *A. salmonicida*

---

```
                              1                5
A. hydrophila GCAT - Ala asp ser arg pro ala phe-
                             *
                              1      *         5
A. salmonicida GCAT- Ala asp thr arg pro ala phe-

                  10                   15           18
-ser arg ile val met phe gly asp ser leu ser-....

                  10                   15           18
-ser arg ile val met phe gly asp ser leu ser-....
```

---

Note: *A. hydrophila* GCAT sequence is in bold face; the asterisk, (*), marks the amino acid at which the proteins differ.

TABLE 4. Codon usage frequencies for the A. hydrophila GCAT gene.

| | | | |
|---|---|---|---|
| UUU-Phe-3 | UCU-Ser-0 | UAU-Tyr-5 | UGU-Cys-0 |
| **UUC-Phe-9*** | **UCC-Ser-8*** | **UAC-Tyr-7*** | **UGC-Cys-2*** |
| UUA-Leu-0 | UCA-Ser-0 | UAA-XXX-0 | **UGA-XXX-1*** |
| UUG-Leu-0 | UCG-Ser-1 | UAG-XXX-0 | UGG-Trp-5 |
| CUU-Leu-0 | CCU-Pro-2 | **CAU-His-3*** | CGU-Arg-3 |
| CUC-Leu-4 | **CCC-Pro-11*** | CAC-His-2 | **CGC-Arg11*** |
| CUA-Leu-0 | CCA-Pro-1 | CAA-Gln-3 | CGA-Arg-0 |
| **CUG-Leu-21*** | CCG-Pro-7 | **CAG-Gln-14*** | CGG-Arg-3 |
| AUU-Ile-0 | ACU-Thr-0 | AAU-Asn-1 | AGU-Ser-0 |
| **AUC-Ile-6*** | **ACC-Thr-7*** | **AAC-Asn-16*** | **AGC-Ser13*** |
| AUA-Ile-2 | ACA-Thr-1 | AAA-Lys-2 | AGA-Arg-0 |
| AUG-Met-6 | ACG-Thr-1 | **AAG-Lys-11*** | AGG-Arg-0 |
| GUU-Val-0 | GCU-Ala-4 | GAU-Asp-6 | GGU-Gly-2 |
| GUC-Val-6 | **GCC-Ala-18*** | **GAC-Asp-10*** | **GGC-Gly15*** |
| GUA-Val-1 | GCA-Ala-2 | GAA-Glu-1 | GGA-Gly-2 |
| **GUG-Val-7*** | GCG-Ala-4 | **GAG-Glu-11*** | GGG-Gly-1 |

*-Codons marked with an asterisk have highest frequency in E. coli non-regulatory genes (Ikemura and Ozeki 1982).

XXX-Stop codons.

The open reading frame of 897 bp encodes a hydrophillic protein of 31.3 kD, with an average (H) 19 hydropathy (Kyte and Doolittle 1982; Eisenberg 1984) of -0.56, and no lengthy hydrophobic subsequences.

No sequences similar to the E. coli -35 and -10 promoter consensus sequences (McClure 1985) were detected. Nor was an obvious Shine-Dalgarno sequence (Shine and Dalgarno 1975) found. The GCAT gene contains only a UGA stop codon, unlike the terminator for the aerolysin gene (Howard et al. 1987) which is comparable to the rho-independent terminator of E. coli.

A catabolite activator protein (CAP)-like binding site was located approximately 150 bp upstream of the initiation codon. The sequence from the region upstream of gcatA differs from the CAP consensus sequence (de Crombrugghe et al. 1984) only at 5 bases (see Figure 8). The bases in the region thought to be the most important for CAP binding, (namely the pentanucleotide TGTGA, de Crombrugghe et al. 1984) are unanimously conserved in the putative site upstream of the GCAT gene. It was noticed that the majority of differences that occurred between this putative CAP binding site and the consensus binding site were in the weakly conserved bases known to vary in many of the cases studied (de Crombrugghe et al. 1984.)

As mentioned above, the first 18 amino-terminal amino acids of the proposed A. hydrophila GCAT (derived from the

FIGURE 8. Putative Identification of a Catabolite Activator Protein Binding Site

---

CAP binding-site
"consensus sequence"
from E. coli*

- N N N N A A N T G T G A N N T N N N N C A N A T T N N N N

  X X X X  X X X X X X X X  X X X X X  X  X X X X X X

- A A G G G G G T G T G A C C G T A A T C G A T T T A A T C

CAP binding-site
from DNA upstream
of A. hydrophila
GCAT

---

* - This sequence represents the region most commonly protected by cAMP-CAP in chemical modification studies of E. coli CAP sites. Where N denotes any base where specific sequence appears unimportant; Underlined bases denote regions that are usually conserved in E. coli. Regions where the A. hydrophila sequence is similar to the E. coli sequence and/or spacing are marked with an X.

DNA sequence) were compared to the first 18 amino-terminal amino acids of A. salmonicida GCAT (derived from peptide sequencing). This comparison revealed that these two proteins differed only at one amino acid at position 3 of the protein. In GCAT from A. hydrophila this residue is a serine, and in A. salmonicida GCAT the residue is a threonine.

The predicted amino acid composition of A. hydrophila GCAT is compared to the experimentally derived amino acid composition of GCAT from A. salmonicida in Table 5. The distinct differences in amino acid compositions of the two proteins and the apparent differences in MW, suggest that although the enzymes from these two organisms appear to be identical in function, they differ distinctly in their primary structure.

SDS-PAGE analysis of the two GCAT molecules has demonstrated that the A. salmonicida GCAT is 23.6 $\pm$ 0.3 kD (mean of 5 determinations $\pm$ S.D.;Buckley et al. 1982) while the A. hydrophila enzyme is predicted to be 35 $\pm$ 0.4 kD (mean of 3 determinations $\pm$ S.D., data not shown), and according to the derived sequence for GCAT from A. hydrophila the protein appears to be approximately 31.3 kD. These analyses of GCAT size further substantiate the implied structural differences in these two related proteins.

TABLE 5. Amino acid Compositions of mature GCAT* from Two Species of Aeromonas.

| Amio Acid | A. hydrophila[b] | A. salmonicida[c] |
|---|---|---|
| Alanine | 26 | 23 |
| Arginine | 17 | 5 |
| Asparagine | 17 | 21[e] |
| Aspartic acid | 16 | |
| Cysteine | 1 | ND[d] |
| Glutamine | 16 | 19[f] |
| Glutamic acid | 12 | |
| Glycine | 19 | 27 |
| Histidine | 5 | 4 |
| Isoleucine | 8 | 5 |
| Leucine | 21 | 15 |
| Lysine | 11 | 8 |
| Methionine | 5 | 3 |
| Phenylalanine | 11 | 5 |
| Proline | 21 | 10 |
| Serine | 22 | 18 |
| Threonine | 8 | 8 |
| Tryptophan | 4 | ND[d] |
| Tyrosine | 12 | 7 |
| Valine | 11 | 18 |
| Molecular weight | 31303 d | 23600 d |

*[a]Reported in mol/mol GCAT. [b]Determined by nucleotide sequence of gcatA. [c]Determined by chemical analysis. [d]ND not done. [e]Both Asp and Asn determined as Asp. [f]Both Glu and Gln determined as Glu.

## Sequence similarities with other lipases

A search of the protein sequence library of the National Biomedical Research Foundation (Lipman and Pearson 1985) did not identify any sequences with extended regions similar to GCAT. Recently however, many similarities between a variety of lipases have been pointed out (Maraganore and Henrikson 1986; Komaromy and Schotz 1987; Wion et al. 1987). The results in Figure 9 show that GCAT contains two distinct regions that may share sequence similarities with the proposed active sites and the lipid binding sites of other lipases (Maraganore and Heinrikson 1986).

## The Signal Sequence of GCAT

The first 54 nucleotides of the open reading frame of the GCAT gene code for a sequence 18 amino acid residues in length which is likely the signal sequence (Figure 6). As with other reported signal sequences, (von Heijne 1983), there is a positively charged amino acid, in this case lysine, adjacent to the initiation codon, followed by a core of hydrophobic amino acids. The cleavage site for signal peptidase is between the two alanine residues -1 and +1 (Figure 6).

FIGURE 9. Sequence Similarities with Other Lipases.

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**9A: LIPID BINDING DOMAIN**

Microbial GCAT
$^{9}$Arg.Ile.Val.Met.Phe.Gly.Asp.Ser.Leu.Ser

R.Hepatic Lipase
$^{261}$Ser.Val.His.Leu.Phe.Ile.Asp.Ser.Leu.Gln

Human LPL
$^{244}$Ser.Ile.His.Leu.Phe.Ile.Asp.Ser.Leu.Leu

R.Lingual lipase
$^{163}$Lys.Ile.His.Tyr.Val.Gly.His.Ser.Gln.Gly

Human LCAT
$^{173}$Pro.Val.Phe.Leu.Ile.Gly.His.Ser.Leu.Gly

PPL
$^{145}$Asn.Val.His.Val.Ile.Gly.His.Ser.Leu.Gly

**9B: ACTIVE SITE DOMAIN**

GCAT
$^{232}$Trp.Lys.Pro.Phe.Ala.Ser.Arg.Ser.Ala.Ser.Thr.Asp. .Ser.Gln

PPL
$^{107}$Trp.Lys. .Gly.Gly.Ser.Arg.Thr.Gly.Tyr.Thr.Glu.Ala.Ser.Gln

LCAT
$^{213}$Trp. . .Gly.Gly.Ser.Ile.Lys.Pro.Met.Leu.Val.Leu.Ala.Ser

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9A: Similarities within the lipid binding domains of PPL and other lipases.
9B: Comparison with the proposed active sites of PPL and a similar region of human LCAT.

See the text for more details. The numbers refer to amino acid positions.

To determine the cellular location of cloned GCAT, the various cellular fractions of osmotically shocked cells that harboured plasmids bearing the GCAT gene were assayed for the presence of the enzyme.

Localization of cloned GCAT in E. coli-HB101

Acyltransferase activity was recovered in the shock fluid of HB101 containing pHEc1 or pHEc2.2 (see table 6). No detectable GCAT activity was present in culture supernatants of either clone except at late culture times when there was a concomitant release of the periplasmic enzymes β-lactamase and RNAase, and the cytoplasmic enzyme glutamate dehydrogenase. When GCAT was produced under inducing conditions from the plasmid pJT22, activity was only detected in the culture supernatants when the cellular marker enzymes were released. These results indicated that intact HB101 cannot export GCAT.

Localization of cloned GCAT in A. salmonicida As440-rif-1

The wide host range plasmids bearing the GCAT gene, pJT22, and pJTP6, were transferred into As440-rif-1 through conjugation with HB101 strains bearing the plasmids. The resulting transconjugants were selected on the basis of plasmid-mediated ampicillin resistance, and slightly increased haemolysis on HBA. To rule out possible mutants which might have displayed a similar phenotype, putative

TABLE 6. Distribution of GCAT and marker enzymes in *E. coli*.

| Organism | GCAT | Glutamate dehydro-genase | RNAase | Beta-lac-tamase |
|---|---|---|---|---|
| A. | | | | |
| HB101 | | | | |
| supernatant | 0 | 0 | 0 | ND |
| wash | 0 | 0 | 0.18 | ND |
| shock fluid | 0 | 0 | 0.94 | ND |
| shocked cells | 0 | 0.48 | 0.34 | ND |
| B. | | | | |
| HB101-pHEc1 | | | | |
| supernatant | 0 | 0 | 0 | ND |
| wash | 0 | 0 | 0.12 | ND |
| shock fluid | 5.5 | 0 | 1.22 | ND |
| shocked cells | 1.8 | 0.36 | 0.27 | ND |
| C. | | | | |
| HB101-pHEc2.2 | | | | |
| supernatant | 0.1 | 0 | 0 | ND |
| wash | 0 | 0 | 0.12 | ND |
| shock fluid | 7.0 | 0.06 | 0.8 | ND |
| shocked cells | 0.8 | 0.32 | 0.42 | ND |
| D. | | | | |
| HB101-pJT22 | | | | |
| i) | | | | |
| supernatant | 1.2 | 0.09 | ND | 0.1 |
| wash | 0 | 0.03 | ND | 0.06 |
| shock fluid | 21.8 | 0.13 | ND | 0.67 |
| shocked cells | 3.8 | 0.34 | ND | 0.08 |
| ii) | | | | |
| supernatant | 18.7 | 0.21 | ND | 0.63 |
| wash | 1.1 | 0.05 | ND | 0.13 |
| shock fluid | 38.5 | 0.19 | ND | 0.84 |
| shocked cells | 5.9 | 0.67 | ND | 0.07 |

All activities are expressed as Units per ml sample of (see Methods and Materials). All organisms except HB101-pJT22 were harvested at an $O.D._{600}$ of 1.0. HB101-pJT22 was harvested at an $O.D._{600}$ of 1.0, (i), and 4.0, (ii). For more experimental details see Methods and Materials. Both HB101-pJT22 cultures were induced with IPTG at $O.D._{600}$ of 0.5.

GCAT transconjugants were tested for the presence of the recombinant plasmids by electrophoresis and by the ability of the respective plasmid preparations to transform HB101 into a GCAT producer.

When As440-rif-1-pJT22 transconjugants were grown under inducing conditions, plasmid-encoded GCAT appeared in the cell free culture supernatants at early times during growth (Table 7; see expression data below). GCAT activity continued to be mainly extracellular (>95%) at all times throughout growth. When these transconjugants were grown in the absence of IPTG the GCAT levels measured were the same as in the control culture of As440-rif-1 without plasmid. In contrast to the HB101 transformants, the release of GCAT was not due to cell disruption because β-lactamase activity remained associated with the cells throughout growth (Table 7). The periplasmic and cellular fractions of A. salmonicida containing the plasmids pJT22 and pJTP6 consistently contained less than 5% of the total GCAT activity observed (Table 7). As A. salmonicida does not release appreciable amounts of endogenous GCAT under the growth conditions used for this experiment, (Table 7), it is evident that virtually all of the acyltransferase activity detected in the culture supernatants was due to the plasmid-encoded A. hydrophila GCAT.

TABLE 7. Distribution of GCAT and marker enzymes in *A. salmonicida* As440.

| | GCAT | Beta-lactamase |
|---|---|---|
| A. As440 | | |
| supernatant | 2.0 | 0.09 |
| wash | 0 | 0 |
| shock fluid | 0.05 | 1.08 |
| shocked cells | 0.02 | 0.26 |
| B. As440-pJT22 | | |
| i) | | |
| supernatant | 50.2 | 0.10 |
| wash | 0 | 0 |
| shock fluid | 2.3 | 0.98 |
| shocked cells | 1.2 | 0.17 |
| ii) | | |
| supernatant | 223 | 0.16 |
| wash | 1.3 | 0.02 |
| shock fluid | 12.1 | 1.53 |
| shocked cells | 6.7 | 0.32 |

All activities are expressed as Units per ml of sample (see Methods and Materials). All organisms except As440-pJT22-(ii) were harvested at an $O.D._{600}$ of 1.0. As440-pJT22-(ii) was harvested at an $O.D._{600}$ of 2.5. For more experimental details see Methods and Materials.

## Overexpression of GCAT by A. hydrophila

In order to examine the effects of GCAT overexpression in A. hydrophila the cosmid pHEc1 was transferred by conjugation from E. coli HB101 into A. hydrophila-Ah65-rif. As previously determined, HB101-pHEc1 displayed only low levels of GCAT activity in the periplasm and no extracellular GCAT activity was detected without prior lysis of the cells (see Tables 6 and 7 above). The total GCAT activity in HB101-pHEc1 periplasmic fractions at ~$10^9$ cells $ml^{-1}$ only reached 7.3 U $ml^{-1}$.

When cell-free culture supernatants of Ah65-rif-pHEc1 (pG1) were assayed for GCAT, they were found to contain up to 10 times more total GCAT activity than did control cultures of Ah65-rif. The pG1 transconjugant at ~$10^9$ cells $ml^{-1}$ produced GCAT to 50 U $ml^{-1}$, while Ah65-rif at the same culture density produced negligible amounts of GCAT. Also, when comparing the levels of aerolysin (another extracellular protein produced by A. hydrophila) between Ah65-rif and pG1, it was noticed that pG1 displayed a 10 fold increase in aerolysin activity in comparison to that of the Ah65-rif culture at peak production times (Figure 10).

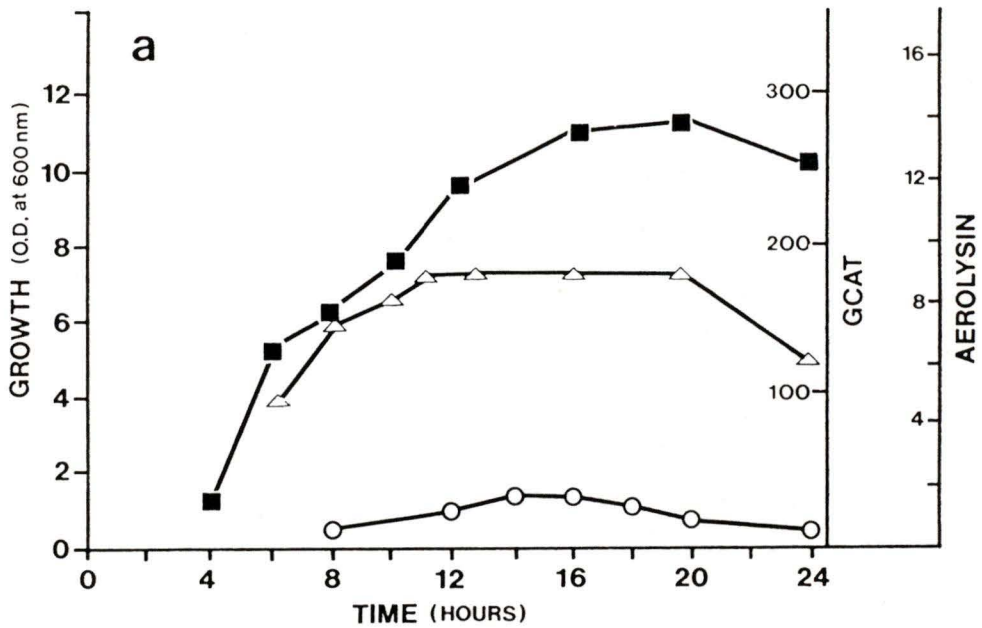
Due to this increase in aerolysin activity it was necessary to determine if the aerolysin gene was also in pHEc1. Southern dot blots of pHEc1 as well as of other plasmids containing the aerolysin gene (Figure 11) were performed using an oligonucleotide complimentary to an 18

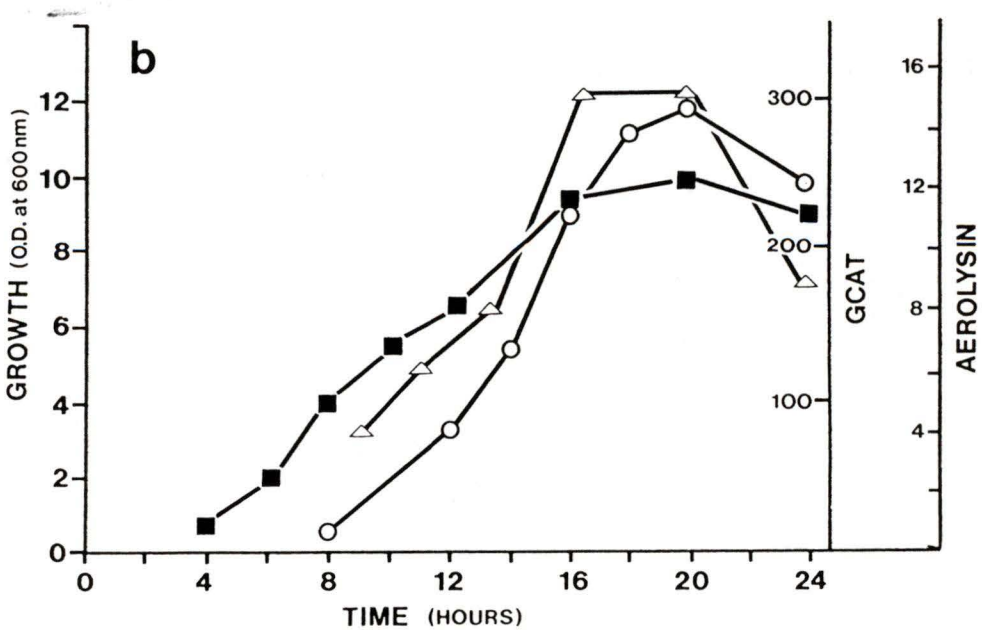
FIGURE 10. Growth, GCAT production, and aerolysin production by A. hydrophila-Ah65-rif (A), compared to Ah65-rif-pHEc1 (B).

= optical density at 600nm.

= GCAT activity (U/ml culture supernatant)

= Aerolysin activity (see methods and materials)


a
GROWTH (O.D. at 600nm)
TIME (HOURS)
GCAT
AEROLYSIN
0
2
4
6
8
10
12
0
4
8
12
16
20
24
100
200
300
4
8
12
16


b
GROWTH (O.D. at 600nm)
TIME (HOURS)
GCAT
AEROLYSIN
0
2
4
6
8
10
12
0
4
8
12
16
20
24
100
200
300
4
8
12
16

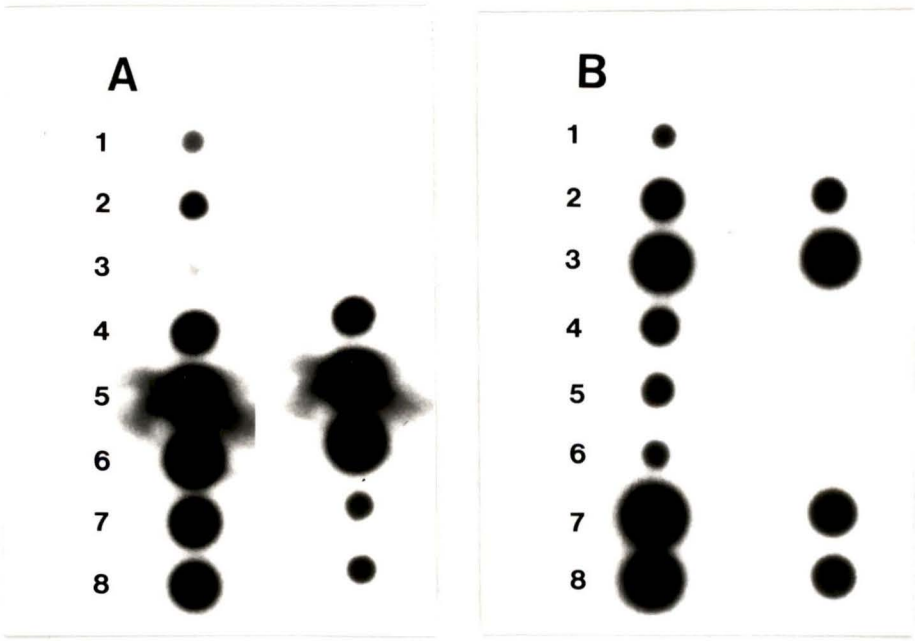



FIGURE 11. Southern dot blots of plasmids containing gcatA and aerA.

The negative control is pBR322 (1), and both Aeromonas hydrophila Ah65 and A. salmonicida As440 chromosomal DNA's are also included (7 and 8 respectively). pHEc1 (2) and pHEc2.2 (3) contain the GCAT gene gcatA. pPH4 (4), pPH30 (5), and pPH114 (6) all contain the aerolysin gene aerA, (Howard 1985).

**A** has been probed with an oligonucleotide specific for the aerolysin sequence. **B** has been probed with an oligonucleotide specific for the GCAT sequence.

In each blot (A and B), the panel on the left is after a low stringency wash and the panel on the right is after a high stringency wash.

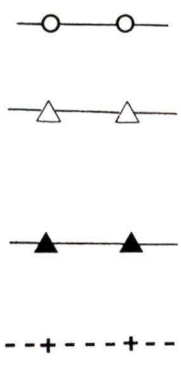
nucleotide sequence within the aerolysin gene (Howard et al. 1987). In a control experiment, an oligonucleotide homologous with an 18 nucleotide sequence of the GCAT gene was also used to blot the various plasmids (Figure 11). The blots confirmed that the aerolysin gene was not present within the pHEc1, or pHEc2.2 inserts.Despite the large increase in the extracellular production of both GCAT and aerolysin by Ah65-pG1, only a very slight reduction in the growth rate was observed when compared to wild type Ah65 (Figure 10). This reduction is represented by an increase in doubling times from ~45 min for Ah65-rif to ~60 min for pG1. In contrast to this, low level production of GCAT alone seems to cause HB101 to lyse (Table 5).

## Expression of GCAT from tacP expression vectors and the effects of GCAT on bacterial growth

### Expression of GCAT from *E. coli* HB101

To examine the expression and/or excretion of GCAT, HB101 containing the *tacP* recombinant plasmids pJT22 and pJTP6 were grown in the presence and absence of the *tacP* inducer IPTG. The viable cell counts, GCAT activity, and β-lactamase activity were measured. The subcellular location of these enzymes was also determined (Figure 12).

FIGURE 12. The expression of GCAT from tacP plasmids in E. coli. For details see the text.
**12a:** HB101; **12b:** HB101-pJT22; **12c:** HB101-pJTP6;
**12d:** HB101 + IPTG; **12e:** HB101-pJT22 + IPTG;
**12f:** HB101-pJTP6 + IPTG

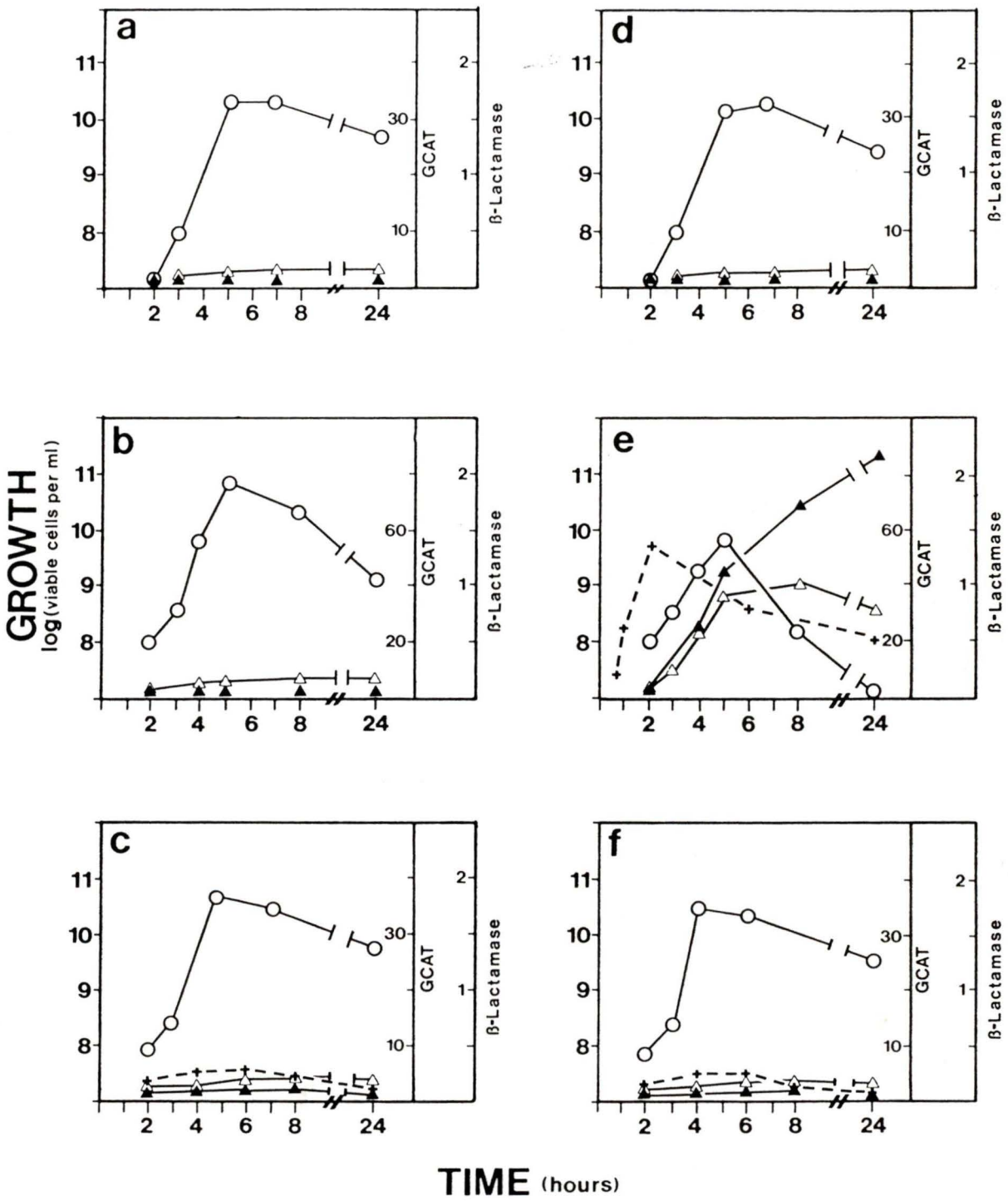


—o—o— = log [viable cells per ml].

—△—△— = beta-lactamase (U/ml) in the culture supernatant.

—▲—▲— = GCAT activity in the culture supernatant.

--+---+-- = GCAT activity in the periplasm.


a
b
c
d
e
f
GROWTH
log (viable cells per ml)
GCAT
β-Lactamase
TIME (hours)
2
4
6
8
24

GCAT Expression in HB101 from pJT22

When the structural gene, gcatA, was cloned into pMMB66 with a minimal amount of excess DNA upstream of the GCAT gene as in pJT22, the production of GCAT was under complete control of the tac promoter in HB101. This can be readily discerned by comparing Figure 12b with Figure 12e. As shown in Figure 12b, no GCAT activity was associated with HB101-pJT22 throughout the entire growth curve. But as is shown in Figure 12e, induction of the tac promoter by the addition of IPTG to a final concentration of 1 mM caused GCAT to be produced. As can be seen in Figure 12e, the level of GCAT expressed reached a maximum after 3 to 5 hours of induction with IPTG. The enzyme activity was entirely confined to the periplasm of HB101, until at 6-7 hr post induction (Figure 12e) when significant GCAT activity was detected in the culture supernatant. At this time, β-lactamase activity became associated with the culture supernatant and cell viability began to drop suggesting cell lysis was occurring. This suggests that the expression of GCAT is in some way harmful to HB101, causing the cells to lyse. Also demonstrated is, not surprisingly, that the promotion of gcatA requires more information than that provided by the 39 bp that exists between the GCAT start codon and the PstI restriction site used for the initial cloning.

GCAT Expression in HB101 from pJTP6

The plasmid pJTP6 contains approximately 750 bp of DNA upstream of gcatA. It was thought that, unless gcatA was part of an operon, this region would contain the promoter sequence of the GCAT gene, gcatP.

To determine if this 750 bp segment of DNA contained a promoter capable of directing GCAT synthesis, cells containing pJTP6 were grown with and without IPTG. If a promoter was present GCAT would be produced even in the absence of IPTG, if on the other hand, no promoter was present, GCAT would only be produced in the presence of IPTG assuming that no strong terminator sequences were present upstream. When the plasmid pJTP6 was transformed into E. coli HB101 the resulting colonies were very weakly haemolytic on HBA. Unlike HB101 bearing the plasmid pJT22, in which the expression of GCAT is completely under the control of the tac promoter, HB101 bearing pJTP6 show no induction of GCAT expression when grown in the presence of IPTG. In fact, gcatA is expressed only to a very small degree in HB101-pJTP6, both with and without IPTG in the growth media (Figures 12c and 12f), suggesting the presence of a very weak promoter upstream. The maximum levels of GCAT activity obtained from HB101-pJTP6 periplasmic fractions were one to two orders of magnitude below the maximum levels obtained with pJT22 in HB101. This low level production of GCAT by HB101-pJTP6 appeared to have little to

no effect on growth and viability of HB101 as can be seen by comparisons of Figures 12a, 12d, 12c, and 12f.

**GCAT Expression from A. salmonicida As440-rif-1**

To examine the expression and/or secretion of GCAT, As440-rif-1 containing the tacP recombinant plasmids pJT22 and pJTP6 were grown in the presence and absence of the tacP inducer IPTG. The viable cell counts, GCAT activity, and β-lactamase activity were measured. The subcellular location of the various enzymes was also determined (Figure 13).

GCAT Expression in As440-rif-1 from pJT22

The expression of gcatA in AS440-rif-1-pJT22 was under complete control of the tac promoter of the vector (Figures 13b, 13e) as it was in E. coli. Acyltransferase activity was detected at times as early as 45 minutes post induction and the enzyme continued to appear extracellularly throughout growth (Figure 13e) reaching maximal levels in 4 to 5 hours post induction.

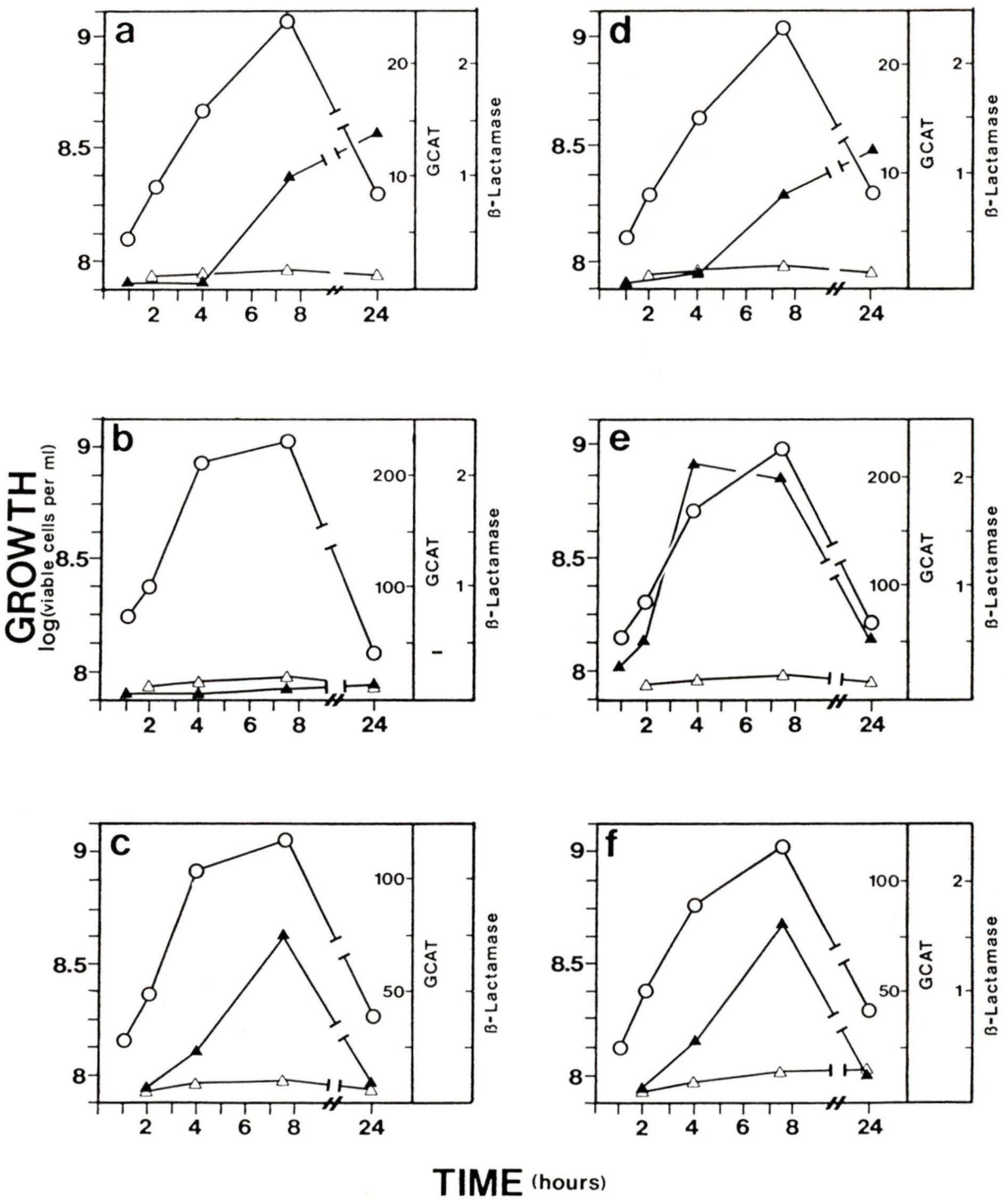
When the plasmid was not induced with IPTG, the levels of GCAT produced by As440-rif-1-pJT22 were comparable to the extremely low levels of endogenous GCAT seen in the culture supernatants of control As440 cultures grown with the same culture conditions (Figure 13a). This verified that all inducible GCAT activity was of plasmid origin, and that there was no expression of gcatA without prior induction of

FIGURE 13. The expression of GCAT from tacP plasmids in A. salmonicida. For details see the text. **13a:** As440-rif1; **13b:** As440-rif1-pJT22; **13c:** AS440-rif1-pJTP6; **13d:** AS440-rif1 + IPTG; **13e:** As440-rif1-pJT22 + IPTG; **13f:** AS440-rif1-pJTP6 + IPTG.

= log [viable cells per ml].

= beta-lactamase (U/ml) in the culture supernatant.

= GCAT activity in the culture supernatant.


a
b
c
d
e
f
GROWTH
log(viable cells per ml)
9
8.5
8
2
4
6
8
24
GCAT
ß-Lactamase
20
10
200
100
50
1
2
TIME (hours)

tacP in pJT22. In contrast to E. coli, the overexpression of GCAT has no detectable deleterious effects on the growth and viability of A. salmonicida. This can be seen by comparing Figures 13a, 13d, 13b, and 13e with the corresponding figures for E. coli.

Expression of GCAT in As440-rif-1 from pJTP6

Transconjugants of As440-rif-1 bearing the plasmid pJTP6 were distinguished from As440-rif-1 without plasmid as being only slightly more haemolytic on HBA. In contrast toAs440-rif-1-pJT22, As440-rif-1-pJTP6, like HB101-pJTP6 displayed no change in GCAT activity upon induction (Figures 13c and 13f). However, the clones of As440 containing pJTP6 did display extracellular GCAT activities much higher than those seen in both HB101-pJTP6 (Figures 12c and 12f), and As440-rif-1 (Figures 13a and 13d) grown with the same culture conditions, although the levels were still lower than those seen in IPTG-induced cultures of As440-rif-1-pJT22. This suggests that if an intact promoter is present in the upstream DNA, gcatA is expressed constitutively by this promoter at a lower level than the level of expression that tacP is capable of promoting.

## Aerolysin Expression from the plasmid pJTPA7

As was shown in Figure 4, the plasmid pJTPA7 is the result of a fusion of the aerolysin structural gene, aerA, and the putative GCAT promoter gcatP. An appropriate

transconjugant of this plasmid was constructed in As440-rif-1 in order to examine if gcatP was capable of directing the transcription of a gene other than gcatA. The expression of aerolysin from plasmid pJTPA7 was assayed in the culture supernatants of As440-rif-1-pJTPA7. The culture conditions were the same as for As440-rif-1 bearing the GCAT plasmids pJT22 and pJTP6.

When As440-rif-pJTPA7 was compared to As440-rif-1, As440-rif-1-pJTP6, and the aerolysin producers: AS440-rif-1 pKW200 (aerolysin gene with promoter), and As440-rif-1-pNB5 (aerolysin gene without promoter); (J.T. Buckley, unpublished results), it was demonstrated that aerolysin was constitutively produced at a level considerably lower than that of the constitutive expression of pKW200, but somewhat higher than the expression seen in pNB5 in the absence of IPTG (Figure 14). These results suggest that the upstream DNA of pJTP6 is capable of directing transcription, although only at a very low level.

**Immunological Similarities in the GCAT Family of Proteins**

It is evident that the GCAT molecules from two species of Aeromonas, namely A. hydrophila and A. salmonicida, are related on the basis of the striking similarities found in the first 18 amino terminal amino acid residues. But, based on the large discrepancy in their apparent MW, (As440 GCAT is 23.6 kD whereas Ah65 GCAT is 35 kD), the question that arises is how much of the two protein's sequence and

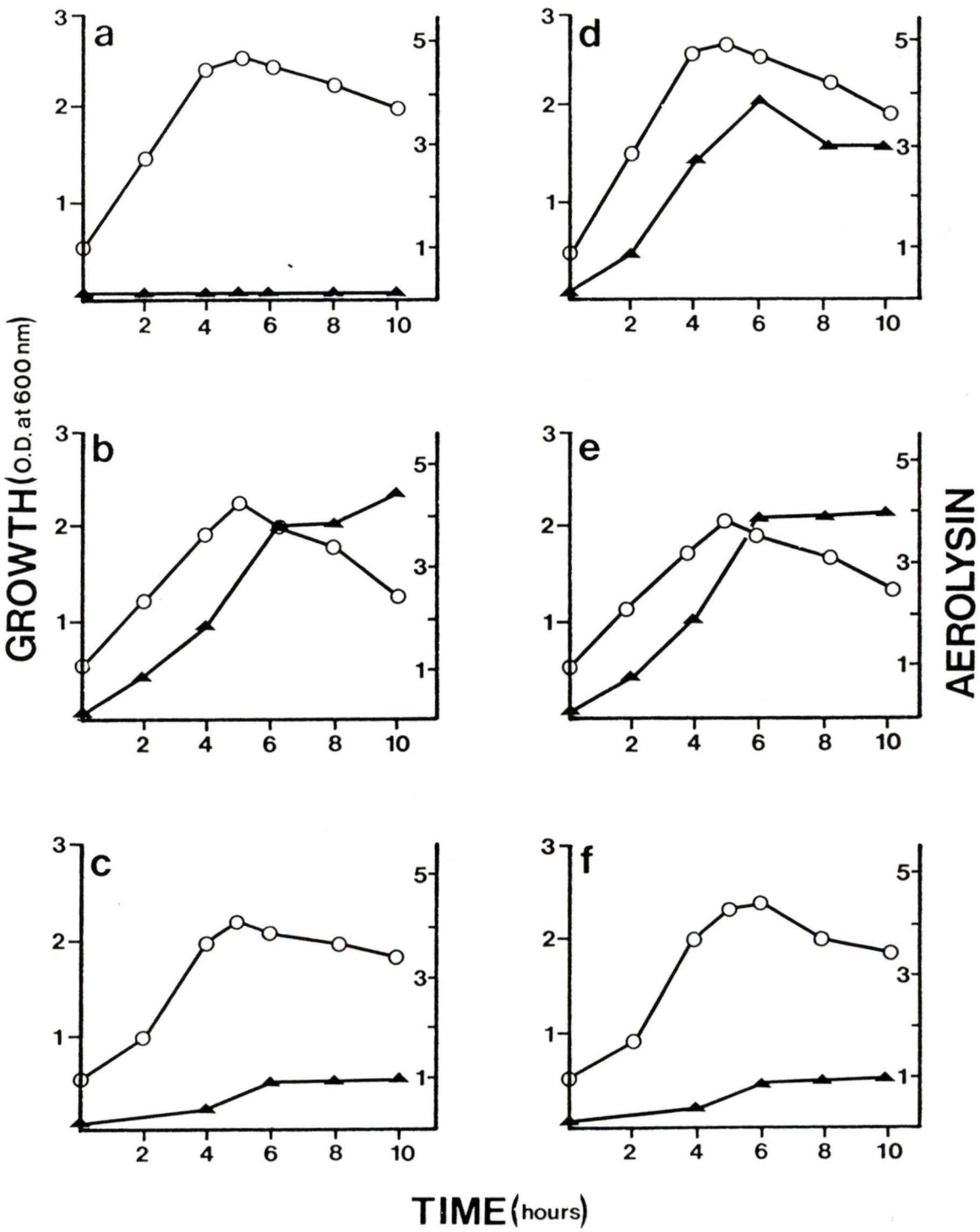
FIGURE 14. Aerolysin expression from the plasmid pJTPA7.

The expression of aerolysin from the putative GCAT promoter gcatP in pJTPA7 was compared to the expression of aerolysin due to tacP and aerP.

**14a:** As440-rif1-pNB5; **14b:** As440-rif1-pKW200; **14c:** As440-rif1-pJTPA7; **14d:** As440-rif1-pNB5 + IPTG; **14e:** As440-rif1-pKW200 + IPTG; **14f:** As440-rif1-pJTPA7 + IPTG.

—○—○— = Growth (Optical density at 600 nm.)

—▲—▲— = Aerolysin activity.


a
b
c
d
e
f
GROWTH (O.D. at 600 nm)
AEROLYSIN
TIME (hours)
2
4
6
8
10
1
2
3
1
3
5

secondary structure are conserved.

Polyclonal antibodies to both proteins were raised in rabbits, and used in both ELISA and immunoblotting experiments. It was demonstrated that both antisera cross-reacted with the two species of GCAT (Figure 15, Figure 16),

SDS-PAGE, followed by immunoblotting of the proteins, suggested that both antisera could react with both proteins after SDS denaturation of the proteins (Figure 16).

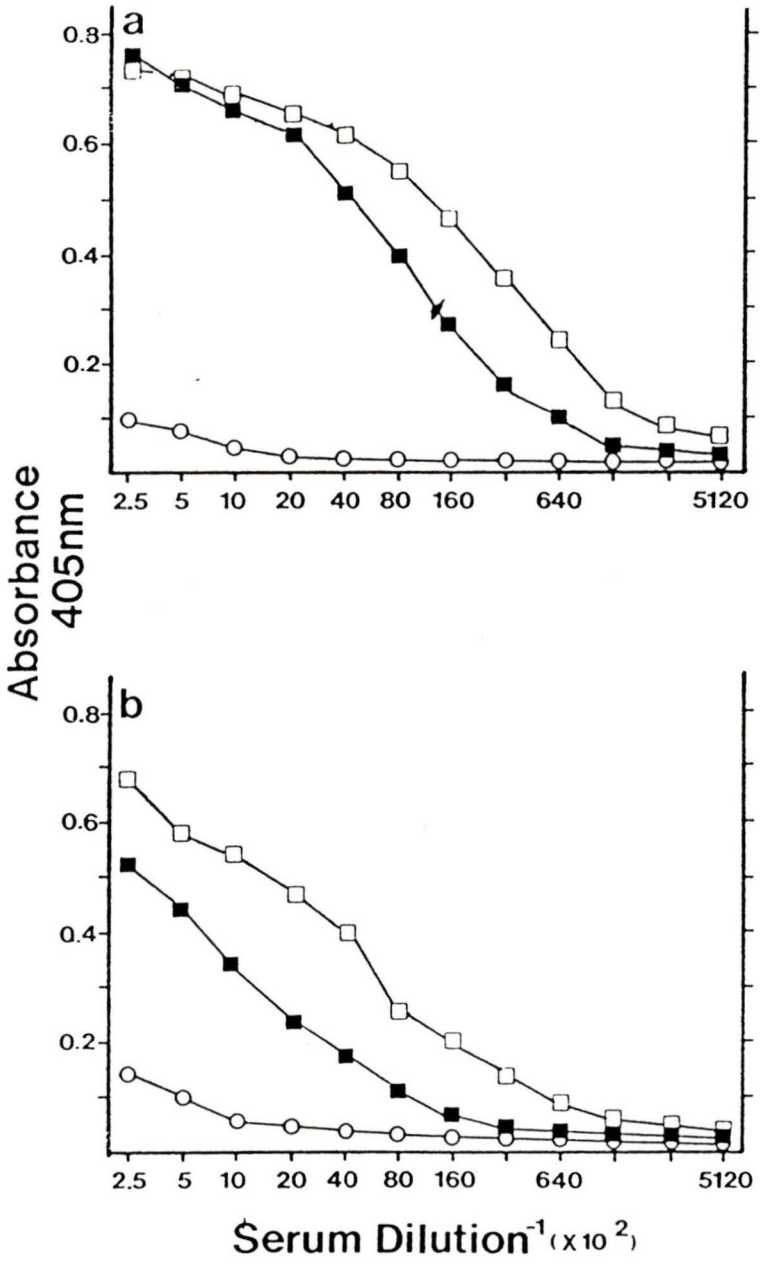



FIGURE 15. Enzyme Linked Immunosorbent Assay (ELISA).

15a. Ag = A. hydrophila GCAT; Ab (rabbit sera),
1) ■ = anti-A. hydrophila-GCAT sera;
2) □ = anti-A. salmonicida-GCAT sera;
3) O = negative control, (Ag = BSA, Ab = anti-A. hydrophila-GCAT sera).

15b. Ag = A. salmonicida GCAT; Ab (rabbit sera),
1) ■ = anti-A. hydrophila-GCAT sera;
2) □ = anti-A. salmonicida-GCAT sera;
3) O = negative control, (Ag = BSA, Ab = anti-A. salmonicida-GCAT sera).

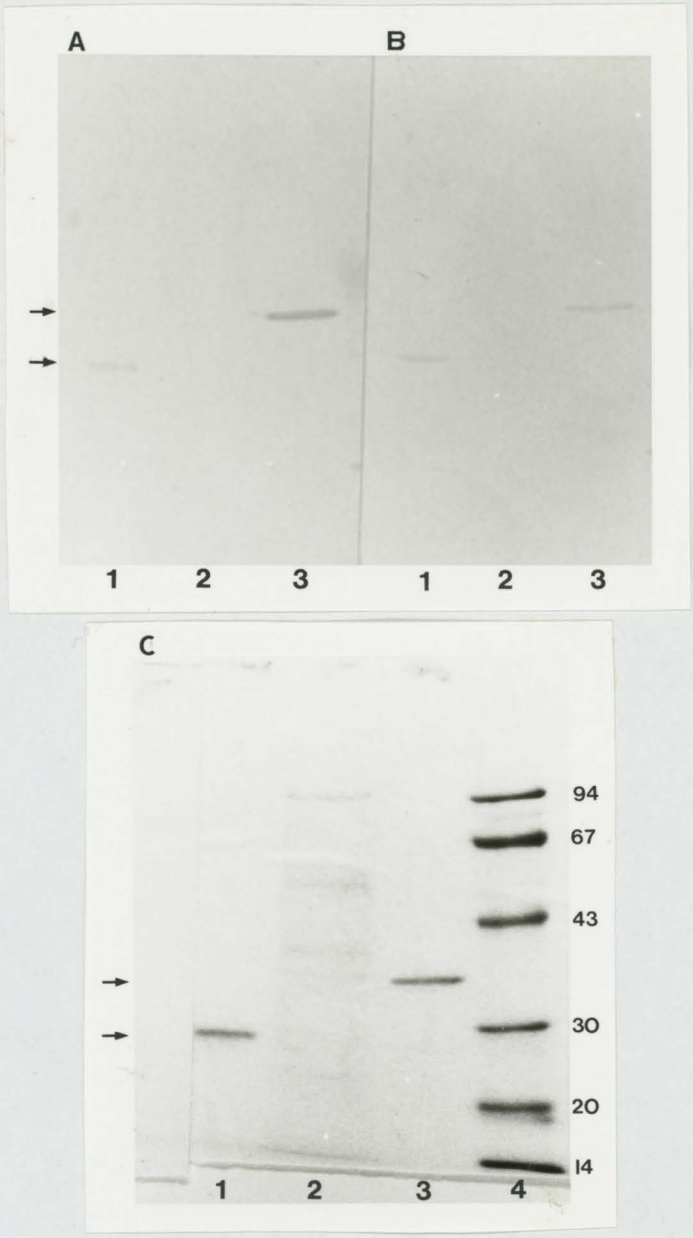



FIGURE 16. Western Immunoblots of A. hydrophila and A. salmonicida GCAT.

A, 1st Ab = anti A. hydrophila-GCAT sera;
B, 1st Ab = anti A. salmonicida-GCAT sera;
C, Coommassie blue stained SDS-PAGE.

**Lane 1** contains 4 µg As440 GCAT, (apparent MW 23.6 kD); **lane 2** contains 20 µl Ah65 18 hr culture supernatant; **lane 3** contains 4 µg Ah65 GCAT, (apparent MW = 35 kD); and **lane 4** (gel C only), contains MW standards, at ~6 µg each.

Small arrows indicate the position of the two GCAT molecules, top arrow = Ah65 GCAT, bottom arrow = As440 GCAT.

## DISCUSSION

This study revealed that the GCAT gene, gcatA, from A. hydrophila encodes a 31 kD enzyme capable of acyltransfer. This enzyme, which is normally extracellular in cultures of A. hydrophila (MacIntyre and Buckley 1978), is also released by the related bacteria A. salmonicida containing the A. hydrophila GCAT gene. In contrast, E. coli clones bearing gcatA only secrete GCAT to the periplasmic space.

Examination of the nucleotide sequence upstream of the GCAT gene failed to reveal a typical promoter, although a sequence with partial similarity to the CAP binding-site consensus sequence of E. coli (de Crombrugghe et al. 1984) was identified. This suggested that the production of GCAT by A. hydrophila could be under the control of a system analogous to the catabolite repression system of E. coli.

The sequence of the structural gene gcatA contained two regions that share similarity with other lipases (Figure 9). These regions are believed to be the active site and the interfacial lipid-binding site of the other lipases (Maraganore and Heinrikson 1986).

Polyclonal antibodies directed against GCAT from both A. hydrophila and A. salmonicida cross-reacted with both proteins in ELISA and in immunoblots (Figures 15 and 16). This suggests that although the molecular weights and amino

acid compositions of the two related proteins are quite different (see Table 5), some of the epitopes of GCAT are the same in these two proteins.

**Analysis of the GCAT Sequence**

The sequencing of the A. hydrophila GCAT gene, gcatA, revealed an open reading frame that codes for a mature protein of 31.3 kD. This molecular weight differs significantly from that of 35 kD determined for A. hydrophila GCAT by SDS-PAGE (Figure 16). It has been previously demonstrated that GCAT from A. salmonicida is quite resistant to denaturation by detergents and by organic solvents (MacIntyre, thesis 1981). A resistance to denaturation such as this is one possible explanation for the two different molecular weight determinations, as molecular weight determination from SDS-PAGE relies on the assumption that all proteins are denatured equally well by SDS thus leading to the assumption that all samples have an equal charge to mass ratio. But as some proteins are clearly not completely denatured by SDS (see below), this method of molecular weight determination is fallible. Certainly, the most probable explanation is that GCAT, like many membrane or lipophilic proteins (Nakamura and Mizushima 1976), contains a high degree of beta-conformation that is not completely removed by the standard denaturing conditions used for SDS-PAGE sample preparations. Examples of other bacterial proteins that resist detergent denaturation and

thus display aberrant electrophoretic mobility are the E. coli detergent-resistant phospholipase A (Tamori et al. 1979), and the porins (Nakamura and Mizushima 1976; Tokunaga et al. 1979).

The primary sequence of GCAT revealed an 18 amino acid signal sequence typical of most exported proteins. This signal sequence contains the small neutral amino acids valine and alanine at positions -3 and -1 respectively, and the large polar amino acid glutamine at position -2. Position +1, (cleavage by signal peptidase is between -1 and +1), is occupied by an alanine residue. According to von Heinje (1984) these amino acids are commonly found in these positions.

Similarities between GCAT from two species of Aeromonas

The amino terminus of the functional protein from A. hydrophila differed by only one amino acid from the A. salmonicida enzyme within the first 18 amino acids (Figure 7). Despite this apparent similarity, both the molecular weights (Table 5; Figure 16), and the amino acid compositions (Table 5) differed radically between the two enzymes. These differences indicate either sequence divergence downstream of the amino termini of the two proteins, or as suggested already, that GCAT from A. hydrophila normally has proteolytic post-translational processing but remains unprocessed in A. salmonicida. Howard and Buckley (1985) have demonstrated that one

A. hydrophila extracellular protein (aerolysin) is activated by post-translational removal of approximately 20 amino acids from the carboxy terminus. However, post-translational processing of GCAT could not be a step in activation, as the high molecular weight form observed in the culture supernatants of E. coli and A. salmonicida (bearing the GCAT plasmids) appears to be active. It is not beyond reason to assume that the enzyme from A. hydrophila contains some internal or carboxyl terminal domain that does not affect GCAT activity.

As the ELISA results demonstrate, the anti-A. salmonicida-GCAT antibodies bound very well to both GCAT species, while the anti-A. hydrophila-GCAT antibodies cross-reacted to a lesser degree with the A. salmonicida protein. These results may indicate that most immunogenic sites that exist within the A. salmonicida-GCAT are also present within the A. hydrophila-GCAT.

It is evident from the ELISA and immunoblot results that the immunodominant epitopes of both proteins are at least partially conserved. Two such regions could be those containing the active site and lipid-binding site. The only sequence data available for A. salmonicida GCAT shows that the first 18 amino acids of the amino terminus are the same as the first 18 amino acids of A. hydrophila GCAT in all but one residue. It is within this region that the lipid-binding domain is proposed to be.

## Sequence similarity with Other Lipases

GCAT from A. hydrophila contains two short regions that appear to share some degree of sequence similarity with several mammalian lipases. These regions and the proposed similar sites from the other lipases are depicted in Figure 9.

The region of similarity in Figure 9A represents the region that Verger (1984) suggests is the interfacial lipid-binding site. Verger proposes that when this site is bound to lipid, it is responsible for the dramatic activation observed by Wells (1974) when certain lipases are presented with aggregated substrates (substrates at concentrations above their critical micellar concentration).

These lipid binding domains of the various lipases are identified on the basis of similarity to the region surrounding Ser152 of Porcine Pancreatic Lipase, (PPL), as it has been demonstrated that when this amino acid is modified by diethyl-p-nitrophenyl phosphate, PPL becomes inactive toward substrate aggregates such as micelles. However, the enzyme does retain some activity towards monomolecular substrates such as soluble short chain lecithins or p-nitrophenyl esters (Reddy et al. 1986; Verger 1984), and this preference for soluble monomolecular substrates by the modified PPL is similar to the substrate specificity of the PPL zymogen reported by Pieterson et al. (1974). These workers found that the PPL zymogen was

capable of hydrolysing short chain lecithin at a rate equal to that of PPL when the substrate was present below its critical micellar concentration, but when the substrate concentration was increased to above the critical micellar concentration the zymogen activity did not display the characteristic interfacial activation of PPL. This suggests that the lipid-binding domain of PPL is in some way inactivated by the extra amino acids present on the zymogen resulting in the same net effect as the modification of Ser152 by diethyl-p-nitrophenyl phosphate. Verger (1980) proposes that this interfacially acting site is required to bring the exact geometries of the aggregated substrate and enzyme active site together to allow for the site specific bond cleavage.

The proposed active sites of PPL and LCAT (Maraganore and Heinrikson 1986), in comparison with a region of GCAT that shares some sequence similarity, are depicted in Figure 9B. From this figure it appears that the GCAT sequence displays more similarities with the PPL sequence than with the corresponding LCAT sequence.

It has previously been demonstrated that LCAT is sensitive to the serine poison diisopropylphosphofluoroidate (DFP) (Juahiainen and Dolphin 1986), suggesting that an active site serine plays an important role. Levels of DFP that completely inhibit LCAT activity, however, have no effect on either PPL (Juahiainen and Dolphin 1986), or GCAT

(Buckley, unpublished). Both GCAT and PPL have serine residues in the region thought to contain the active site, but it appears that in these active sites the serines are either not accessible to DFP, or are not in the correct chemical environment to react with DFP.

Farooqui et al. (1988) have isolated a cyanogen bromide fragment of DFP treated LCAT and identified the serine residue modified by DFP. Their results indicate that blocking of the serine at position 181 of LCAT results in destruction of all activity. In light of the fact that Ser181 of LCAT is located in the domain that appears to share similarity with the lipid binding domain of other lipases as reported by Maraganore and Heinrikson (1986), it seems that LCAT is either unable to degrade even soluble substrate when the lipid binding domain serine is modified, or that similarity alone is insufficient when determining domain function.

Serine residues within the active and lipid-binding sites are features common to other lipases capable of carrying out acyl transfer. Enzymes such as this are PPL, capable of catalysing the acylation of alcohols (Brockerhoff and Jensen 1974), and rat lung lysophospholipase (RLL), which transfers palmitate from 1-palmitoyl-lysophosphatidylcholine to a second molecule of lysophosphatidylcholine (Paul et al. 1979). In the case of RLL, Paul et al. found that the reaction proceeded via a

covalent acyl-enzyme intermediate in which the acyl group is bound to a serine residue.

Maraganore and Heinrikson (1986) propose that two classes of serine residues are involved in lipase activity. It appears that although some of the lipases are not inactivated by DFP the tri-peptide sequence of Gly-X-Ser and the penta-peptide sequence of Trp-X-Gly-Gly-Ser are usually conserved within many lipases. These authors also point out that in order to determine which serine functions as the nucleophile in the catalytic mechanism, it is necessary to first isolate peptide fragments of the enzymes that contain the nucleophilic serine either covalently attached to an acyl moiety, or covalently modified by a serine reactive species such as DFP or diethyl-p-nitrophenyl phosphate.

Thus it appears on first inspection that the active site of GCAT is quite similar to PPL both in sequence and in chemical characteristics, but the preference of GCAT for cholesterol as an acyl acceptor (Buckley 1982), leads one to believe that the active site of GCAT is more similar to that of LCAT on a functional basis. Further studies using serine-reactive reagents and/or site directed mutagenesis are necessary to determine if these proposed lipid-binding and active sites of GCAT participate in the GCAT reaction mechanism.

**The Expression of Cloned GCAT**

The results regarding the expression of GCAT from the different recombinant plasmids suggest that the sequence upstream of gcatA contains a promoter capable of directing the synthesis of GCAT constitutively at a low level in the culture conditions used. It is likely that the expression observed from the plasmid pJTP6 was due to this A. hydrophila-derived control region. Also, when the aerolysin functional gene was cloned downstream of this possible control region as in pJTPA7, a very low level of aerolysin was produced extracellularly by A. salmonicida strains bearing the fusion plasmid. The level of aerolysin produced was equal to approximately 4 HU $ml^{-1}$, or 0.015 mg $ml^{-1}$ at a culture density of ~2 x $10^9$ cells $ml^{-1}$. This is considerably lower than the level of 10.5-11 HU $ml^{-1}$ or approximately 3.8 mg $ml^{-1}$ when HB101-pKW200 is grown under the same conditions to the same culture density.

The plasmid pJT22, which only expressed GCAT under induction of the vector based tac promoter, (a hybrid of the promoters from the lac operon and the trp operon that is identical to the E. coli concensus sequence), did not contain sufficient upstream DNA to allow for the presence of the true GCAT promoter, gcatP. From this reasoning, and from work on the pBR322 based promoters (Stuber and Bujard 1981), it appears that the level of expression observed in pHEc2.2 is due to promotion of gcatA by the pBR322 based bla

promoters P1 and P2. Restriction enzyme mapping shows that gcatA is in the correct orientation for this promotion to occur.

Expression of GCAT from pHEc1 could be due to both the promoter of the pVK102 based tetracycline promoter as well as the endogenous gcatP.

The promotion of GCAT and aerolysin due to the promoter gcatP, as in pJTP6 and pJTPA7, appears to be much more efficient in Aeromonas spp. than in E. coli (see Figures 12c, 12f, 13c, 13f, and 14).

It has also been demonstrated that the promoter for the aerolysin gene, which bears a strong resemblance to the E. coli consensus sequence, does not function in E. coli but does function well in A. salmonicida (J.T. Buckley unpublished results).

The phenomenon of species specificity of promoters has been recently addressed by a few authors, and specificity appears to be common, although in most cases it occurs between promoters from Gram negative and Gram positive bacteria. For example: the promoter of the delta endotoxin gene of Bacillus thuringiensis is not recognized in E. coli (Haider et al. 1987); the lux genes from Photobacterium leiognathi are expressed only weakly in some strains of E. coli (Delong et al. 1987); a transcriptional start site from a Methylomonas clara plasmid that has two overlapping divergent promoters, is only expressed in one direction by

E. coli, while M. clara can initiate transcription in both directions. This last example is especially intriguing, as both promoters share a large degree of similarity with the established consensus sequences for promoters, but only one of the promoters is utilized by E. coli (Metzler et al. 1988); the cellulase genes of Cellulomonas fimi are only expressed in E. coli at one tenth of the level observed in C. fimi or in Brevibacterium lactofermentum (Paradis et al. 1987).

The promoters of Streptomyces spp. have been examined extensively (Bibb and Cohen 1982; Jaurin and Cohen 1985). From these studies it was demonstrated that very few Streptomyces promoters can function in E. coli, but, of the many E. coli promoters examined in Streptomyces, most were completely functional. This suggests that Streptomyces contains at least two distinct RNA polymerase holoenzymes that differ in their binding site recognition (Buttner and Brown 1987).

The fact that there is no obvious match between the probable GCAT promoter region and the E. coli consensus sequence (McClure 1985) suggests that the expression of this gene will be poor when cloned in E. coli. It has been demonstrated that the promoters from Streptomyces spp. that do not function in E. coli (Jaurin and Cohen 1985) all lack similarity with the E. coli consensus sequences. In fact, the only Streptomyces promoters that do function in E. coli

are those that share some degree of similarity with the consensus sequence (Buttner and Brown 1987).

Contrary to this evidence that suggests that consensus sequence resemblance is all that is necessary for promoters to function in E. coli, Metzler et al. (1988) have demonstrated that a promoter from M. clara, which bears a striking resemblance to the consensus sequence, has no detectable promotional activity when cloned upstream of a chloramphenicol acyltransferase (CAT) cartridge. Although in this case it has not been determined whether or not other control regions exist in this segment of M. clara DNA.

It is well known that the promoters of eukaryotic genes bear no resemblance to prokaryotic promoters (McClure 1985), and likely this is the reason that these genes are not expressed by their natural promoters in E. coli. Evidence such as that from Streptomyces and the results reported in this manuscript, suggest that promoter consensus sequences may vary considerably throughout all organisms, and that expression of the genes downstream of these unique consensus sequences may be regulated by either unique RNA polymerases or by unique $\sigma$-subunits, the subunits of RNA polymerase responsible for recognition and binding of the promoter site.

The possibility that gcatA could be under the control of an activator or repressor protein resulted in the identification of a possible catabolite activator protein

(CAP) binding site located approximately 150 nucleotides upstream of the GCAT start codon. According to the consensus sequence for CAP binding (de Crombrugghe et al. 1984), the site upstream of gcatA aligns exactly in 8 of the 13 bases that are usually conserved in CAP-binding sites. Of the 8 matches, 5 make up the TGTGA pentamer seen in most CAP-binding sites (Figure 8). de Crombrugghe (1984) has identified this sequence of 5'-TGTGA-3' in approximately 80% of the CAP-binding sites identified, with the terminal GA from this occurring in 100% of the CAP-binding sites. In many of the CAP sites a site that loosely resembles an inverted repeat of this pentamer is found just downstream, separated by a spacer of 6 nucleotides, although in some cases, such as galE and araBAD (de Crombrugghe et al. 1984), the inverted repeat only matches in 2 and 3 instances respectively. The pseudo-inverted repeat in the proposed CAP-binding site of gcatP matches in 2 positions. This CAP-binding site upstream of GCAT, according to the data compiled by de Crombrugghe et al. (1984), is far enough upstream of the start of the gcatA gene to be grouped with those CAP-binding sites whose physiological roles are unknown. CAP-binding sites whose roles are known have all been identified within 100 nucleotides of the start site for the functional gene being regulated. If GCAT is utilized by A. hydrophila for nutrient sequestering purposes, it is not unreasonable to assume that a catabolite repression system

similar to that of the CAP system of E. coli could control GCAT synthesis in Aeromonas.

## Extracellular Secretion of GCAT

Possibly the most intriguing results of this work are that A. salmonicida recognizes the A. hydrophila promoter better than E. coli, and in contrast to E. coli, A. salmonicida also secretes, and apparently correctly processes, A. hydrophila GCAT. It appears from the growth curves in Figures 12 and 13 (with IPTG induction of tacP), that not only does A. salmonicida export GCAT, but it is capable of actively secreting GCAT until culture supernatant levels are 10 to 20 times higher than the levels of endogenous GCAT which wild type A. hydrophila or A. salmonicida are capable of producing at similar cell densities (Figures 10 and 13). These levels of secretion have no significant effect on growth rate or cell viability. Upon induction of gcatA in E. coli, GCAT activity accumulates in the periplasm, causing reduced growth rates and a significant amount of cell lysis (Table 6; Figure 12). This suggests that E. coli does not contain the necessary machinery to carry out the translocation of GCAT across its outer membrane. This is in agreement with the results obtained for the following cloned proteins: aerolysin from A. hydrophila (Howard and Buckley 1986; Chakraborty et al. 1986); the alpha-amylase from A. hydrophila (Gobius and

Pemberton 1988); the DNase from V. cholerae (Focareta and Manning 1987), and many others as discussed in the introduction.

These results, along with the virtual absence of GCAT in the periplasm of A. salmonicida (Table 7), indicate that the A. hydrophila and A. salmonicida species used in this study contain very similar machinery for the extracellular export of GCAT.

Howard and Buckley (1983) isolated pleiotropic export mutants of A. hydrophila that accumulated extracellular proteins in the periplasm. Contrary to the mode of Pseudomonas exotoxin A secretion proposed by Lory et al. (1983), this indicates that protein translocation across the inner membrane can in some cases occur independently of translocation across the outer membrane. Aeromonas spp. may possess specialized secretory mechanisms in their outer membranes that recognize some structural or sequence related feature of the molecule being excreted (eg. GCAT), and effect its export. As virtually all members of the family Vibrionaceae are known to release GCAT, it would be interesting to see how similar the secretory systems are within this family. Also, as A. salmonicida appears capable of secreting A. hydrophila aerolysin (Figure 14; from J.T. Buckley, unpublished results), it would be intriguing to examine if these two proteins, GCAT and aerolysin, are secreted by independent mechanisms that are shared by both

species, or if these two proteins are exported by a common mechanism that relies on some general feature of protein structure rather than on a specific sequence within the exported protein, as in the export of the E. coli haemolysin (Springer and Goebel 1980; Mackman et al. 1985; Micaud et al. 1986).

The availability of the genes for these two extracellular A. hydrophila proteins on wide host range cloning vectors makes it possible to create the necessary transconjugants in various members of the Vibrionaceae to facilitate such a study.

When the cosmid pHEc1 was transferred into A. hydrophila to create pG1, a 10 fold increase in extracellular GCAT levels was observed. Surprisingly, along with the increase of GCAT, a 10 fold increase in the level of aerolysin production was also detected (Figure 10). Southern blot analysis has proven the absence of the aerolysin gene on pHEc1, thus the increase of aerolysin production is not due to an increase in the copy number of the aerolysin gene within the bacteria. It appears that some factor of pHEc1 origin is intimately involved in the control of aerolysin production.

Few systems for the study of protein export in bacteria other than E. coli exist. Due to E. coli's inherent inability to export proteins to the exterior, it seems surprising that few other systems, for the cloning and

expression of secretory proteins in different genera of Gram negative bacteria, have been developed. Some of the work presented here represents a model of this type of system.

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