A Genetic and Functional Analysis of Type IV Pili Produced by *Aeromonas* Bacteria

by

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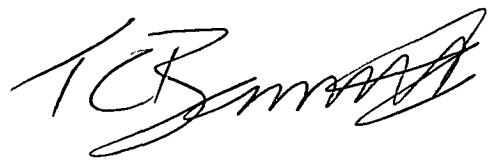

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Much of the work described in this thesis has been published, is in an advanced state of preparation, or has been presented at conferences.

Refereed Journals

1. **Barnett, T. C.**, Kirov, S. M., Strom, M. S. and Sanderson, K. 1997. *Aeromonas* spp. possess at least two distinct type IV pilus families. Microb. Pathog. 23: 241-47.
2. **Barnett, T. C.** and Kirov, S. M. 1998. The type IV *Aeromonas* pilus (Tap) gene cluster is widely conserved in *Aeromonas* species. Microb. Pathog., 26: 77-84
3. Kirov, S. M., **Barnett, T. C.**, Pepe, C. M., Strom, M. S. and Albert, M. J. 1999. Investigation of the role of the type IV *Aeromonas* pilus (Tap) in the pathogenesis of *Aeromonas* gastrointestinal infection. Infection and Immunity, in press.

Conference Abstracts

1. Sanderson, K., **Barnett T. C.** and Kirov S. M. 1996. Type IV pilus adhesins and *Aeromonas* pathogenesis. Microbiol. Aust. 17(4): A69.
2. **Barnett, T. C.**, Sanderson, K. and Kirov, S. M. 1996. Genetic analysis of an *Aeromonas* type IV pilin gene. Microbiol. Aust. 17(4): A83.
3. Kirov, S. M., **Barnett, T. C.** and Strom, M. S. 1997. Pili of diarrhoea-associated *Aeromonas* species. Fourth Australian Conference on Molecular Analysis of Bacterial Pathogens, Kiama, NSW.
4. **Barnett, T. C.** and Kirov, S. M. 1998. Distribution of the type IV *Aeromonas* pilus gene cluster in *Aeromonas*. Microbiol. Aust. 19(4): A102.
5. **Barnett, T. C.** and Kirov, S. M. 1999. Expression and function of the cloned type IV *Aeromonas* pilus (Tap). Sixth International *Aeromonas/Plesiomonas* Symposium, Chicago, USA.
6. **Barnett, T. C.** and Kirov, S. M. 1999. Investigations into the role of the type IV *Aeromonas* pilus (Tap) in the pathogenesis of *Aeromonas* infection. Fifth Australian Conference on Molecular Analysis of Bacterial Pathogens, Victor Harbor, SA.

Abstract

Bacteria belonging to the genus *Aeromonas* are ubiquitous water-borne organisms that are also present in many foods. Some strains are human gastrointestinal pathogens. However, the disease-causing mechanisms of these bacteria are not well understood. This is particularly true for intestinal colonisation, which is a critical step in the disease process. When this thesis commenced, there was evidence that filamentous surface structures (type IV pili) purified from diarrhoea-associated species had an important role in colonisation. These pili were designated "bundle-forming pili" (Bfp) because of their tendency to form bundles linking bacteria.

The initial aim of this thesis was to clone the genes encoding Bfp pili from a diarrhoeal isolate of *Aeromonas veronii* biovar sobria (strain BC88). Although traditional approaches (transposon mutagenesis, screening of libraries with degenerate oligonucleotide probes, DNA probes, and a Bfp antiserum) were unsuccessful at achieving this, a small region of a gene encoding the Bfp pilin protein was ultimately cloned using a degenerate PCR approach. Moreover, a second type IV pilus gene cluster was also cloned from this strain. Characterisation of this newly discovered pilus family (designated Tap, for "type IV *Aeromonas* pilus") was then undertaken.

The *tap* gene cluster from *A. veronii* biovar sobria BC88 was shown to comprise four genes that were all transcribed in the same direction. These genes exhibited high sequence homology to genes encoded by similar gene clusters, identified by other investigators, from strains of *A. hydrophila*, *Vibrio cholerae* and *V. vulnificus*.

To assess the significance of Tap pili for *Aeromonas* virulence, the distribution of the *tap* genes was determined in *Aeromonas* reference strains (including both pathogenic and non-pathogenic species), and in a range of clinical and environmental isolates of those species most commonly associated with human gastrointestinal disease. The *tap* cluster was present in all *Aeromonas* strains tested. A defined mutation in *tapA* from *A. veronii* biovar sobria BC88 was then constructed. Inactivation of this gene did not alter the ability of this strain to adhere to epithelial or intestinal cells *in vitro*, or to colonise the intestinal tract of infant mice (colonisation and competition experiments).

Furthermore, the *tapA* mutant strain was not attenuated in its ability to cause disease in rabbits (removable intestinal tie adult rabbit diarrhoeal model; performed by Dr. M. J. Albert, International Center for Diarrheal Disease Research, Dhaka, Bangladesh).

To investigate the expression and assembly of Tap pili, an antiserum was constructed against a recombinant protein produced by overexpression of *tapA* in *Escherichia coli*. Western blot analysis showed that TapA was expressed. However, Tap pili on the cell surface were not seen by immune electron microscopy. (Previous studies in our laboratory have demonstrated that Bfp pili are the predominant structures expressed on the surface of *Aeromonas* bacteria during growth *in vitro*.)

Hence, this thesis identified a second family of type IV pili in *Aeromonas* species. The function and expression results obtained to date suggest that Tap pili are not as significant as Bfp pili for colonisation of the intestinal tract. However, the widespread distribution of the *tap* genes in *Aeromonas* species, as well as in other bacterial pathogens, suggests that they are important for some aspect of the biology of these organisms.

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Abbreviations

Ω	omega interposon
σ54	sigma 54
°C	degrees celcius
Amp	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Bfp	bundle-forming pilus (*Aeromonas*)
BFP	bundle-forming pilus (enteropathogenic *E. coli*)
BHIB	brain heart infusion broth
BLAST	basic local alignment search tool
bp	base pairs
cAMP	cyclic adenosine triphosphate
cGMP	cyclic guanidine triphosphate
CHO cells	Chinese hamster ovary cells
Cml	chloramphenicol
CO_2	carbon dioxide
CSPD	disodium 3-(4-methoxyspiro{1.2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
CT	cholera toxin
CTAB	hexadecyltrimethylammonium bromide
CTXϕ	cholera toxin phage
d	days
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanidine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
FCS	foetal calf serum
GCAT	glycerophospholipid::cholesterol acyltransferase
h	hours
HG	hybridisation group
HUS	haemolytic uraemic syndrome
IPTG	isopropylthiogalactopyranoside
Kan	kanamycin
kb	kilobases
kDa	kilodaltons
l	litres
LB	Luria-Bertani medium
LD_{50}	50% lethal dose
LF pili	long-flexible pili

LPS	lipopolysaccharides
M	molar
MEM	minimal essential medium
min	minutes
MMM	minimal maintenance medium
MSHA	mannose-sensitive haemagglutinin
MW	molecular weight
OMP	outer membrane protein
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PMSF	phenylmethylsulphonyl fluoride
RITARD	removable intestinal tie adult rabbit diarrhoeal model
rRNA	ribosomal ribonucleic acid
r-TapA	recombinant TapA
s	seconds
SC	subcutaneous
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Spec	spectinomycin
Stx	Shiga-like toxin
Tap	type IV *Aeromonas* pilus
TB	Terrific broth
TBS	tris buffered saline
TCP	toxin coregulated pilus
Tet	tetracycline
TSAY	tryptone soya agar with 0.6% yeast extract
TSBY	tryptone soya broth with 0.6% yeast extract
TTBS	tris buffered saline with 0.3% tween-20
UK	United Kingdom
USA	United States of America

Chapter 1

Literature review

"How the use of molecular techniques has resulted in an increased understanding of the taxonomy and virulence mechanisms of *Aeromonas* bacteria"

Chapter 2

Materials and methods

Chapter 3

Identification of two distinct families of type IV pili in *Aeromonas* species associated with gastroenteritis

Chapter 4

Distribution of the *tap* gene cluster in the genus *Aeromonas*

Chapter 5

The role of Tap pili in the adhesion and colonisation of intestinal tissue by *Aeromonas veronii* biovar sobria

Chapter 6

Concluding discussion

Chapter 1

Literature review

"How the use of molecular techniques has resulted in an increased understanding of the taxonomy and virulence mechanisms of *Aeromonas* bacteria"

1.1. Introduction

Aeromonas bacteria are a major component of the aerobic bacterial population in surface water supplies worldwide. It is widely recognised that some species (psychrophilic and mesophilic) are major pathogens for salmonid fish. In addition, some mesophilic species appear to be able to cause disease in humans. Such diseases include severe extraintestinal infections, primarily affecting immuno-compromised individuals, in addition to gastrointestinal illnesses, which primarily affect children less than 5 years of age, the elderly and the immuno-compromised. There also appears to be a species-associated disease spectrum (Table 1.1). Of the 17 species currently recognised, only eight are associated with human disease.

Over recent years, there have been major advances in the understanding of the taxonomy and disease mechanisms of this genus, although to date, there is no way to recognise definitively whether a particular isolate is pathogenic. Identification to species level is probably not sufficient, since it is likely that only a subset of strains within a disease-associated species are pathogenic. Therefore, current approaches to solve this problem rely on identifying particular bacterial components involved in the disease process. To date, numerous toxins and colonisation factors have been identified, but none of these are possessed more frequently by strains of clinical origin than by strains of environmental origin. Hence, they have not proven very useful as markers of pathogenic strains.

Colonisation of the intestine by diarrhoeal pathogens is an essential part of the disease process and is usually mediated, at least in part, by filamentous structures termed "pili'. Type IV pili are essential colonisation factors for many bacterial pathogens, whether they infect intestinal or extra-intestinal sites. At the commencement of this thesis, type IV pili had been purified from several strains of *Aeromonas* species (Hokama *et al.*, 1990; Hokama and Iwanaga, 1992; Iwanaga and Hokama, 1992; Kirov and Sanderson, 1996; Nakasone *et al.*, 1996). Although there was substantial indirect evidence that type IV pili are major colonisation factors for *Aeromonas*, the genes encoding them had not been cloned and mutation analysis of

Table 1.1. Association of *Aeromonas* species with human disease[a]

Species[b]	Disease
Common clinical association[c]	
A. hydrophila HG1	gastroenteritis haemolytic uraemic syndrome[d] septicaemia meningitis peritonitis wound infections pneumonia
A. caviae HG4	gastroenteritis septicaemia wound infections
A. veronii biovar sovria HG8/10	gastroenteritis septicaemia wound infections meningitis
Less frequent clinical association [b]	
A. hydrophila HG3	gastroenteritis septicaemia wound infections
A. veronii biovar veronii HG8/10	gastroenteritis septicaemia
A. jandaei HG9	septicaemia cellulitis
A. schubertii HG12	septicaemia cellulitis
A. trota HG14	gastroenteritis

[a] Modified from Janda and Abbott (1998).

[b] HG, hybridisation group (see section 1.2.2).

[c] Based on frequency of isolation from clinical materials.

[d] *A. hydrophila* has been documented as a rare cause of haemolytic uraemic syndrome (see section 1.4.6).

function had not been performed. Furthermore, morphological studies indicated that there were several different types of pili on *Aeromonas* species.

Therefore, the aims of this thesis were to characterise the type IV pili produced by *Aeromonas* bacteria at both the molecular and functional level. The literature review to follow will provide essential background information on the taxonomy and virulence determinants of *Aeromonas* species, with particular emphasis on how molecular studies are contributing to knowledge that will help identify pathogenic strains. The review is divided into three major sections, which discuss the taxonomy, toxins and adhesins possessed by members of this genus.

1.1.1. Background

Aeromonas bacteria are Gram-negative organisms that are ubiquitous in the aquatic environment and, as such, are a common contaminant of foods and water supplies. Members of this genus have been shown to cause a variety of diseases acquired by ingestion of contaminated food or water, or by infection following some form of physical trauma (Table 1.1). There is also a substantial body of evidence suggesting that at least some strains are significant enteropathogens. However, the association of *Aeromonas* species with gastrointestinal disease is somewhat more controversial since Koch's postulates have not been fulfilled, and the supporting evidence is primarily epidemiological.

Part of this controversy also lies in the fact that no outbreaks caused by a single strain of *Aeromonas* have been documented. Although several smaller food-, and water-borne outbreaks have been reported, these have often failed to perform essential epidemiological investigations, such as the identification of identical strains from the source of infection and from the individual patients stools (Krovacek *et al.*, 1995). In studies where this type of analysis has been performed, the strains isolated from the proposed source of infection and the patient specimens are often different (de la Morena *et al.*, 1993; Davin-Regli *et al.*, 1998; Talon *et al.*, 1998).

Despite such controversies, there is still significant epidemiological data implicating some species of *Aeromonas* as diarrhoeal pathogens. Over the years there

have been several large-scale case-control studies on the isolation of *Aeromonas* strains from diarrhoeal and non-diarrhoeal stools. These studies have either supported or contradicted the idea that *Aeromonas* bacteria are enteropathogens. A majority of the large scale investigations conducted have shown a statistically higher isolation rate of *Aeromonas* bacteria from diarrhoeal stools when compared to asymptomatic stools (Gracey *et al.*, 1982a; Burke *et al.*, 1983; Agger, 1986). However, other studies have not found this trend (Figura *et al.*, 1986; Kuijper *et al.*, 1987; Geiss *et al.*, 1988).

More intensive epidemiological investigations of *Aeromonas* bacteria in gastrointestinal disease have also been performed. These studies have involved the isolation of *Aeromonas* in pure culture as the sole potential pathogen from infected stools, O-antigen or cytotoxin reactivity in patient acute and convalescent sera, and the observation that resolution of disease is accompanied by a disappearance of *Aeromonas* from stool specimens (recently reviewed in Janda and Abbott, 1998). Taken together, these observations support the idea that some strains of *Aeromonas* bacteria are enteropathogenic. Of the 17 species currently recognised, only three predominate in clinical specimens (Table 1.1).

The clinical presentation of *Aeromonas* gastrointestinal disease is diverse. This observation probably reflects the immune status of the host as well as the different pathogenic mechanisms of the organism involved. Most cases present as a mild, self-limiting, watery diarrhoea resembling disease caused by enterotoxigenic *Escherichia coli*. Less frequently, they are associated with chronic dysentery and severe invasive colitis (Gracey *et al.*, 1982a; Gracey *et al.*, 1982b). In rare instances, diseases resembling cholera (Champsaur *et al.*, 1982), and haemolytic uraemic syndrome are seen (Bogdanovic *et al.*, 1991; Robson *et al.*, 1992). These rarer diseases may reflect the production of a cholera toxin-like enterotoxin (see section 3.1.5.) and possibly Shiga-like toxins (see section 3.1.6.), respectively, by the infecting strain.

Current laboratory-based methods used to identify pathogenic bacteria from clinical and environmental sources involve taxonomic classification (usually biochemical), which may be followed by the identification of a specific virulence-

associated property, such as serotyping O antigens, or detection of specific toxins. As described above, identification of *Aeromonas* isolates to species level is not sufficient, and since the virulence mechanisms of these bacteria remain undefined, further investigation of pathogenic strains is not possible. Therefore, current research is focusing on the search for particular combinations of virulence-associated properties unique to pathogenic strains.

Despite limitations, taxonomic identification will still form an essential part of the identification of pathogenic *Aeromonas* bacteria in the clinical laboratory, and in laboratory-based investigations into *Aeromonas* virulence factors. In the following section, the taxonomy of the *Aeromonas* genus will be discussed.

1.2. Taxonomy

The taxonomy of the genus *Aeromonas* is complex, although there have been significant advances over recent years, particularly in identification schemes for clinical strains. New, simplified biotyping schemes are being developed in an attempt to simplify classification of an isolate to a particular species. Currently, the only way to assign an isolate definitively is through complex molecular procedures. Consequently, a major problem with much research into this genus over the last 15 years has been the over-simplification of taxonomy and the use of outdated classifications in published works. Given that there appears to be a species-associated disease spectrum for *Aeromonas* species (Table 1.1), and that many species have unique antibiotic resistance patterns, it is vital to speciate isolates correctly. This is particularly true for studies of pathogenesis since it may be that different species possess different mechanisms of disease production.

The last edition of Bergey's Manual of Systematic Bacteriology (published in 1984) classified the genus *Aeromonas* within the family Vibrionaceae (Popoff, 1984). However, in the newest version of this manual, the genus *Aeromonas* has been assigned to a new family (the Aeromonadaceae), based on 16S and 5S rRNA sequence analysis as described by Colwell and co-workers (Colwell *et al.*, 1986; Amy Carnahan, personal communication). Bergey's manual describes members of

this genus as Gram-negative, rod-shaped to coccoid cells. They are facultative anaerobes that are both oxidase- and catalase-positive, and able to reduce nitrate to nitrite. The mesophilic species are motile by means of a polar flagellum whereas the single psychrophilic species (*A. salmonicida*) is non-motile under standard *in vitro* culture conditions (Popoff, 1984). Some strains also produce peritrichous (lateral) flagella as described in section 1.10.1.

The first major study into the taxonomy of *Aeromonas* was in 1976, when Popoff and Véron applied numerical taxonomy to classify 68 mesophilic strains on the basis of 50 variable biochemical, physical and metabolic characters (Popoff and Véron, 1976). This study divided these strains into two groups, which the authors proposed as two separate species, *A. hydrophila* (with biovars X1 and X2) and *A. sobria* (biovar Y).

1.2.2. Molecular studies

Popoff and colleagues followed up their initial study using DNA:DNA hybridisation (Popoff *et al.*, 1981). They then designated three species; *A. hydrophila* (biovar X1 from the numerical taxonomy study), *A. caviae* (biovar X2) and *A. sobria* (biovar Y). These authors also noted, however, that each of these species could be divided into two or three distinct DNA hybridisation groups (HGs; termed genospecies, or genomospecies), which could not at that time be separated phenotypically (such phenotypic groups are termed phenospecies). Therefore, the *A. hydrophila* phenospecies contained HGs 1 to 3, phenospecies *A. caviae* contained HGs 4 to 6, and phenospecies *A. sobria* contained HGs 7 and 8.

Expanding phenotypic typing since this time has led to the naming of the majority of these HGs (Allen *et al.*, 1983; Popoff, 1984; Fanning *et al.*, 1985; Hickman-Brenner *et al.*, 1987; Hickman-Brenner *et al.*, 1988; Schubert and Hegazi, 1988; Carnahan *et al.*, 1991a; Carnahan *et al.*, 1991c; Carnahan and Joseph, 1991; Martinez-Murcia *et al.*, 1992; Esteve *et al.*, 1995; Huys *et al.*, 1997). Currently there are 17 recognised genospecies as indicated in Table 1.2 (A. Carnahan, personal communication).

Table 1.2. Currently recognised *Aeromonas* genospecies[a]

HG	Genospecies	Phenospecies	Reference
1	*A. hydrophila*	*A. hydrophila*	Popoff, 1984
2	*A. bestiarum*	*A. hydrophila*-like	Popoff, 1984
3	*A. salmonicida*	*A. salmonicida* subspecies salmonicida masoucida achromogenes	Popoff, 1984
3	*A. salmonicida*	*A. smithia*	-
3	Unnamed	*A. hydrophila*	
4	*A. caviae*	*A. caviae*	Popoff, 1984
5A	*A. media*	*A. caviae*-like	
5B	*A. media*	*A. media*	Allen *et al.*, 1983
6	*A. eucrenophila*	*A. eucrenophila*	Schubert & Hegazi, 1988
7	*A. sobria*	*A. sobria*	Popoff, 1984
8	*A. veronii*	*A. veronii* biovar sobria	Hickman-Brenner *et al.*, 1987
9	*A. jandaei*	*A. jandaei*	Carnahan & Joseph, 1991; Carnahan *et al.*, 1991a
10	*A. veronii*	*A. veronii* biovar veronii	Fanning *et al.*, 1985
11	Unnamed	*Aeromonas* spp. (ornithine positive)	Fanning *et al.*, 1985
12	*A. schubertii*	*A. schubertii*	Hickman-Brenner *et al.*, 1988
13	*Aeromonas* Group 501	*A. schubertii*-like	Hickman-Brenner *et al.*, 1988
14	*A. trota*	*A. trota*	Carnahan *et al.*, 1991b
15	*A. allosaccharophila*	*A. allosaccharophila*	Martinez-Murcia *et al.*, 1992
16	*A. encheleia*	*A. encheleia*	Esteve *et al.*, 1995
17	*A. popoffii*	*A. popoffii*	Huys *et al.*, 1997

[a] Taken from A. Carnahan (personal communication).

1.2.3. Taxonomy of strains associated with gastrointestinal disease

Genetic studies conducted on large numbers of faecal isolates have shown that *A. hydrophila* HG1, *A. caviae* HG4 and *A. veronii* biovar sobria HG8 predominate in *Aeromonas*-associated gastrointestinal disease (Kuijper *et al.*, 1989; Altwegg *et al.*, 1990; Janda, 1991; Janda and Kokka, 1991). Other species which have been associated with human gastroenteritis are *A. hydrophila* HG3, *A. jandaei* HG9, *A. veronii* biovar veronii HG10, and *A. trota* HG14 (Kirov, 1997).

All *Aeromonas* strains used in this thesis have been biotyped using the schemes of Kirov *et al.* (1986), based on a small biochemical screen described by Popoff and Véron (1976), or the Aerokey II dichotomous key described by Carnahan *et al.* (1991b). Some strains used have also been characterised genetically to genospecies level. All strains used in this thesis, their source, and their method of taxonomic classification are listed in Appendix 1.

1.3. Overview of the pathogenic mechanisms of *Aeromonas* species

Based upon studies conducted to date, the pathogenesis of gastrointestinal disease caused by *Aeromonas* bacteria appears to be multifactorial, involving a variety of adhesive and toxic mechanisms which may, and probably do, vary between the different species and individual strains. Potential virulence factors identified to date suggest that the majority of pathogenic strains (particularly *A. hydrophila* and *A. veronii* biovar sobria) cause disease by an adhesive-toxic mechanism resembling the pathogenesis of enterotoxigenic *Escherichia coli* and *Vibrio cholerae*. However, there are likely to be differences in the mechanisms used by these two species as they appear to possess different adhesive mechanisms and, indeed, colonise different sites of the intestine as determined in a mouse model of colonisation (Sanderson *et al.*, 1996). *A. veronii* biovar sobria is more commonly associated with dysenteric illness and, therefore, may also be invasive, although there have been very few studies investigating *Aeromonas* invasion. *A. caviae* strains may act by an alternative mechanism resembling enteropathogenic or enteroinvasive *E. coli*, since the majority

of strains either do not produce exotoxins under the same conditions as *A. veronii* biovar sobria and *A. hydrophila*, or they are unable to produce them (Singh and Sanyal, 1992b; Wang *et al.*, 1996; Heuzenroeder *et al.*, 1999) *A. caviae* strains have been shown to adhere to, and invade HEp-2 cells (Shaw *et al.*, 1997).

The underlying problem with the investigation of *Aeromonas* virulence factors involved in the production of gastrointestinal disease is the lack of a suitable animal model that reliably reproduces diarrhoeal symptoms. Throughout the remaining review several different models of virulence factor characterisation will be described. They include models of the fish disease furunculosis, LD_{50} assays, and models measuring specific virulence mechanisms such as haemolysis of erythrocytes and adhesion to cell lines. However, it should be noted that while these methods may be suitable for defining putative functions, their relevance to human disease is more difficult to establish. Therefore, some care should be taken when interpreting the results from many of these studies with respect to the pathogenesis of gastrointestinal disease.

The next section will review *Aeromonas* virulence determinants studied to date with a particular emphasis on how their molecular analysis has contributed to current insights in their association with disease.

1.4. Toxins

The ability of pathogenic bacteria to exert effects characteristic of their particular disease relies on the interplay of one or more virulence factors that damage host tissue via a toxic, invasive, or enteropathogenic mechanism. Although *Aeromonas* species have been reported to be invasive, no particular invasin molecules have been described which promote this process. Similarly, no cell-contact dependent enteropathogenic mechanism has been reported. For this reason, toxic mechanisms are the major focus of this review.

1.4.1. Historical aspects

Toxins are by far the most widely studied virulence factors of *Aeromonas* species. Many terms have been used to describe these toxins based on their functional activity (e.g. cytotoxic, cytotonic, enterotoxic and haemolytic activities), and properties (e.g. heat-labile or heat-stable). The early studies on *Aeromonas* toxins were performed with cell-free culture supernatants containing "soups" of exported proteins, which were subsequently characterised by their activity on a variety of cell lines and in several animal models (Thelestam and Ljungh, 1981; Stelma *et al.*, 1988; Singh and Sanyal, 1992a). However, these approaches had major pitfalls regarding the interpretation of results. For example, the presence of relatively strong toxins would mask the presence of other toxins with more subtle effects. Likewise, many strains are known to produce a variety of extracellular proteases, which have been shown to be involved in the activation of some toxins. Consequently, the so-called heat-lability of a particular toxin may simply be a reflection of the stability of the protease required for activation.

The use of molecular techniques have helped to resolve much of the confusion surrounding *Aeromonas* toxins, so much so that aerolysin, the major haemolytic toxin secreted by *Aeromonas* species, is one of the best characterised bacterial haemolytic toxins.

1.4.2. Aerolysin

Table 1.3 summarises toxic proteins that have been identified to be secreted by *Aeromonas* bacteria. The genetic determinant encoding a haemolytic toxin from *Aeromonas* was first cloned from an isolate of *A. hydrophila* by Howard and colleagues (Howard and Buckley, 1986; Howard *et al.*, 1987). Genes encoding similar toxins have since been cloned from numerous other strains, each of which has been designated one of several different names (e.g. aerolysin, cytolysin, Asaotoxin, β-haemolysin, cytolytic- and cytotoxic enterotoxin). By comparing the primary amino acid sequences and molecular weights of these toxins, they can broadly be

Table 1.3. Toxins and proteases identified in *Aeromonas* species

Toxin	MW (kDa)	Gene cloned	Homologues
Haemolytic toxins			
Aerolysin	53.8-54.7	Yes	*P. aeruginosa* haemolysin *S. aureus* α-toxin *C. perfringens* ε-toxin *C. perfringens* perfringolysin 0
Haemolysin	~64	Yes	*V. cholerae* HlyA
GCAT	37.4	Yes	*V. cholerae* lecithinase *V. parahaemolyticus* lecithinase *V. mimicus* lecithinase
Enterotoxins			
Cholera toxin-like enterotoxin	?	No	*V. cholerae* cholera toxin
Alt	44	Yes	*A. hydrophila* phospholipase A1
Cytotonic enterotoxin	70	No	*A. hydrophila* lipase
Other toxins			
Shiga-like toxin 1	unknown	No	Enterohaemorrhagic *E. coli* Stx1
Proteases			
Zinc protease	19	No	*P. aeruginosa* LasA
Metalloprotease	37-38	Yes	*A. oryzae* neutral protease *P. atrinum* penicillolysin
Metalloprotease	34	No	*V. cholerae* haemagglutinin / protease
Serine protease	64-68	Yes	*H. vulgaris* protease *A. aegypti* protease
Serine protease	22	No	unknown

divided into two types, those homologous to the original toxin cloned by Howard and colleagues (1987) and those homologous to haemolysin (HlyA) from *Vibrio cholerae* (Table 1.3.). Wong *et al.* (1998) have proposed that the terms aerolysin (AerA) and haemolysin (HlyA) be used to describe these proteins, respectively. This nomenclature will be used throughout this thesis.

1.4.2.1. Sequence analysis

Alignment of the predicted amino acid sequences of the aerolysin gene products suggests that there are at least two different variants of this toxin. The toxins designated AHH3 and AHH5 (Hirono *et al.*, 1992), and Act (Chopra *et al.*, 1993) exhibit a high degree of identity with the aerolysin cloned by Howard and colleagues (1987). In contrast, those designated ASA1 (Hirono *et al.*, 1992), AerA (Husslein *et al.*, 1988), and ASH3 (Hirono and Aoki, 1993) exhibit considerable sequence divergence from the family of toxins described above in their N- and C-terminal regions, but show a high degree of identity with each other. All of the aerolysin-like gene products described to date, however, appear to have a molecular weight ranging from 53.8 to 54.7 kDa. Preproaerolysin is encoded by *aerA*, which is clustered with two other genes, *aerB* and *aerC* (Chakraborty *et al.*, 1986). These genes are involved in the modulation of aerolysin activity and in the expression of *aerA*, respectively.

Chopra *et al.* (1993) cloned a cytotoxic enterotoxin gene from a diarrhoeal isolate of *A. hydrophila*, which they designated "Act" to differentiate it from aerolysin. The reason for this differentiation was the fact that substitution of two amino acid residues shown to be crucial for the haemolytic activity of aerolysin did not affect the biological activity of Act. Although still the subject of some debate (Buckley and Howard, 1999), Act is probably analogous with aerolysin since both toxins have high sequence homology (93% identity), and similar molecular weights, pore sizes, and mechanisms of action and activation.

Although the primary amino acid sequence of aerolysin appears to be fairly unique, a short (31 amino acid) region does exhibit homology with *Pseudomonas*

aeruginosa haemolysin, *Staphylococcus aureus* α-toxin, and *Clostridium perfringens* ε-toxin and perfringolysin O (Parker *et al.*, 1996).

1.4.2.2. Mechanism of action

Preproaerolysin is secreted from the cell, and is activated to form aerolysin following the removal of a small C-terminal peptide (~ 40 amino acids), by one of a number of proteases (e.g. trypsin, chymotrypsin, and metalloproteases), which may be either bacterial or host in origin (Abrami *et al.*, 1998a; van der Goot *et al.*, 1992). Binding of the aerolysin dimer to the host receptor increases its local concentration and initiates a process referred to as oligomerisation (Parker *et al.*, 1996). This process involves the dissociation of the soluble toxin into monomers and the formation of an extremely stable heptamer. Subsequently, the hydrophobic region near the C-terminus is thought to promote insertion into the target cell membrane (Parker *et al.*, 1996).

Recently, it has been suggested that aerolysin does not cause cell death simply by allowing the leakage of intracellular components. Abrami *et al.* (1998b), have proposed a more elaborate mechanism involving selective permeabilisation of the target cell to small ions, such as potassium, which causes depolarisation of the plasma membrane. This, in turn, results in dramatic vacuolation of the endoplasmic reticulum, and selective alteration of the early biosynthetic membrane pathway by inhibiting transport of newly synthesised membrane proteins to the plasma membrane.

1.4.2.3. Virulence studies

Early experiments with purified aerolysin and culture filtrates from β-haemolytic *A. hydrophila* isolates provided the first evidence that aerolysin exhibited haemolytic, cytotoxic and enterotoxic activities, and that it may be an important virulence determinant for *Aeromonas* species. Asao *et al.* (1984) purified a haemolytic protein with a molecular weight (MW) of approximately 50 kDa, consistent with the MW of aerolysin. This haemolysin lysed rabbit erythrocytes,

elicited cytotoxic activity to Vero cells, and caused fluid accumulation in infant mouse intestines and in ligated rabbit ileal loops. A subsequent study demonstrated that culture filtrates from 19 of 21 (90%) β-haemolytic isolates of *A. hydrophila* were enterotoxic in rabbit ileal loops, whereas those from non-haemolytic strains did not cause fluid accumulation (Stelma *et al.*, 1986). Moreover, antiserum raised against the toxin purified by Asao *et al.* (1984), abolished this activity from four isolates. Taken together, these studies provided the initial indirect evidence that aerolysin played an important role in *Aeromonas* pathogenesis. However, proof was still lacking that aerolysin is important, or indeed whether it was produced, during the infection process.

Such proof was provided when isogenic mutants in the aerolysin genes were constructed and analysed in animal systems. Chakraborty *et al.* (1987), constructed a deletion-insertion mutation in the *aerA* and *aerB* genes from a strain of *A. hydrophila*. In a mouse toxicity model (intraperitoneal injection), this mutant had a LD_{50} $>8 \times 10^8$ compared with LD_{50} values of approximately 7×10^7 and 5×10^7 for the wild type and genetically reconstituted strains, respectively. Furthermore, the wild type strain was recovered from the blood, liver and spleen of infected animals whereas the mutant was never recovered from these sites. In addition, specific neutralising antibodies against aerolysin were detected in mice surviving this intraperitoneal challenge, demonstrating that aerolysin was produced during systemic infection with *A. hydrophila*.

A more recent study by Wong *et al.* (1998), investigated the role of aerolysin and haemolysin (see section 1.4.3) in an infant mouse assay. Inactivation of *aerA* caused a nine-fold, although not statistically significant, increase in LD_{50} following oral challenge. A statistically significant 20-fold increase in LD_{50} was observed only when both *aerA* and *hlyA* were inactivated. It was only in the double mutant that haemolytic and cytotoxic activities were abolished, demonstrating that, at least in this strain of *A. hydrophila*, more than one haemolytic protein may be involved in virulence.

1.4.3. Haemolysin

Genes encoding β-haemolytic toxins distinct from aerolysin have been cloned from several strains of *Aeromonas*. These toxins, termed haemolysin (HlyA), are larger (~ 64 kDa) than aerolysin (~ 54 kDa) and have only 17.8 to 19.6% amino acid sequence identity with the aerolysin cloned by Howard *et al.* (1987). They do, however, exhibit homology (41% identity and 54% similarity for AHH1; Hirono and Aoki, 1991) with HlyA from *Vibrio cholerae* El Tor (Alm *et al.*, 1988).

To date, there has been limited work aimed at elucidating the expression, mechanism of action, or role in virulence of HlyA from *Aeromonas* ($HlyA^{Aero}$). However, a hydrophobic N-terminal region has been proposed as a signal sequence, and $HlyA^{Aero}$ could be activated by trypsinisation (Hirono and Aoki, 1991). These observations are consistent with the mechanisms known for the secretion and activation of both HlyA from *Vibrio cholerae* ($HlyA^{Vc}$) and aerolysin (Alm *et al.*, 1988; van der Goot *et al.*, 1992). $HlyA^{Vc}$ has been shown to form pores in lipid bilayers with a pore size of ~ 0.7 nm (Menzl *et al.*, 1996). Therefore, given the sequence similarity between $HlyA^{Aero}$ and $HlyA^{Vc}$, it is not unreasonable to assume that the former is also a pore-forming toxin (Wong *et al.*, 1998).

The only report to date investigating the role of $HlyA^{Aero}$ in *Aeromonas* virulence was the study by Wong *et al.* (1998) described above (section 1.4.2.3). Wong *et al.* (1998) did not observe a decrease in haemolytic or cytotoxic activity of a *hlyA* mutant compared to a wild type strain. Moreover, following oral challenge of infant mice, no increase in LD_{50} was observed for a *hlyA* mutant. This lack of attenuation could be explained by the fact that the strain examined possessed a functional *aerA* gene. The results of Wong and colleagues (section 1.4.2) indicated that while aerolysin is the predominant toxin responsible for haemolytic activity in *Aeromonas*, haemolysin may also be an important virulence determinant for some strains, or that the presence of both haemolytic toxins is a requirement for virulent strains of *A. hydrophila* (Wong *et al.*, 1998).

1.4.4. Glycerophospholipid:cholesterol acyltransferase (GCAT)

Both *Aeromonas* and *Vibrio* species produce a novel lipase that has the unusual ability to produce cholesterol esters. This 37.4 kDa enzyme was named glycerophospholipid:cholesterol acyltransferase (GCAT) based on the first reaction it was shown to catalyse (Buckley, 1982). GCAT is capable of catalysing the hydrolysis of a number of lipids, and destroys host cells by digesting their cell membranes. For this reason, GCAT is also a haemolytic toxin. The structural gene encoding GCAT has been cloned and sequenced from two strains of *A. hydrophila* and one strain of *A. salmonicida* (Thornton *et al.*, 1988).

GCAT is believed to be a critical virulence determinant in the pathogenesis of the fish disease, furunculosis, caused by strains of the psychrophilic, non-human pathogen *A. salmonicida*. However, a role for GCAT in mammalian disease is much less likely, as GCAT is significantly less efficient at lysing mammalian cells. This probably reflects higher amounts of sphingomyelin in the latter, which is not a substrate for GCAT (Buckley, 1982).

In summary, three different haemolytic toxins from *Aeromonas* species have been genetically characterised. Although all are capable of causing cell lysis, only aerolysin and haemolysin are likely to play roles in human disease. In addition, there are probably other toxins that remain to be characterised, such as the toxins with α-haemolytic activities described by Ljungh *et al.* (1981), Rodriguez *et al.* (1992), and Thelestam and Ljungh (1981).

1.4.5. Cytotonic enterotoxins

Cytotonic enterotoxins differ from the toxins described above in that they cause intestinal fluid accumulation without cell lysis. The principal mechanism by which cytotonic enterotoxins act is by increasing intracellular cyclic AMP (cAMP), cyclic GMP (cGMP) and/or prostaglandin E_2 (PGE_2) levels. Cytotonic enterotoxins have been identified in many strains of *Aeromonas* by their ability to cause intestinal fluid accumulation in one of several animal models, or by their ability to elongate

Chinese hamster ovary (CHO) cells. Cytotonic enterotoxins from several strains have been shown to cross-react immunologically with cholera toxin (CT), and these CT-cross reactive toxins were all heat stable (56°C, 20 min) and able to elevate cAMP up to a level ~20% that produced by CT (Schultz and McCardell, 1988). Furthermore, Schultz and McCardell (1988) identified CT homologous DNA sequences in several *A. hydrophila* strains with DNA probes directed against the genes encoding the A_2 and B subunits of CT. However, despite the fact that all of these studies were performed more than 10 years ago, no CT homologues have been cloned or purified from *Aeromonas* species.

To date, the genes encoding three cytotonic enterotoxins have been cloned from *Aeromonas* species (Chakraborty *et al.*, 1984; Chopra *et al.*, 1994), although published DNA sequences exist for only one of these. Chopra *et al.* (1994) cloned two cytotonic enterotoxin genes from *A. hydrophila*. One gene encoded a heat-stable toxin (56°C, 20 min) while the other encoded a heat-labile toxin. The gene encoding the heat labile toxin (*alt*) was subsequently sequenced and its product (Alt) biologically characterised. Alt exhibited significant homology at the amino acid level with phospholipase A1 (Pla1; 96% identity, 97% similarity) from *Aeromonas hydrophila* (Merino *et al.*, 1999), although Alt did not exhibit phospholipase activity. Sequence alignment with Alt demonstrates that the majority of the Alt protein (amino acids 2 to 327, of 368) aligns with an internal region (amino acids 334 to 669, of 805) of Pla1, suggesting that Alt is possibly an N- and C-terminal truncated variant of Pla1.

The enterotoxic activity of Alt resulted from its ability to increase intracellular cAMP and PGE_2 to levels approximately half of those obtained with CT, although Alt did not exhibit immunological cross-reactivity or sequence similarity with cholera toxin. The *alt* gene product had a predicted molecular weight of 38 kDa, slightly less than Alt purified from *A. hydrophila* (44 kDa), suggesting that it may be post-translationally modified. This was supported by the fact that native Alt from *A. hydrophila* had higher activity than recombinant Alt. A possible role for Alt during

infection was demonstrated in a mouse ligated ileal loop assay. Mice immunised with Alt exhibited a 39% decrease in fluid accumulation when compared with non-immunised animals (Chopra *et al.*, 1994). However, a definitive role for Alt in *Aeromonas* virulence awaits the testing of isogenic mutants defective in Alt production.

A 70 kDa cytotonic enterotoxin was purified from a strain of *A. hydrophila* by McCardell *et al.* (1995). This heat stable toxin caused elongation of CHO cells, which did not occur as a result of increased cAMP, cGMP or PGE_2 levels. Although no homologous proteins were identified in the Genbank or SwissProt databases, the N-terminal sequence does exhibit 61% identity and 77% similarity with amino acids 17 to 36 of an extracellular lipase (Lip) from *A. hydrophila* (Chuang *et al.*, 1997), characterised after the study of McCardell *et al.* (1995). Unprocessed Lip had a MW (79.9 kDa) similar to the purified cytotonic enterotoxin (70 kDa). Therefore, it is possible that this enterotoxin is a variant of a lipase, which would explain why it failed to elevate cAMP, cGMP or PGE2 levels as part of its cytotonic activity.

In summary, *Aeromonas* species have the ability to produce several types of cytotonic enterotoxin. All *Aeromonas* cytotonic enterotoxins, which have been characterised at the molecular level, appear to have been derived from lipases. These toxins presumably act by insertion into, and disruption of, the cell membrane. However, other enterotoxins remain to be characterised genetically, and at least one of these may be analogous to the major enterotoxin produced by pathogenic strains of *V. cholerae.*

1.4.6. Shiga-like toxins

There are several reports implicating strains of *Aeromonas* as a causative agent of haemolytic uraemic syndrome (HUS) (Bogdanovic *et al.*, 1991; Robson *et al.*, 1992). This disease is traditionally caused by strains of enterohaemorrhagic *Escherichia coli.* HUS occurs as a result of the production of one or more toxins of the Shiga-like toxin (Stx) family, and attaching and effacing lesions similar to those produced by enteropathogenic *E. coli.* The Stx family from enterohaemorrhagic *E.*

coli consists of two major, immunologically non-cross-reactive groups termed Stx1 and Stx2. These toxins consist of an enzymatically active 32 kDa A subunit linked to five identical 7.7 kDa B subunits that are responsible for binding to target cell membranes. The toxicity of the A subunit results from its ability to remove a single adenine residue from 28S rRNA, thereby inhibiting protein synthesis (Nataro and Kaper, 1998). Epidemiological studies have indicated that Stx1 is more important than Stx2 in the development of HUS (Nataro and Kaper, 1998).

A recent study has demonstrated the presence of Stx1 in a limited number of *Aeromonas* species (Haque *et al.*, 1996). Culture filtrates of 4 of 39 isolates (3 clinical *A. hydrophila* and 1 environmental *A. caviae*) produced cytopathic effects on Vero cells that could be neutralised by *E. coli* O157:H7 Stx1 antiserum. Furthermore, DNA from these strains could be amplified with Stx1 specific primers, and the genes encoding Stx1 in *Aeromonas* were localised to a 2.14 kb plasmid. Brown *et al.* (1997) demonstrated that plasmid DNA is uncommon in *Aeromonas* species. Therefore, the significance of Stx1 in *Aeromonas* pathogenesis remains to be answered, and the presence of *stx* genes in *Aeromonas* strains associated with HUS will need to be confirmed. The possibility that plasmid or phage DNA, containing *stx* genes, is integrated into the *Aeromonas* genome has not been explored.

1.4.7. Proteases

Several proteases have been identified from strains of *Aeromonas* species following protein purification or gene cloning experiments (Table 1.3). Although for most of these there is little or no evidence of direct involvement in pathogenicity, they do induce tissue damage and cause cell death. However, this is probably not their primary purpose, as microbial proteases have been shown to have several other roles (Howard *et al.*, 1996). Many bacterial toxins, such as Shiga-like toxins, cholera toxin, and at least three toxins produced by *Aeromonas* (aerolysin, haemolysin and GCAT) are activated by proteolytic cleavage (Hilton *et al.*, 1990; Hirono and Aoki, 1991; van der Goot *et al.*, 1992; Gordon and Leppla, 1994). Furthermore, proteases are able to degrade immunoglobulins and components of the complement system,

which would facilitate the initial propagation of a pathogen, and proteases may also supply amino acids during infection (Gordon and Leppla, 1994). Therefore, while not directly responsible for disease production, proteases are involved in enhancing the pathogenesis of many diseases. Proteases identified to date fall into two broad classes, metalloproteases (inhibited by ethylenediaminetetraacetic acid; EDTA) and serine proteases (inhibited by phenylmethylsulphonyl fluoride; PMSF). Representatives of each of these groups have been identified in *Aeromonas* species.

Of the proteases identified to date, only two serine proteases have been shown to have any role in pathogenesis, and both are involved in the infection of salmonid fish. Both *A. hydrophila* and *A. salmonicida* have been shown to produce an unusually high molecular weight, 64 to 68 kDa serine protease, which is thought to be an important virulence determinant, along with GCAT, in the pathogenesis of furunculosis by *A. salmonicida.* Purified serine protease administered intramuscularly along with GCAT, results in the production of classic furuncles in salmonid fish (Ellis, 1991).

A second serine protease (22 kDa) has also been identified by protein purification from a single strain of *A. hydrophila* (Rodriguez *et al.*, 1992). Although no DNA or protein sequences have been obtained for this protein, it has been shown to be toxic in rainbow trout (LD_{50} of 150 ng/g).

1.4.8. Distribution of toxin genes

Despite all of the studies described in section. 1.4.1 to 1.4.7, there are still very few advances in our ability to identify pathogenic strains. The simplest approach to accomplish this is to examine the distribution of putative virulence factors in pathogenic and non-pathogenic isolates. Here, again, problems arise because of the difficulties in determining whether a given *Aeromonas* isolate is capable of causing disease in simple animal models. For this reason, these studies have often relied on the use of isolates of clinical and environmental origin.

There have been few studies examining the distribution of toxin genes in *Aeromonas* species. Husslein *et al.* (1991; 1992) examined 257 strains from clinical

and environmental sources with gene probes comprising the aerolysin gene (*aerA*), and the *aerA* promoter region. This study detected *aerA* in virtually all *A. veronii* biovar sobria and *A. hydrophila* isolates and in about half of all *A. caviae* strains, regardless of whether these strains were isolated from clinical or environmental sources. A particularly interesting finding of this study was that the *aerA* promoter probe hybridised with only a small, biochemically-clustered group of atypical *A. veronii* biovar sobria strains. These strains have since been designated as a new species, *A. trota*.

Recently, a study by Heuzenroeder *et al.* (1999), surveyed the presence of *hlyA* and *aerA* genes in clinical and environmental isolates of *Aeromonas*. The *hlyA* gene was detected in 96% of *A. hydrophila*, 12% *A. veronii* biovar sobria and 35% *A. caviae*, while 78% of *A. hydrophila*, 97% *A. veronii* biovar sobria, and 41% of *A. caviae* isolates tested possessed *aerA*. These observations supported previous studies investigating the distribution of *aerA* (Hirono *et al.*, 1992) and *hlyA* (Hirono and Aoki, 1991), in that both genes were present in the majority of strains tested, and that this distribution did not correlate with the source of isolation. All "pathogenic" isolates of *A. hydrophila*, as determined by LD_{50} values for infant mice, possessed both *hlyA* and *aerA*. This observation supported previous experiments conducted by these authors, investigating the virulence of *aerA* and *hlyA* mutants of *A. hydrophila* in an infant mouse model (Wong *et al.*, 1998). Interestingly, *hlyA* was rarely detected in *A. veronii* biovar sobria isolates, despite this species being haemolytic. In contrast, *hlyA* was detected in 41.1% of *A. caviae* isolates, an organism rarely reported as being haemolytic. Heuzenroeder *et al.* (1999) proposed that both *aerA* and *hlyA* are required for full virulence of *A. hydrophila*. However, *hlyA* is less likely to be important for *A. veronii* biovar sobria due to its sporadic distribution in this species. This observation supports the idea mentioned earlier that the mechanisms of disease production by *A. hydrophila* and *A. veronii* biovar sobria are likely to differ.

There are at least two further studies that have investigated the distribution of the aerolysin gene in *Aeromonas* species, although very few strains were tested in each study (Lior and Johnson, 1991; Pollard *et al.*, 1990). Both studies involved the

use of PCR to detect *aerA* using primers designed from the sequence published by Howard *et al.* (1987). Using this procedure, *aerA* was detected for all haemolytic *A. hydrophila* strains, but not from haemolytic strains of any other *Aeromonas* species, suggesting that the primers were specific only for *aerA* from *A. hydrophila*.

Hence, while a large number of toxin genes have been identified and cloned, very little is known about the distribution of each of these throughout the genus *Aeromonas*. Of the small number of studies of this type that have been published, it appears that for most of these toxins, only a subset of strains possess the genes encoding them. Further work investigating the distribution of each toxin in clinical and environmental isolates is necessary to determine whether any of these genes have potential for use as a marker of pathogenic strains.

In summary, despite all of the studies described above in section 1.4, it appears that simply investigating the ability to produce toxins is insufficient to identify pathogenic *Aeromonas* strains. Therefore, effort has shifted to investigate the mechanisms of colonisation possessed by these bacteria, as adhesion to host cell surfaces is a necessary first step in the pathogenesis of most bacterial pathogens.

1.5. Colonisation factors

The investigation of adhesive mechanisms is more complex than the investigation of toxins. Pathogenic bacteria often possess multiple distinct colonisation factors, which may be expressed at different times during the infectious process, or by different strains of the same species. Moreover, the different colonisation factors may fulfil redundant roles, and the effect of mutating one factor may be masked by the presence of another.

For *Aeromonas* species, the colonisation process is also likely to involve multiple colonisation factors. For example, experiments where filamentous surface structures have been removed by mechanical shearing, or enzymatic treatment, have dramatically reduced adhesion levels (~80%), but not abolished it. There are also likely to be species-associated differences, since cell line and animal colonisation experiments have indicated that different species colonise different cell surfaces

(Kirov *et al.*, 1995a; Sanderson *et al.*, 1996). This is also true for other pathogens, such as the different *E. coli* pathotypes. Throughout the remainder of this review, many terms will be used to describe these colonisation factors. "Adhesins" are proteins that mediate adhesion to host cells. Similarly, "colonisation factors" mediate colonisation of host tissues. However, colonisation factors are not necessarily adhesins and vice-versa. For example, the toxin co-regulated pilus (TCP) is the major colonisation factor for *V. cholerae* although, to date, this structure has not been shown to mediate adhesion to intestinal tissue. Instead, TCP appears to mediate bacterium-bacterium interactions that facilitate microcolony formation (Attridge *et al.*, 1996; Tamamoto *et al.*, 1998; Fullner and Mekalanos, 1999).

The next sections will discuss the outer membrane-associated adhesins identified in *Aeromonas* species, followed by a detailed discussion of the filamentous surface structures that have formed the basis for the studies described in this thesis.

1.6. Outer membrane-associated adhesins

1.6.1. Lipopolysaccharides

Lipopolysaccharides (LPS) are amphipathic molecules consisting of three subunits; lipid A, core oligosaccharide and the O-antigen side chain. Lipid A is responsible for the toxicity of bacterial endotoxin, and consists of a hydrophobic region of six fatty acid side chains that anchor the LPS molecule into the outer membrane. The core oligosaccharide consists of 10 to 15 sugar units that anchor the O-antigen side chain to lipid A. The O-antigen side chain, also known as the somatic antigen, consists of many repeating oligosaccharide units. The somatic antigen is responsible for the unique O serotype used to classify many bacterial species. For some pathogens, this classification is useful for identifying pathogenic strains (e.g. *V. cholerae* O1 and *E. coli* O157). Strains expressing somatic antigen are often referred to as "smooth" strains due to their colony morphology. Likewise, strains not expressing somatic antigen are often referred to as "rough" strains.

Although a serotyping scheme has been developed for use with *Aeromonas* species (Sakazaki and Shimada, 1984; Cheasty *et al.*, 1988; Thomas *et al.*, 1990), it is not in widespread use, and is used much less than the schemes currently used for the identification of pathogenic *E. coli* and *V. cholerae* strains. Despite these limitations, however, there have been a few studies investigating the prevalence of particular serotypes in clinical specimens and the role of LPS in virulence. For *Aeromonas*, serogroup O:34 appears to be the most common serogroup associated with disease, and one study has found that it accounts for 26.4% of all *Aeromonas* infections (Merino *et al.*, 1997a). Serogroup O:11 is also commonly associated with human disease, accounting for 10-30% of infections (Kokka *et al.*, 1991). Despite the fact that the chromosomal locus encoding LPS biosynthesis has not been cloned or sequenced, there are several reports documenting a possible role for LPS in *Aeromonas* pathogenesis.

Several studies have suggested that strains expressing somatic antigen (smooth LPS strains) are more virulent than rough LPS strains. For example, smooth LPS strains belonging to serotype O:34 exhibit higher adhesion to cell lines and intestinal tissue, and have lower LD_{50} values than rough strains (Francki and Chang, 1994; Aguilar *et al.*, 1997; Merino *et al.*, 1998). Likewise, *A. hydrophila* serogroup O:34 strains grown at 20°C (expressing smooth LPS) were shown to be more than twice as adhesive to HEp-2 cells than strains grown at 37°C (rough LPS). When Tn5 mutants were isolated with defects in the *rfb* gene, which is responsible for O antigen biosynthesis, adhesion of the resulting rough LPS strains was low, regardless of growth conditions (Merino *et al.*, 1996a). Similar results were also obtained in a germ-free chicken gut colonisation model (Merino *et al.*, 1996b). However, some caution must be observed when analysing defects in LPS biogenesis genes as defects in protein export can also occur. For example, *rfb* mutants of *V. cholerae* are defective in the translocation of toxin co-regulated pilus subunit proteins across the outer membrane (Iredell and Manning, 1997a; Iredell and Manning, 1997b; Iredell *et*

al., 1998). Therefore, these defects in adhesion may be due, at least in part, to pleiotrophic defects in the secretion of outer membrane proteins.

Of particular interest, recent investigations by Aguilar *et al.* (1997) and Merino *et al.* (1998) have demonstrated that the defect in O antigen biosynthesis observed when strains were grown at 37°C could be reversed when strains were grown under high osmolarity conditions (Luria broth + 300 mM NaCl). This switch from rough to smooth LPS production occurred concurrently with increased haemolytic and cytotoxic activities, increased adhesion to HEp-2 cells, and lowered LD_{50} values for rainbow trout and mice following intraperitoneal injection. These results indicate not only that LPS might be an important adhesin for serogroups O:13, O:33, O:34, and O:44, but that the environmental conditions required for smooth LPS biosynthesis may be responsible for the global regulation of many *Aeromonas* virulence factors.

1.6.2. Outer membrane proteins

The protein component of the Gram-negative outer membrane is responsible for numerous functions, including the transport of nutrients and by-products of metabolism in and out of the cell in addition to the adherence of bacterial cells to host receptors. Jeanteur *et al.* (1992) purified and determined the N-terminal amino acid sequences of six outer membrane proteins (OMPs) from a strain of *A. hydrophila*, and these were found to be homologous to OMPs from several other Gram-negative bacteria, some of which have roles in virulence (Table 1.4.). Subsequent studies by Quinn *et al.* (1993; 1994) identified three carbohydrate-reactive OMPs that may be involved in the association of *Aeromonas* bacteria to host tissues. Two of these proteins were shown to be homologous with proteins I and II from the study of Jeanteur *et al.* (1992)

Protein I, a 47 kDa protein homologous with LamB from *E. coli*, also appears to be the same as the 43 kDa protein identified from *A. veronii* biovar sobria by Nandapalan and Chang (Nandapalan and Chang, 1989). Monoclonal antibodies raised against this protein inhibited mannose-sensitive haemagglutination and adhesion of *A. veronii* biovar sobria to HEp-2 cells.

Table 1.4. Outer membrane proteins identified in *Aeromonas* species

Outer membrane protein[a]	**MW** (kDa)	**Homologue**	**Function**
Protein I	47	*E. coli* LamB	Uptake of maltose *A. hydrophila* adhesion
Protein II	39	*E. coli* OmpF / OmpC	Osmoregulation *S. flexneri* invasion *A. hydrophila* adhesion
Protein III	36	*E. coli* PhoE	Polyanion porin
Protein IV	24	*V. cholerae* OmpK	Unknown
Protein V	30	*E. coli* OmpA	*E. coli* serum resistance
Protein VI	25	*V. cholerae* OmpW	? Adhesion
FkpA	28.7	*S. typhimurium* FkpA	Invasion
IlpA	22.4	*E. coli* SlyD	Unknown

[a] Proteins I to VI designated according to Jeanteur *et al.* (1992).

Protein V from the study of Jeanteur *et al.* (1992) was a 30 kDa protein homologous with OmpA from members of Enterobacteriaceae. This protein has been shown to contribute to serum resistance and pathogenicity of *E. coli* K1 (Weiser and Gotschlich, 1991). A protein homologous with protein V has been purified from *A. salmonicida*, and this protein elicited protective immunity in rainbow trout following intraperitoneal immunisation (Lutwyche *et al.*, 1995). Two, tandemly located, genes encoding OmpA have since been cloned from *A. salmonicida*, and these were shown to be widespread among *Aeromonas* species by PCR analysis (Costello *et al.*, 1996).

Another two outer membrane proteins from *Aeromonas* species, which have been genetically characterised, are the immunophilin-like proteins, IlpA and FkpA, cloned from *A. hydrophila* A6 (Wong *et al.*, 1997). These genes were cloned using a polyclonal rabbit antiserum that protected against oral infection of infant mice by this strain. FkpA was homologous to FkpA from *Salmonella typhimurium*, and a mutation in *fkpA* from this species resulted in a decreased ability to invade macrophages (Horne *et al.*, 1997). A mutation constructed in the *Aeromonas fkpA* gene, however, resulted in no loss in virulence. However, a role for this protein during systemic infection was not investigated, nor was a mutation in *ilpA* (Wong *et al.*, 1997).

In summary, both the LPS and OMPs may play significant roles in the adhesion to, and colonisation of, host tissues by *Aeromonas* species, and yet these roles remain to be elucidated. The initial contact between a bacterial pathogen and the host cell surface, however, is almost always mediated by filamentous surface structures, which are the major focus of this thesis. Therefore, the following sections will present a detailed review of these structures as they contribute to the pathogenicity of bacteria in general, with particular focus on enteropathogenic bacteria, followed by a review on what was known about *Aeromonas* filamentous surface structures.

1.7. Filamentous surface structures

Bacteria are known to produce numerous different types of filamentous structures on their surface, and these can broadly be divided into flagella, pili, and filamentous bacteriophages.

Flagella are long, thick bacterial organelles, which have either a polar or peritrichous location on the cell surface. Flagella are the primary structures responsible for chemotaxis and motility. Each flagellum is driven by a membrane-located motor that rotates as a consequence of proton or sodium flux. Coupled to the motor is a protein hook structure connected to the long, flagellin polymer. These structures are often composed of multiple flagellin subunits, which are sometimes responsible for adhesion to host receptors.

Pili (synonymous with "fimbriae"), are thinner (< 10 nm) than flagella (≥ 20 nm), and almost always have a peritrichous location on the cell surface. Pili are currently classified into several morphological and genetic types, of which types I and IV are the most widely distributed amongst pathogenic bacteria. These pili types will be discussed in more detail in the following sections. Pili are known to mediate the initial binding of almost all Gram-negative bacterial pathogens to host cell surfaces, and are also involved in inter-bacterial interactions and microcolony formation. Throughout this thesis the term "pili" will be used in preference to "fimbriae', being the most commonly used term to describe these structures in the *Aeromonas* literature.

Filamentous bacteriophages are virus particles shed as long filaments from the cell surface. For this reason they are often indistinguishable, morphologically, from pili and are often mistaken for these structures. Bacteriophages often form an important part of the biology of their host, as they may encode genes, such as toxins and colonisation factors, beneficial for the host bacterium.

1.8. Flagella

In addition to their role as motility organelles, flagella have been implicated as important virulence factors for several bacterial pathogens in their ability to mediate adhesion to, and invasion of, epithelial and intestinal cell surfaces. For example, the polar flagellum is essential for full invasive ability of *Campylobacter jejuni* (Grant *et al.*, 1993), and for full adhesion of *Vibrio cholerae* to intestinal tissue (Attridge and Rowley, 1983; Benitez *et al.*, 1997).

As mentioned above, the flagellin polymer often consists of several distinct flagellin subunits that have varying roles in adhesion. The diarrhoeal pathogen *Campylobacter jejuni*, for example, possesses two flagellin genes *flaA* and *flaB*, and mutation of *flaA*, but not *flaB*, reduced the ability of this organism to invade INT407 intestinal cells (Grant *et al.*, 1993).

Recently, five flagellin genes have been found to be possessed by the human pathogen *Vibrio cholerae* (Klose and Mekalanos, 1998). While isogenic mutations in each of these genes have not been investigated in animal models of experimental cholera, Attridge and Rowley (1983) have suggested a role for the *V. cholerae* flagellum in adherence. These authors demonstrated that spontaneous non-motile (non-flagellated) strains, and motile strains sheared of filamentous surface structures (non-flagellated) adhered at much lower levels (~ 1 to 5%) to mouse intestinal tissue than a wild-type motile (flagellated) strain.

Flagella also appear to be involved in intestinal colonisation by *Salmonella typhimurium*, although non-flagellated mutants were not attenuated in a murine model of typhoid fever (Lockman and Curtiss, 1990). In this same model, however, *fim fli* double mutants, which lack both type I pili and flagella, respectively, were significantly attenuated, while single *fim* or *fli* mutations alone did not affect virulence (Lockman and Curtiss, 1992). These results suggest that the fimbriae and flagellum have redundant roles during the process of intestinal colonisation by *S. typhimurium*.

Several bacteria, such as *V. parahaemolyticus,* possess lateral flagella in addition to the polar flagellum. For this organism, the polar system is used for swimming in liquid environments and the lateral system is used for swarming motility over surfaces (Sar *et al.*, 1990). The latter is believed to allow growth on surfaces and in biofilms. Both polar and lateral systems are driven by reversible motors embedded in the cytoplasmic membrane. However, the polar flagellum is driven by a sodium-motive force, whereas the lateral flagellum is driven by a proton-motive source (Atsumi *et al.*, 1992). Expression of lateral flagella is initiated, along with a concurrent elimination of polar flagellum expression, following sensing of a high viscosity environment. This differential expression is controlled by sensing a decrease in torque of the polar flagellum (Kawagishi *et al.*, 1996).

In summary, it can be seen that flagella are not only important virulence factors for diarrhoeal pathogens in their role as motility organelles, but also in their ability to mediate adhesion to the intestinal mucosa. Several bacterial pathogens possess two distinct flagellar systems, which have been called the "polar" and "lateral" flagellar systems. *Aeromonas* species also appear to possess both of these systems, and these will be discussed in more detail in section 1.10.1.

1.9. Pili

1.9.1. Type I pili

Type I pili were originally described in *Salmonella* species and were defined as bacterial surface filaments that caused mannose-sensitive agglutination of guinea pig erythrocytes. Since that time they have been described on numerous Gram-negative bacteria, and are often referred to as "common pili". These structures have been found to mediate adhesion to a variety of different cell types such as erythrocytes, leukocytes, enterocytes, respiratory epithelial cells, protozoa, yeasts, fungal hyphae and plant root hairs (Duguid and Old, 1994). Although the term "type I pilus" refers to the common pili produced by *E. coli* and *Salmonella* species, the pili produced by

several other bacterial species exhibit significant homology to the type I pili and will also be discussed in this section.

Pili structurally and genetically homologous to the common type I pili described above are known colonisation factors for many pathogenic bacteria, including uropathogenic and enterotoxigenic *E. coli* (ETEC), *Haemophilus influenzae* and *Bordetella pertussis*. To date, numerous pili of this type have been identified in *E. coli*, with at least 14 currently known to be produced by various strains of ETEC alone (Nataro and Kaper, 1998). In this organism, the production of a specific pilus type confers the species specificity of the pathogen. For example, K88 pili mediate binding to pigs, K99 pili are specific for calves and lambs in addition to pigs, while CFA/I, CFA/II, CFA/III and CFA/IV pili are all specific for human intestinal tissues. Similarly, the *H. influenzae* pilus confers specificity to buccal and bronchial epithelial cells (Gilsdorf *et al.*, 1997).

Type I pili are composed of a major repeating subunit as well as minor components responsible for their adhesive nature. The *E. coli* P pilus, which mediates binding of uropathogenic *E. coli* to the urogenital tract epithelium, is composed of a major repeating subunit protein, PapA, anchored into the outer membrane via PapH. PapA is guided to the cell surface by a conserved chaperone molecule (PapD) and an outer membrane usher (PapC). At the distal end of the pilus is the tip fibrillin, which is composed of PapE, and the tip adhesin, PapG. Morphologically this structure appears as a rigid rod with a short, thin fibrillin at the tip (Finlay and Falkow, 1997). Type I and *H. influenzae* pili are assembled via a similar pathway, and are similar to P pili, except that they are flexible structures.

In summary, pili homologous to the *E. coli* type I pili are important colonisation factors for many diarrhoeal and extraintestinal pathogens. Similar structures have been identified on strains of *Aeromonas* species, and although they have not been characterised in great detail, they do appear to exhibit structural and sequence homology to the *E. coli* type I pilus, and will be discussed further in section 1.10.2.2.

1.9.2. Type IV pili

Type IV pili are assembled by a different pathway than type I pili, which involves components of the main terminal branch of the general secretion pathway (type II secretion pathway). These pili have been found in many diverse Gram-negative bacterial species, including *Pseudomonas aeruginosa*, *Neisseria* species, *Moraxella* species, *E. coli* and *V. cholerae*. The experiments described in this thesis are concerned with the investigation of the type IV pili produced by *Aeromonas* bacteria. Therefore, the structure, genetics, and function of these structures will now be reviewed in detail.

1.9.2.1. Structure of type IV pili

Broadly, type IV pili are divided into two classes (Strom and Lory, 1993). "Classical" type IV pili (type IVA pili) have a short N-terminal leader sequence usually composed of 6 amino acids preceding a conserved phenylalanine residue. Type IVB pili are bundle-forming pili, which have been found in *V. cholerae* (toxin co-regulated pilus; TCP), enteropathogenic *E. coli* (bundle-forming pilus; BFP) and enterotoxigenic *E. coli* (longus pilus). These structures have a longer leader sequence of 13 to 25 amino acids and the N-terminal amino acids of the mature pilins are either a methionine or a leucine. However, recent evidence obtained for *V. cholerae* has demonstrated that TCP is a filamentous bacteriophage assembled via the type IV pilus biogenesis pathway. Although, to date, this has only been shown for the TCP pilus, it is possible that the other type IVB pili may also be filamentous bacteriophages. Indeed, all of the genes required for assembly of the enteropathogenic *E. coli* (EPEC) BFP are clustered in a similar fashion to the *tcp* gene cluster (Donnenberg *et al.*, 1997), whereas the genes involved in assembly of the type IVA pili, at least for *P. aeruginosa*, are dispersed throughout the chromosome (Alm and Mattick, 1997).

As described above, type IV prepilin proteins possess a short, positively charged leader sequence that is generally 6-7 amino acids in "classical" type IV pili and 13-25 amino acids long in type IVB pili. This leader sequence has been shown to

be a substrate for an enzyme termed a "prepilin peptidase" in *P. aeruginosa* (PilD; Nunn and Lory, 1991), *N. gonorrhoeae* (PilD; Dupuy and Pugsley, 1994),*V. cholerae* (TcpJ; Kaufman *et al.*, 1991) and *A. hydrophila* (TapD; Pepe *et al.*, 1996). These enzymes also process prepilin-like proteins ("pseudopilins"), which comprise components of the type II secretion pathway (e.g. XcpTUVW from *P. aeruginosa*; Nunn and Lory, 1993).

Type IV pilin proteins can be divided into two structural regions, a conserved domain and a variable domain, based on amino acid sequence similarity. The conserved domain spans approximately 40 amino acids and consists of the leader sequence, a domain highly conserved among all type IV pili, and a slightly less well conserved domain which is generally conserved only within a particular species (Strom and Lory, 1993; Tennent and Mattick, 1994). Critical amino acid residues include a glycine at -1, which is required for cleavage, and a glutamate at +5, which is essential for assembly and efficient methylation of the mature protein (Strom and Lory, 1991). Towards the C-terminus there is another region of homology containing two cysteine residues (12 to 28 amino acids apart) which are involved in the formation of a disulphide loop within the subunits of *N. gonorrhoeae* MS11 and *P. aeruginosa* PAK and PAO. *D. nodosus* contain an additional two cysteine residues which form another disulphide loop near the end of the conserved amino terminus (Mattick *et al.*, 1991; Tennent and Mattick, 1994).

Type IV pili are approximately 6 nm in diameter and can be several μm in length (Tennent and Mattick, 1994). Most type IV pili are composed of a single repeating subunit ranging in size from 145 to 160 amino acids (15-18 kDa) which show a high degree of N-terminal amino acid sequence similarity among different species (Strom and Lory, 1993). The exceptions are the type IV pili produced by *Neisseria* species, which appear to possess a tip-located adhesin (PilC; Rudel *et al.*, 1995).

1.9.2.2. Functions of type IV pili

Type IV pili are involved in the colonisation of mucosal surfaces by diverse bacterial species. In addition, these structures have also been demonstrated to have other roles, such as twitching motility and their ability to act as receptors for bacteriophages (Tennent and Mattick, 1994).

1.9.2.2.1. Adhesion

A number of studies have shown that type IV pili mediate binding to epithelial cells. Type IV pili from *P. aeruginosa* (Woods *et al.*, 1980; Doig *et al.*, 1988), *N. gonorrhoeae* (Pearce and Buchanan, 1978), *N. meningitidis* (Virji *et al.*, 1993); *V. cholerae* (Attridge *et al.*, 1996; Taylor *et al.*, 1987) and enteropathogenic *E. coli* (Giron *et al.*, 1991) have all been shown to mediate adhesion to epithelial cells and/or colonisation of host mucosal surfaces. Although the target ligand is not known for most type IV pili, it has been shown that the *P. aeruginosa* type IV pilus binds to specific glycoproteins from buccal epithelial cells (Doig *et al.*, 1990; Schweizer *et al.*, 1998). The binding of type IV pili to host receptors has been shown to be host and pilus specific, at least for the *M. bovis* type IV pilus, since expression of *M bovis* pili by *P. aeruginosa* allows this strain to adhere to cultured bovine lens epithelial cells (Ruehl *et al.*, 1993). Monoclonal antibody studies on the *P. aeruginosa* type IV pilus indicated that the adhesin moiety lies near the C-terminal disulphide loop (Doig *et al.*, 1990). In contrast, similar studies on the *N. gonorrhoeae* type IV pilus indicated that the adhesin lies towards the C-terminus of the N-terminal conserved region (Rothbard *et al.*, 1985).

1.9.2.2.2. Twitching Motility

Twitching motility is a flagellar-independent motility manifested as a jerky motion of the bacterial cell when viewed in suspension, and as rapidly spreading colonies on agar surfaces incubated under humid conditions. Such motility appears to be important for full virulence of *P. aeruginosa*, as non-twitching, hyperpiliated mutants are non-infectious (Hazlett *et al.*, 1991). Electron microscopic studies of twitching motility mutants, and the pattern of bacteriophage binding, led to the

suggestion that twitching motility is a process mediated by pilus extension and retraction, presumably operating via a pilin subunit assembly-disassembly cycle (Bradley, 1980). It has been suggested that twitching motility, protein secretion and DNA uptake all occur via rotation of a basal complex which spans the inner and outer membranes. This complex would consist of several proteins with type IV leader sequences, and would be driven by energy provided by nucleotide binding proteins, such as XcpR or ComG-1 in *P. aeruginosa* (Mattick and Alm, 1995).

1.9.2.2.3. Other functions of type IV pili

Several other functions have also been attributed to type IV pili, including their role as bacteriophage receptors and in mediating competence for DNA uptake. These roles may enhance pathogenicity by indirect mechanisms, such as by the acquisition of new virulence determinants. In one case, this has indeed been shown to be the case. Production of the toxin-co-regulated pilus (TCP) by strains of *V. cholerae* is a prerequisite for this bacterium to cause cholera. TCP has been shown not only to be the major virulence determinant required for colonisation of the intestinal tract (Attridge *et al.*, 1996; Taylor *et al.*, 1987), but it also acts as a receptor for a filamentous bacteriophage that encodes cholera toxin, the major toxin responsible for the production of cholera dysentery (Waldor and Mekalanos, 1996). As described earlier, it has recently been demonstrated that TCP itself is a bacteriophage (Karaolis *et al.*, 1999). Therefore, in order to become fully pathogenic, certain strains of *V. cholerae* must first have become infected with the TCP phage, and the subsequent expression of this pilus (phage) enables the bacterium to colonise the host intestine and to become toxigenic (Karaolis *et al.*, 1998).

In summary, type IV pili have been described on a wide variety of Gram-negative bacterial pathogens. These structures are characterised by a number of conserved molecular features, particularly by their N-terminal amino acid sequences. Although the role of type IV pili in pathogenesis is poorly understood for many species, these structures are known to perform several functions, including adhesion to host cell surfaces and twitching motility. Type IV pili have been identified on

several strains of *Aeromonas* species, and what was known regarding these structures at the commencement of this thesis will be discussed in detail in section 1.10.2.2.

1.10. *Aeromonas* filamentous surface structures

1.10.1. *Aeromonas* flagella

With regard to diarrhoeal disease, very little is known regarding the role of the *Aeromonas* polar or lateral flagellum systems. It has been demonstrated that the possession of polar flagella is essential for the ability of *A. hydrophila* O:34 strains to adhere to, and invade fish cell lines (Merino *et al.*, 1997b). Two tandemly located flagellin genes, *flaA* and *flaB*, have been cloned from a strain of *A. salmonicida* (Umelo and Trust, 1997), but the adhesive and invasive ability of isogenic mutant strains has not been examined. Although *A. salmonicida* is generally considered to be non-motile, due to the lack of a polar flagellum, growth at 30 to 37°C has been shown to induce a motile phenotype due to the production of these structures (McIntosh and Austin, 1991).

The presence of peritrichous, or lateral, flagella has also been noted in *Aeromonas* species (Shimada *et al.*, 1985; Thornley *et al.*, 1997). The lateral flagellin has been purified, and the N-terminal amino acid sequence of this protein exhibited significant homology with the lateral flagellin of *Vibrio parahaemolyticus* (Thornley *et al.*, 1997). Two genes (*lafA1* and *lafA2*) have been cloned that encode the lateral flagellin proteins, as well as a gene (*lafB*) predicted to encode a flagellar hook protein (J. G. Shaw, personal communication). To date, the genes encoding lateral flagella have only been found in ~ 35 to 50% of strains tested. The role(s) of these structures in colonisation of the gastrointestinal tract are currently under investigation in our laboratory, and in the laboratories of other investigators.

1.10.2. *Aeromonas* pili

The majority of work investigating *Aeromonas* pili has involved protein purification and morphological studies. At the commencement of this thesis, there

was only one study published investigating an *Aeromonas* pilus at the genetic level (Ho *et al.*, 1992). The remainder of this review is divided into two major sections discussing these morphological and protein purification studies.

1.10.2.1. Morphological studies

Studies of the filamentous structures produced by *Aeromonas* species using electron microscopic techniques have painted a complex picture of these appendages. Numerous different morphological types have been observed and these vary with the species, source and growth conditions of a particular isolate.

Broadly, *Aeromonas* pili described in the literature have been divided into two different morphological types, the "short-rigid" (S/R) and the "long-flexible/wavy" (L/W) pili. However, this nomenclature is somewhat over-simplified, since within each morphological group, a variety of pilus types have been observed. L/W pili of varied widths (4–10 nm), may occur singly or in bundles (Carrello *et al.*, 1989; Ho *et al.*, 1990; Kirov *et al.*, 1995b; Kirov *et al.*, 1998). S/R pili (7–10 nm in width) have also been observed, as have filamentous networks and aggregates that do not clearly emanate from any one point on the bacterium (Kirov *et al.*, 1995b). The latter are morphologically similar to *E. coli* curli (Olsen *et al.*, 1989), and the *V. cholerae* Type B fimbriae identified in the study by Hall *et al.* (1988).

The filamentous structures described in the study by Kirov *et al.* (1995b) were increased in number when bacteria were grown at low temperatures (≤ 20°C) in liquid medium, and this observation correlated with increased cell-line adhesion levels for strains grown under such conditions. Similarly, strains sheared of their surface structures exhibited decreased adhesion to cell lines. The long-flexible and short-rigid pili have been characterised further by protein purification studies.

1.10.2.2. Protein purification studies

1.10.2.2.1. Short-rigid (S/R) pili

Short-rigid pili have been purified from three strains of *A. hydrophila* (Sato *et al.*, 1989; Ho *et al.*, 1990; Honma and Nakasone, 1990). The 17 kDa subunit protein

exhibited high N-terminal amino acid sequence homology with the type I pilin proteins from *Serratia marcescens*, *Haemophilus influenzae*, and the P pilus of uropathogenic *E. coli* (Ho *et al.*, 1990). However, in contrast with the latter, the *Aeromonas* pili did not haemagglutinate human or guinea pig erythrocytes (Ho et al. 1990). Furthermore, these pili did not bind to intestinal cells (Sato *et al.*, 1989; Honma and Nakasone, 1990). Therefore, the role, if any, of these pili in intestinal colonisation remains unknown. However, short-rigid pili are involved in autoaggregation of bacteria and, as such, may assist in microcolony formation (Honma and Nakasone, 1990).

1.10.2.2.2. "Mini pilin"

The long-flexible pilus characterised by Ho *et al.* (1990) was an entirely novel, flexible "pilus" identified on a strain of *A. hydrophila*. This pilus was composed of a unique 46 amino acid hydrophobic polypeptide repeating subunit, and had an N-terminal sequence that exhibited no homology with any other known pilus proteins. This pilus was maximally expressed under defined environmental conditions (growth at 22°C vs 37°C, iron limiting conditions, and liquid medium) and haemagglutinated erythrocytes in a fucose, mannose and galactose resistant manner. The gene encoding mini pilin (*fxp*) was subsequently cloned and localised to a 7.6 kb plasmid (Ho *et al.*, 1992). However, sequences homologous with *fxp* were not identified by Southern hybridisation in any of 66 other *Aeromonas* strains analysed in this study. The authors proposed that *fxp* encoded a unique structure, possibly widely distributed in nature, that was carried on a genetic element rapidly lost from most strains during *in vitro* cultivation (Ho *et al.*, 1992). This suggestion was supported by the study of Brown *et al.* (1997), who demonstrated that plasmid DNA is uncommon in *Aeromonas* species.

An alternative possibility for the true identity of "mini pilin" was revealed in 1996 following the discovery of a filamentous bacteriophage (CTXϕ). This phage encodes the cholera, Ace and Zot toxins possessed by epidemic and pandemic strains of *V. cholerae* (Waldor and Mekalanos, 1996). The major repeating subunit of CTXϕ

(Cep) was similar to "mini pilin" (Fxp) with respect to size (50 and 47 amino acids, respectively), hydrophobicity, and amino acid sequence homology (27.5% identity, 64% similarity). Moreover, the replicative form of CTXϕ DNA was 7 kb, comparable to the size of the "mini pilin" plasmid (7.6 kb). Therefore, it seems likely that the flexible pilus characterised by Ho *et al.* (1990; 1992) is actually a filamentous phage particle. This fact may also give some clues as to the promiscuous nature of *fxp*. In *V. cholerae*, CTXϕ uses the toxin co-regulated pilus (TCP) as a receptor, and uptake of the phage particle not only relies on the recipient strain possessing TCP genes, but also that they are expressed. In *Aeromonas*, the receptor for Fxp, like TCP, may only be expressed by a limited subset of strains, for example, those belonging to a specific species and serogroup, during growth in defined environmental conditions. Hence, the distribution of strains possessing *fxp* would, at most, be limited to these strains.

1.10.2.2.3. Long-flexible pili

The "mini-pilin" described above has often been confused with other L/W pili produced by *Aeromonas* species. Clearly, this structure is different from other pilus structures purified from the surface of several strains of *Aeromonas* bacteria, and these pili have been studied in more detail at the protein level than the structures described above. L/W pili are the predominant type produced by *Aeromonas* strains (particularly *A. veronii* biovar sobria) recovered from diarrhoeal stools (Carrello *et al.*, 1988; Kirov *et al.*, 1995b). Examination of these strains by electron microscopy revealed L/W pili existing as isolated structures and as rope-like bundles (Kirov *et al.*, 1995b). However, subsequent immunogold labelling of surface pili on a strain of *A. veronii* biovar sobria, with an antiserum raised against the purified subunit protein, revealed that both isolated and bundles of pili were composed of the same subunit protein (Kirov and Sanderson, 1996). Due to their tendency to form bundles, these pili have been referred to as bundle-forming pili (Bfp). It should be noted, however, that these structures belong to the type IVA class of pili, and are different to the type IVB bundle forming pili of enteropathogenic *E. coli*. The observed difference in pilus

morphologies composed of the same subunit protein is reminiscent of the type IV pili produced by *Neisseria meningitidis*. For this species, similar morphological changes in surface pili have been shown to occur as a consequence of primary amino acid sequence changes producing alternate glycosylation sites (Virji *et al.*, 1993).

L/W pili have been purified from the surface of several *Aeromonas* isolates, and N-terminal amino acid sequencing has revealed that they all belong to the type IV class of pili (Hokama *et al.*, 1990; Hokama and Iwanaga, 1992; Iwanaga and Hokama, 1992; Kirov and Sanderson, 1996; Nakasone *et al.*, 1996; Kirov *et al.*, 1998). These pili were all 19 to 23 kDa in size and approximately 7 nm in diameter. The N-terminal sequences of the *Aeromonas* pili were most similar to the mannose-sensitive haemagglutinin (MSHA) pilus of *Vibrio cholerae* (Fig. 1.1.).

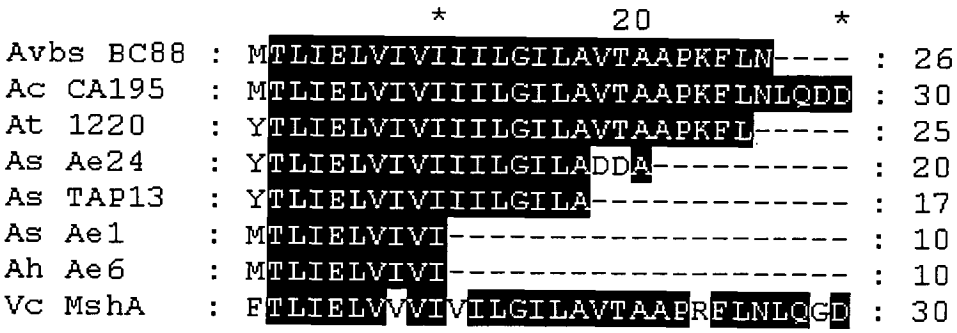
Although the N-terminal sequences of these pili are highly homologous, there does appear to be significant antigenic diversity between the pili of different strains. Large-scale antigenic screening of several culture collections have identified few cross-reactive isolates (Hokama *et al.*, 1990; Hokama and Iwanaga, 1992; Iwanaga and Hokama, 1992; Kirov and Sanderson, 1996). This observation presumably reflects significant sequence divergence in the primary amino acid sequence of the C-terminal regions of the pilin proteins. Antigenic variation may also be aided by alternate glycosylation states as described above (Virji *et al.*, 1993).

1.10.2.2.4. Role as colonisation factors

The *Aeromonas* L/W pili appear to mediate adhesion to intestinal tissue. All purified pili examined to date have been shown to adhere to either formalin-fixed rabbit intestinal tissue (Hokama and Iwanaga, 1992; Iwanaga and Hokama, 1992; Nakasone *et al.*, 1996; Kirov *et al.*, 1999), or to human intestinal tissue obtained as surgical specimens (Hokama *et al.*, 1990; Kirov *et al.*, 1999). Furthermore, pre-treatment of the intestinal tissue with purified pili, or pre-treatment of the bacteria with the Fab fraction of immune antisera, dramatically reduced adhesion levels. Levels of reduction of 50% (pili pre-treatment) and 80% (antisera pre-treatment) have been reported (Nakasone *et al.*, 1996; Kirov *et al.*, 1999). These pili either failed to haemagglutinate human erythrocytes (Iwanaga and Hokama, 1992; Kirov

Fig. 1.1. Amino acid sequence alignment of type IV pilin proteins purified from *Aeromonas* species.

Conserved residues are shaded black. Species abbreviations and the pilin subunit designations are as follows: Bfp pili; Avbs BC88, *A. veronii* biovar sobria BC88, Sfp (Kirov and Sanderson, 1996); Ac CA195, *A. caviae* CA195, Cfp (Kirov *et al.*, 1998); As Ae24, '*A. sobria*' Ae24 (Hokama and Iwanaga, 1992); As TAP13, '*A. sobria*' TAP13 (Iwanaga and Hokama, 1992); As Ae1, '*A. sobria*' Ae1 (Hokama and Iwanaga, 1991); Ah Ae6, *A. hydrophila* Ae6 (Hokama *et al.*, 1990); At 1220, *A. trota* 1220 (Nakasone *et al.*, 1996); Vc MshA, Vibrio cholerae O1 El Tor Phil 6973, MshA (Jonson et al., 1994); Ah Ah65.


* 20 *
Avbs BC88 : MTLIELVIVIIILGILAVTAAPKFLN---- : 26
Ac CA195 : MTLIELVIVIIILGILAVTAAPKFLNLQDD : 30
At 1220 : YTLIELVIVIIILGILAVTAAPKFL----- : 25
As Ae24 : YTLIELVIVIIILGILADDA---------- : 20
As TAP13 : YTLIELVIVIIILGILA------------ : 17
As Ae1 : MTLIELVIVI------------------- : 10
Ah Ae6 : MTLIELVIVI------------------- : 10
Vc MshA : FTLIELVVVIVILGILAVTAAPRFLNLQGD : 30

and Sanderson, 1996; Nakasone *et al.*, 1996), or did exhibit haemagglutination in a galactose- and mannose-sensitive, fucose-resistant manner (Hokama *et al.*, 1990; Hokama and Iwanaga, 1992). The latter is identical to the sugar sensitivity exhibited by the MSHA pilus of *V. cholerae* (Jonson *et al.*, 1991).

The adhesive ability of the purified pili supports results obtained in several more indirect studies. Maximal adhesion to cell lines has been shown to occur when strains are grown under conditions that promote the expression of flexible pili. Furthermore, removal of the structures by mechanical or enzymatic methods has been shown to decrease adhesion levels by 60 to 80% (Carrello *et al.*, 1988; Kirov *et al.*, 1995a).

1.11. Overall summary

In summary, although still the subject of some controversy, it is widely believed that some strains of *Aeromonas* bacteria are significant enteropathogens. The use of molecular biology techniques has dramatically increased the knowledge of the taxonomy and virulence mechanisms of *Aeromonas* species. Three species of *Aeromonas* are commonly associated with human gastroenteritis, and the pathogenesis of diarrhoeal disease caused by each of these is likely to involve different virulence mechanisms. However, there are still many unresolved questions regarding *Aeromonas* pathogenesis and it is not possible to identify pathogenic strains definitively. It is likely that such strains possess a combination of virulence factors that allow the production of diarrhoeal disease symptoms. Adhesion and colonisation of the gastrointestinal tract is an important initial step in this process and filamentous surface structures are likely be involved in this initial binding. Type IV pili have been purified from several strains of *Aeromonas* and work to date suggests that these structures are important colonisation factors for all three species commonly associated with gastrointestinal disease. However, the role of these structures has not been established definitively. The genetic analysis and role of these structures in *Aeromonas* gastrointestinal infection will be investigated in this thesis.

1.12. Aims of this thesis

The initial aim of this thesis was to clone the genes encoding the type IV pili expressed by a strain of *A. veronii* biovar sobria (Bfp). In the course of this investigation, the genes encoding a second family of type IV pili (Tap) were cloned, demonstrating that *Aeromonas* species encode two families of type IV pili (Ch 3). The genes encoding Tap pili were found to be widely conserved in the genus *Aeromonas* (Ch 4). The expression of the encoded Tap pili, and an analysis of the ability of wild type and mutant strains to adhere to cell lines, and to colonise and produce diarrhoeal symptoms in animal models was investigated to determine the significance of Tap pili as colonisation factors (Ch 5).

Chapter 2

Materials and methods

2.1. Bacterial strains and plasmids

Aeromonas isolates used in this thesis were obtained from the culture collection stored at the Discipline of Pathology, University of Tasmania, or were gifts from other laboratories. A detailed list of all *A. veronii* biovar sobria, *A. hydrophila* and *A. caviae* strains is given in Appendices 1a, 1b and 1c, respectively. *Aeromonas* type strains, and any strains not belonging to the above species are listed in Appendix 1d. *E. coli* laboratory strains are listed in Appendix 1e. A list of plasmids used in this thesis is given in Appendix 2.

2.2. Bacterial maintenance and growth conditions

All bacterial strains were stored in glycerol storage medium (Appendix 3.1.2) at -80°C and as stabs in minimal maintenance medium (MMM, Appendix 3.1.1).

All bacterial strains, except for *E. coli* laboratory strains, were routinely cultured in tryptone soya broth containing 0.6% yeast extract (TSBY, Appendix 3.1.3) or on tryptone soya agar containing 0.6% yeast extract (TSAY, Appendix 3.1.4).

E. coli strains were routinely cultured in Luria-Bertani (LB) broth (Appendix 3.1.5) or on LB agar (Appendix 3.1.6).

Antibiotics and other additives were added to growth media, when required, at the concentrations listed in Appendix 4.

2.3. Animals

Animals used in this thesis were housed in appropriate cages, either within the Discipline of Pathology Animal holding facility (mice) or at the Central Animal House, University of Tasmania (rabbits). Rabbits used for the removable intestinal tie adult rabbit diarrhoeal (RITARD) model were held at the Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh. All animals were obtained and used with permission of the University of Tasmania Ethics Committee (Animal Experimentation).

Mice used in this thesis were BALB/c inbred mice. Rabbits used for antiserum production were New Zealand white rabbits.

2.4. Enzymes and immunoconjugates

Enzymes and immunoconjugates used in this thesis are listed in Appendix 5.

2.5. DNA extraction procedures

2.5.1. Preparation of genomic DNA

Genomic DNA was prepared from *Aeromonas* strains using a modification of the procedure described by Ausubel *et al.* (1994). In brief, overnight cultures (10 ml) were pelleted by centrifugation (3000 × g, 15 min) and resuspended in 5.7 ml TE buffer. Sodium dodecyl sulphate (SDS) and proteinase K were added to final concentrations of 0.5% w/v and 100 μg/ml, respectively. Following incubation at 37°C for 1 h, NaCl was added to a final concentration of 0.8 M, followed by 0.8 ml CTAB solution (Appendix 6.1.2.), and the mixture was mixed gently by inversion and incubated at 65°C for 10 min. CTAB, exogenous polysaccharides and proteins were removed by one to three extractions with chloroform. DNA was recovered following the addition of 0.7 volumes of isopropanol and centrifugation (3000 × g, 30 min) at 4°C and resuspended in sterile milliQ water.

2.5.2. Preparation of plasmid DNA

2.5.2.1. Boiling miniprep method

Small scale preparations of small (< 10 kb), high copy number plasmids were prepared using the protocol of Liu and Misra (1995). Briefly, 200 μL overnight cultures in Terrific broth (Appendix 3.1.7) were grown in 1.5 ml Eppendorf tubes with vigorous shaking. Cells were pelleted by brief centrifugation (15000 × g, 2 min) and resuspended in 40 μl STT buffer (Appendix 6.1.3) containing 1 mg/ml lysozyme. The resulting cell suspensions were lysed by placing in a boiling water

bath for 1 min, and cell debris was removed by centrifugation (15000 × g, 10 min). The remaining supernatant was used directly for restriction enzyme digestion and agarose gel electrophoresis.

2.5.2.2. Alkaline lysis

Small and large scale preparations of plasmid DNA were prepared according to the protocol of Birnboim and Doly (1979). Briefly, overnight cultures were prepared in 50 ml LB or Terrific broth. Cells were recovered by centrifugation (3000 × g, 15 min), and were resuspended in 4 ml of Solution I (Appendix 6.1.4.). Solution II (8 ml; Appendix 6.1.5) was added and the mixture was mixed gently by inversion and incubated at room temperature for 5 min. SDS and the majority of chromosomal DNA was precipitated following the addition of 6 ml ice-cold solution III (Appendix 6.1.6), and the resulting mixture was again mixed by inversion. Following incubation on ice for at least 10 min, the flocculent white precipitate was removed by centrifugation (3000 × g, 15 min) at 4°C, and RNA was removed by digestion with RNase A at a final concentration of 5 μg/ml for 10 min. Plasmid DNA was recovered by centrifugation (3000 × g, 30 min) at 4°C, following the addition of 0.7 volumes of isopropanol, and resuspended in sterile milliQ water.

The above procedure was scaled appropriately for larger and smaller culture volumes.

2.5.2.3. Small scale minipreps for sequencing

Small scale plasmid preparations for DNA sequencing were prepared using Qiaprep spin kits (Qiagen, Hilden, Germany), according to the manufacturer's directions.

2.5.2.4. Large scale plasmid preparations

Large scale plasmid preparations were prepared using Qiagen midiprep columns (Qiagen, Hilden, Germany), according to the manufacturers directions.

2.6. Analysis and manipulation of DNA

2.6.1. DNA quantitation, digestion, and electrophoresis

DNA was quantified by measuring absorbance at 260 and 280 nm, digested with restriction enzymes, and separated by agarose gel electrophoresis using TAE buffer (Appendix 6.2.1), as described by Sambrook *et al.* (1989).

2.6.2. Isolation of DNA fragments by agarose gel electrophoresis

DNA fragments separated by agarose gel electrophoresis were purified using an agarose gel DNA extraction kit (Boehringer Mannheim, Castle Hill, Australia). For size-fractionation of chromosomal DNA digests, agarose gel slices were frozen at –80°C, and the DNA was recovered by spinning through a cone of Whatman 3M filter paper placed in a 1.5 ml Eppendorf tube. The DNA was then ethanol precipitated and resuspended in sterile milliQ water.

2.6.3. End-filling with Klenow fragment

DNA polymerase I large (Klenow) fragment was used to end-fill 5' DNA overhangs to allow blunt-end cloning experiments, according to the manufacturers instructions. Briefly, the desired DNA fragment was purified as described in section 2.7.2, precipitated with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ice-cold 95% ethanol, and resuspended in 9 μL of sterile water. The DNA solution was transferred to a 37°C waterbath and 1 μL of Klenow mix (Appendix 6.3.1) was added. Following incubation (37°C for 3 min), 1 μL dNTP mix (Appendix 6.3.2) was added and the mixture was allowed to incubate for a further 5 min at 37°C. The Klenow fragment was inactivated by incubation at 65°C for 10 min, and the resulting mixture used directly for ligation reactions.

2.6.4. *In vitro* cloning

DNA fragments were ligated into plasmid vectors using T4 DNA ligase according to standard methods (Sambrook *et al.*, 1989). The ligation reaction was

allowed to incubate (1 to 4 h) before being transformed into competent *E. coli* cells (see section 2.8.1).

2.6.5. Dye-terminator sequencing

DNA sequencing was performed by the dye-terminator method (Perkin Elmer, Norwalk, U.S.A.) using double-stranded plasmids as template DNA. M13 forward and reverse oligonucleotides (Appendix 7) were used as primers. Cycle sequencing was performed on a PTC100 thermal cycler (MJ Research, Boston, U.S.A.) and analysed on a ABI prism 377 DNA sequencer (Perkin Elmer, Norwalk, U.S.A.) at the Molecular Biology Facility, School of Biomolecular and Biomedical Science, Griffith University, Nathan, Queensland, Australia. The nucleotide sequence of both strands was determined for all inserts. DNA sequences were assembled using Sequence Navigator computer software (Perkin Elmer, Norwalk, U.S.A.). Searches for homologous database sequences were performed using BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information; (Altschul *et al.*, 1990; Gish and States, 1993; Altschul *et al.*, 1997).

2.7. Bacterial transformation and conjugation

2.7.1. Bacterial transformation

To prepare competent *E. coli*, an overnight culture of the appropriate *E. coli* strain was inoculated into 200 ml LB broth and grown to mid-log phase (OD_{650} = 0.6) at 37°C. This culture was cooled briefly (5 min) on ice and cells were pelleted by centrifugation (5000 × g, 15 min, 4°C). The cell pellet was resuspended in 100 ml solution α (Appendix 6.4.1), and the cells were again recovered by centrifugation. The cell pellet was then resuspended in 10 ml solution β (Appendix 6.4.2), and incubated on ice for approximately 2 h. Aliquots (500 µl) of this cell suspension were snap-frozen in liquid nitrogen, and stored at –80°C.

For transformation, 100 µl competent cells were added to the plasmid solution (~10 µl) to be transformed and incubated on ice for 10 min. The cells were heat shocked in a 37°C waterbath for 3 min, and returned to ice for a further 15 min. SOC medium (400 µl; Appendix 3.1.9) was added to each tube, and the cells were incubated at 37°C with shaking (200 rpm) to allow expression of the antibiotic resistance marker. Individual transformants were selected by plating aliquots of each transformation mixture onto LB agar containing the appropriate selective antibiotics. IPTG (1 mM) was added for selection of clones in pZErO-2.1, while IPTG and 50 µg/ml X-gal were added for the selection of clones by blue/white screening.

2.7.2. Bacterial conjugation

Mobilisable plasmids were transferred to recipient cells following conjugal mating on solid media. This system utilised the RP4 mobilisation system present within *E. coli* S17-1*λpir*. Cultures (500 µL) of log phase donor (*E. coli* S17-1*λpir* containing the appropriate donor plasmid) and recipient cells were mixed and the bacteria recovered by centrifugation. The bacterial pellet was gently resuspended (100 µL LB broth), pipetted onto the surface of a LB agar plate, and incubated at 37°C. Following incubation, the conjugation mix was harvested into 5 ml LB broth, and serial dilutions were plated onto LB agar plates containing the appropriate selective and counter-selective antibiotics.

2.8. Preparation of cosmid DNA libraries

A cosmid library of *A. veronii* biovar sobria BC88 DNA was constructed in the cosmid vector pTB023 (see section 3.3.1.1). DNA from strain BC88 (250 µg) was digested in a reaction volume of 300 µL. Following the addition of 15 U *Sau3A* I, the reaction mix was briefly mixed and incubated at 37°C. Aliquots (50 µL) were taken after 0, 15, 30, 60, 90 and 120 s, and the enzyme was heat-inactivated by incubating at 80°C for 15 min. Digestion was monitored by agarose gel electrophoresis, and fractions that resulted in ~ 40 kb fragments were ligated into pTB023. The latter was

prepared by digesting pTB023 with *Bam*H I, after which the enzyme was inactivated by incubation at 80°C for 15 min. Approximately 10 μg of the BC88 DNA fragments were mixed with 1 μg of the vector and ligated overnight at room temperature.

The ligation mixture was packaged directly into bacteriophage λ using Packagene extract (Promega, Madison, U.S.A.). Following incubation at room temperature for 2 h, 400 μL phage buffer (Appendix 6.5.1) was added, and the mixture was used to transduce 2 ml *E. coli* S17-1*λpir* by incubation at 37°C for 30 min. Transductants were selected on LB agar containing 25 μg/ml chloramphenicol.

E. coli S17-1*λpir* used for phage infection was prepared as follows. Following overnight growth at 37°C in LB broth supplemented with 0.2% maltose, a log-phase culture was prepared by adding 1 ml of the overnight culture to 20 ml LB broth supplemented with 0.2% maltose and 2 mM $MgSO_4$, followed by incubation at 37°C for 2 h with shaking. Bacteria in the resulting culture were pelleted and resuspended in 2 ml LB supplemented with maltose and $MgSO_4$, as described above.

Individual cosmid clones were screened for full-sized inserts by replica plating on LB agar containing 12.5 μg/ml tetracycline. As DNA fragments were cloned into the *Bam*H I site, situated within the coding region of the tetracycline resistance gene of pTB023, only Tet^S clones would contain full-length insert DNA.

2.9. Polymerase chain reaction

2.9.1. Synthesis of oligonucleotides

All oligonucleotides used in this thesis were synthesised commercially, and are listed in Appendix 7. The annealing temperature used for each PCR primer is also listed.

2.9.2. Polymerase chain reaction (PCR)

Each PCR mixture (50 μL) consisted of 5 μL 10 × PCR buffer (Appendix 6.6.1), 0.2 mM each of dGTP, dATP, dCTP and dTTP, 2.5 U HotStarTaq DNA

polymerase, 250 ng of each primer; and 1 – 5 μL of template DNA. An initial denaturation step (96°C for 10 min), was followed by 30 - 40 cycles of denaturation, annealing and extension on a PTC100 thermal cycler with a hot bonnet (MJ Research, Boston, USA).

For labelling of DNA probes used in Southern hybridisation experiments, 0.18 mM dTTP and 0.02 mM digoxigenin-11-dUTP (Boehringer Mannheim, Castle Hill, Australia) were added to each reaction tube.

2.10. DNA hybridisation

2.10.1. Southern hybridisation

DNA samples were digested with restriction enzymes and fragments were separated by agarose gel electrophoresis as described in section 2.7.1. The DNA was then depurinated in 0.25 M HCl for 15 min, denatured in 0.5 M NaOH, 1.5 M NaCl for 15 min, and transferred overnight to a nylon membrane (Micron Separations Inc., Westborough, USA), using 0.5 M NaOH, 1.5 M NaCl as described by Sambrook *et al.* (1989). Following transfer, membranes were washed in 2 × SSC for 10 min, and baked at 120°C for 20 min. Membranes were prehybridised for 1 h in hybridisation solution (Appendix 6.7.2, 6.7.3), at 42°C. For high stringency hybridisation, 40% v/v deionised formamide was included in the hybridisation solution. Hybridisation was performed at 42°C overnight in 40 ml hybridisation solution containing the appropriate labelled DNA (25 ng/ml), or oligonucleotide (10 pmol/ml) probe. Subsequently, membranes were washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 0.5 × SSC, 0.1% SDS at 50°C (low stringency), or 65°C (high stringency).

Hybridisation with digoxigenin (DIG)-labelled probes was detected using the DIG luminescent detection kit (Boehringer Mannheim, Castle Hill, Australia) as follows. Membranes were washed briefly in wash buffer (Appendix 6.7.5) and protein binding sites were blocked by incubating in buffer 2 (Appendix 6.7.6) for 30

min at room temperature. The membranes were incubated in a 1:5000 dilution of sheep-anti-DIG-alkaline phosphatase conjugate (in buffer 2) for 1 h, and washed twice in wash buffer for 15 min. Membranes were then equilibrated in buffer 3 (Appendix 6.7.7) for 5 min, and incubated with 1 ml/100 cm^2 CSPD solution (1:100 dilution in buffer 3) for 15 min. Membranes were then sealed in plastic bags, incubated at 37°C for 30 min, and exposed to Cronex orthoTG X-ray film (Sterling Diagnostic Imaging, Newark, U.S.A.). Exposed films were detected using an automatic processor (Kodak, Rochester, U.S.A.), located in the Department of Radiology, Royal Hobart Hospital.

2.10.2. DNA colony blot

For colony blotting experiments, individual bacterial cultures (5 ml) were pipetted onto the surface of nylon membranes and lysed by incubation in lysis buffer (Appendix 6.7.8) for 15 min. Following lysis, the membranes were neutralised by incubating in neutralisation buffer (Appendix 6.7.9) for 5 min and in 2 × SSC for 15 min. Membranes were baked (120°C, 30 min) and washed in 3 × SSC, 0.1% SDS for 3 h. The membranes were then subjected to hybridisation with DIG-labelled probes as described in section 2.12.1.

2.11. Blotting and immunological detection of proteins

2.11.1. Colony blotting

Individual bacterial cultures were either pipetted directly onto the surface of nitrocellulose membranes (broth cultures), or were applied by colony lift procedures (agar cultures), according to standard methods (Sambrook *et al.*, 1989). The bacterial cells were lysed by placing the membrane onto a piece of filter paper soaked with 0.5 M HCl for 5 min. The membranes were then neutralised by washing in 0.05 M Tris-HCl, pH 7.4 for 5 min. Immune detection was performed as described in section 2.13.4.

2.11.2. SDS-PAGE

Protein samples for electrophoresis were prepared by dilution into 1 × Lugtenberg's buffer (Appendix 6.8.1) followed by incubation in a boiling water bath for 5 min.

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by a modification of the protocol of Laemmli (1970), using the Mini-Protean II Dual Slab Cell system (Bio-Rad, Hercules, U.S.A.). Samples were subjected to electrophoresis in a polyacrylamide gel (Appendix 6.8) in 1 × SDS-PAGE running buffer (Appendix 6.8.6). Proteins were visualised by staining in Coomassie blue (Appendix 6.8.7) for 1 h, washed in destain solution (Appendix 6.8.8) until the appropriate coloration was obtained, and rinsed twice (10 min) in milliQ water. Destained gels were preserved by soaking in gel drying solution (Appendix 6.8.9) and pressing between two sheets of cellophane.

2.11.3. Western transfer

Proteins were transferred from polyacrylamide gels to nitrocellulose membranes following the protocol of Towbin *et al.* (1992), using a Mini Trans-Blot system (Bio-Rad, Hercules, U.S.A.). Electrophoretic transfer was conducted in transfer buffer (Appendix 6.9.1) at 30 V, overnight at 4°C.

2.11.4. Immunological detection

Protein binding sites were blocked by incubating the nitrocellulose membranes in blocking solution (Appendix 6.9.4) on an orbital shaker for 30 min. Membranes were then incubated in primary antibody solution (primary antibody diluted into blocking solution) for 2 to 24 h. Unbound primary antibody was removed by washing membranes three times in TTBS (Appendix 6.9.3) and once in TBS (Appendix 6.9.2). Membranes were then incubated in secondary antibody solution (secondary antibody solution diluted into blocking solution). Unbound secondary antibody was removed by washing membranes three times in TTBS and twice in

TBS. Immunodetected proteins were then visualised by incubating membranes in colour development solution (Appendix 6.9.5) until the appropriate level of staining was visible. Membranes were then washed extensively in milliQ water, dried, and sealed in plastic bags.

2.12. Preparation of TapA antiserum

A polyclonal rabbit antiserum raised against TapA from *A. hydrophila* Ah65 was obtained from Dr. M. S. Strom, Northwest Fisheries Science Center, Seattle, U.S.A. An antiserum against TapA from *A. veronii* biovar sobria BC88 was prepared by the procedure described below.

2.12.1. Induction of T7 expression cultures

Expression of recombinant proteins from pET15b was accomplished following induction of the λDE3 lysogen (encoding T7 RNA polymerase) present in *E. coli* BL21(DE3) by a modification of the protocol described by the pET system manual (Novagen, Madison, U.S.A.). *E. coli* BL21(DE3) harbouring the appropriate plasmid was grown to mid-log phase (OD_{600}=0.6) in LB broth containing 100 μg/ml carbenicillin, and the λDE3 lysogen was induced by the addition of 1 mM IPTG. Approximately 30 min after induction, rifampicin (200 μg/ml) was added to inhibit the activity of the host RNA polymerase. The induced culture was allow to incubate for a further 4.5 h at 37°C, with shaking (60 rpm).

2.12.2. Inclusion body isolation

Soluble and insoluble fractions of induced *E. coli* BL21(DE3) cultures were isolated according to the method of Reischl (1998). The induced cells from a 2 l culture were collected by centrifugation (5000 × g, 5 min) and resuspended in 200 ml sonication buffer (Appendix 6.10.1). Lysozyme and PMSF were added to final concentrations of 25 μg/ml and 0.25 mM, respectively, and the cell suspension was sonicated 10 times on ice (30 s burst at full power, 30 s cooling). The insoluble material (crude inclusion bodies) was recovered by centrifugation (4000 × g, 20 min)

and the supernatant (soluble fraction) was retained for analysis. The insoluble material was washed by resuspending in 100 ml sonication buffer and centrifuging as above.

2.12.3. Semi-purification of recombinant TapA

Semi-purification of recombinant TapA (r-TapA) was accomplished by a modification of the protocol described by Reischl (1998). The insoluble material obtained in section 2.13.2, above, was resuspended in suspension buffer (Appendix 6.10.2) containing 4 M urea, and vortexed vigorously for 5 min. The insoluble fraction was recovered by centrifugation (4000 × g, 10 min) and the supernatant saved for analysis. This procedure was repeated four times with suspension buffer containing 6 M urea. The final pellet was dissolved in 5 ml 1 × Lugtenberg's buffer.

2.12.4. Production of antisera

Polyclonal antibodies were generated against the r-TapA protein in New Zealand white rabbits. The r-TapA protein was isolated by SDS-PAGE. The gel slice containing the appropriate protein band was homogenised in 2 ml PBS by passing three times through a 18 gauge needle and three times through a 22 gauge needle. The resulting homogenate (~ 100 μg r-TapA) was emulsified in 1 ml complete Freund's adjuvant and administered subcutaneously at multiple sites to each of three rabbits. The rabbits were boosted at days 21, 35, and 49 with ~100 μg r-TapA emulsified in incomplete Freund's adjuvant. Blood (~ 5 ml) was collected from the rabbits at days 28, 42, and 56, and assessed for antibody production. A final bleed of approximately 20 ml was taken at day 63.

Sera were prepared as follows. Blood samples were allowed to clot, and placed at 4°C for 4 h to allow clot retraction. Clots were then removed carefully using a sterile pasteur pipette, and remaining cells were removed by centrifugation (3000 × g, 15 min, 4°C). Complement was inactivated by incubating the sera at 56°C for 15 min.

2.12.5. Absorption of antisera

Polyclonal antisera were absorbed against whole bacterial cells, bacterial cell lysates immobilised on nitrocellulose membranes, or against an acetone powder of the appropriate bacterial strain.

For whole cell absorptions, a 1 ml culture of the appropriate bacterial strain was pelleted by centrifugation (15000 × g, 1 min) and resuspended in 1 ml of the unabsorbed antiserum containing 0.01% w/v NaN_3. The mixture was incubated 4 to 16 h at 4°C, and the bacterial cells were removed by centrifugation (15 000 × g, 2 min). This procedure was repeated as needed. After the final absorption, the antiserum was sterilised by passing through a 0.2 μm filter (Gelman Sciences, Ann Arbor, U.S.A.).

For absorptions against whole cell lysates, lysates of the appropriate bacterial strain were immobilised on nitrocellulose membranes by western transfer as described in Section 2.13.3. The antiserum to be absorbed was diluted 1 in 5 in blocking solution (Appendix 6.9.4) and incubated with the nitrocellulose membrane sealed in plastic bags overnight, with shaking, at 4°C. This procedure was repeated as required, and the antiserum was sterilised by passing through a 0.2 μm filter (Gelman Sciences, Ann Arbor, U.S.A.).

For absorption against an acetone powder, an acetone powder of the appropriate bacterial strain was prepared as follows. Cells from an overnight culture were pelleted by centrifugation (5000 × g, 10 min) and resuspended in 6 ml of 0.85% saline. Following incubation on ice for 5 min, 24 ml ice cold acetone was added, and the solution was mixed by vortexing. Insoluble material was pelleted by centrifugation (10000 × g, 10 min, 4°C), and resuspended in 10 ml ice cold acetone by vortexing. After incubation on ice for 10 min, the insoluble material was again recovered by centrifugation, transferred to Whatman 3M filter paper, and allowed to dry overnight. The pellet was then transferred to an air-tight container and ground into a fine powder using the blunt end of a sterile glass pasteur pipette. The resulting powder was subsequently used for absorptions by adding to antiserum samples to a

final concentration equivalent to 1% w/v. The mixture was incubated overnight at 4°C with shaking, and the powder was removed by centrifugation (15000 × g, 5 min). This procedure was repeated as needed, and the antiserum was sterilised as described above.

2.13. Transmission electron microscopy

Electron microscopic examinations were performed using a Philips 410 transmission electron microscope housed in the Discipline of Pathology, University of Tasmania.

2.13.1. Negative staining of bacteria

A drop (60 µl) of bacterial broth culture (or a quarter of a loop of agar culture suspended in 500 µl PBS) was placed onto clean dental wax, and a formvar-coated 200 mesh nickel grid was floated on the bacterial suspension for 3 min. The grid was then washed by dipping 5 times into a drop of distilled water, excess fluid was removed by touching a small piece of filter paper to the side of the grid. The grid was floated on a 60 µl drop of uranyl acetate (Appendix 6.11.3) for 30 s, washed extensively with distilled water and allowed to dry on a piece of filter paper.

2.13.2. Immune electron microscopy

For immunogold electron microscopic examination, bacteria on formvar coated grids were prepared as described above, washed briefly in a drop of TTB buffer (Appendix 6.11.2) and floated on a drop of 5% bovine serum albumin in TTB for 15 min to block protein binding sites. The grids were then washed three times in TTB and reacted on a drop of the TapA antiserum (1:10, 1:100 and 1:1000 dilution in TTB) for 60 min, which had been absorbed against an acetone powder of *A. veronii* biovar sobria BC88*tapA*::Ω as described in section 2.12.5. Grids were then washed as before and exposed to goat anti-rabbit IgG conjugated with 10 nm gold particles (BioCell, Cardiff, U.K.) diluted 1:50 in TTB for 60 min. The grids were then washed

and negatively stained, as described above. A 1:100 dilution of Bfp antiserum served as a positive control for all immunogold electron microscopy experiments.

2.14. Characterisation of *Aeromonas veronii* biovar sobria BC88*tapA*::Ω

2.14.1. Crude toxin preparation

Crude toxin preparations consisted of cell-free culture supernatants prepared from each test strain. In brief, following incubation in 5 ml TSBY (37°C, 200 rpm, 20 h) cells were removed by centrifugation (3000 × g, 30 min) and the supernatant was sterilised by passing through a 0.2 mm filter (Gelman Sciences, Ann Arbor, U.S.A.).

2.14.2. Haemolysin production

Rabbit erythrocytes were prepared from New Zealand white rabbits by collecting approximately 2 ml blood into 10 ml Alsever's solution (Appendix 6.12.1) and the cells were recovered by centrifugation (600 × g, 10 min). The cells were then resuspended in PBS (Appendix 6.13.1), centrifuged as above, and the PBS and buffy coat were removed with a sterile pasteur pipette. This procedure was repeated until the PBS supernatant remained clear, and a 1% suspension of cells was prepared by adding 100 µl erythrocytes to 10 ml PBS.

For the assay, 100 µl PBS was added to all wells of a 96-well microtitre tray (U bottom wells; Nalgene, Rochester, U.S.A.), except those in column 2 and rows A (to which 100 µl distilled water was added for a positive control) and B (to which 100 ml TSBY was added for a negative control). Each crude toxin preparation was added, in duplicate, to wells in column 2 except rows A and B. Doubling dilutions of each toxin were prepared from rows 2 to 12 by placing 100 µl of toxin into the following well containing PBS. The final 100 µl from row 12 was discarded. 100 µl of the erythrocyte suspension prepared above was then added to each well and the tray was

incubated at 37°C for 1 h followed by incubation at 4°C for 1 h. After this incubation the tray was centrifuged (600 × g, 10 min, 4°C). The haemolysin titre for each sample recorded as the highest dilution in which 50% haemolysis was observed. *A. veronii* biovar sobria CA110 (titre > 2048) and *A. caviae* CA195 (titre = 0) were included as controls.

2.14.3. Cytotoxin production

A 96-well cell culture plate was seeded with Vero cells as described in section 2.16.1, and incubated (37°C, 5% CO_2) until a confluent monolayer was produced. Serial 2-fold dilutions of each toxin to be assayed were then applied to each well, as described for the haemolysin assay (Section 2.16.2), except that MEM + 5% FCS was used in place of PBS. The plate was incubated for 40 min (37°C, 5% CO_2) after which the medium in each well was replaced with fresh medium. The cytotoxin titre was recorded as the highest dilution in which 75% of cells were dead or rounded.

2.14.4. Enterotoxin production

Enterotoxin production was measured by intestinal fluid accumulation in 2- to 4-day-old suckling mice (Burke *et al.*, 1981). Pontamine sky blue (0.05% w/v) was added to each crude toxin preparation to be assayed in order to aid visualisation during administration into the stomach. Each toxin (100 µl) was injected directly into the stomach of suckling mice via a 29 gauge needle. Mice were incubated in a moist 37°C environment for 2 h after which they were placed on pre-weighed squares of aluminium foil and killed by cervical dislocation. Each mouse was then dissected and the intestines removed. Enterotoxin activity was recorded as a ratio of intestinal weight to the remaining body weight (IW:RBW). A ratio ≥ 0.075 was recorded as positive.

2.15. Cell culture

2.15.1. Maintenance of cell lines

Two cell lines, HEp-2 human laryngeal carcinoma cells (ATCC CCL23) and Henle 407 intestinal cells (ATCC CCL16) were used. All cell lines were cultured in Eagle's minimal essential medium (MEM; Appendix 3.2.1.) containing 5-10% foetal calf serum (FCS) and incubated at 37°C in a 5% CO_2 atmosphere incubator (Heraeus, Plainfield, U.S.A.).

Cell lines to be subcultured were examined with an inverted phase contrast microscope to verify confluence and integrity of the monolayer. The old culture medium was removed and the monolayer was washed with 10 ml PBS, after which 1 ml trypsin solution (Appendix 6.13.2.) was added. Following incubation (1 to 2 min), the cells were dislodged, and 10 ml MEM + 10% FCS was added. The cells were subsequently washed by centrifugation (600 × g, 5 min) and resuspended in 1 ml fresh MEM + 10% FCS. Approximately 0.3 ml of this cell suspension was then added to 10 ml MEM + 10% FCS in a T-25 tissue culture flask (Sarstedt, Leicester, U.K.) and incubated at 37°C in a 5% CO_2 atmosphere.

2.15.2. Freezing and thawing of cell lines

Cells to be frozen were washed, trypsinised and pelleted, as described above, and resuspended in 1 ml MEM containing 10% FCS and 20% v/v dimethylsulphoxide in 2 ml cryogenic vials (Nalgene, Rochester, U.S.A.). The vials were gradually frozen to –80°C using a Cryo-freezing container (Nalgene, Rochester, U.S.A.) and stored under liquid nitrogen.

For recovery, vials of frozen cells were rapidly thawed in a 37°C water bath, and the cell suspension was diluted with an equal volume of MEM + 10% FCS added dropwise over 5 min, followed by a 15 min incubation at room temperature. An equal volume of MEM + 10% FCS was again added dropwise over 5 min, and the cell suspension was allowed to stand for a further 15 min. The cells were washed

three times by centrifugation (200 × g, 5 min) and resuspension in 10 ml MEM + 10% FCS. They were finally resuspended in 10 ml growth medium and incubated at 37°C in a 5% CO_2 atmosphere.

2.15.3. Bacterial adhesion to cell lines

Adhesion to cell lines was assessed by bright field microscopy, as described by Carrello *et al.* (1988). Briefly, 1 ml aliquots (1×10^7) of each strain in MEM + FCS were inoculated onto semi-confluent cell line cultures grown on glass coverslips. Following incubation (37°C, 5% CO_2) for 60 min, non-adherent bacteria were removed by washing four times with 2 ml PBS. Cell monolayers were fixed with 1 ml 3:1 methanol:acetic acid for 5 min, and stained with May-Grunwald and Geimsa stains (BDH, Poole, UK) and mounted for microscopy. A minimum of three coverslips were assayed for each strain in each experiment.

2.15.4. Bacterial adhesion to intestinal tissue

Bacterial adhesion to intestinal tissue was assessed as follows. Briefly, 5×10^6 cfu of each strain to be tested diluted into 1 ml MEM + 10% FCS was inoculated onto intestinal tissue in 24-well tissue culture plates. Following incubation (37°C, 5% CO_2, 1 h), intestinal specimens were washed with PBS, fixed in formalin, embedded in paraffin and sectioned. Sections (10 μm) on glass slides were deparaffinised, hydrated and stained with haematoxylin - eosin for light microscopy.

2.16. *In vivo* animal experiments

2.16.1. Infant mouse colonisation and competition experiments

BALB/c infant mice were inoculated orally with test bacteria according to the protocol of Attridge *et al.* (1993). Log phase cultures of each strain to be tested were diluted to obtain a culture containing ~ 2×10^8 cfu/ml. Blue food colouring (5 μl/ml) was added to facilitate monitoring of the inoculation procedure. The number of bacteria administered was determined retrospectively by plate counts. Three- to 5-

day-old infant mice were removed from their mothers 4 h prior to infection. They were inoculated by intragastric lavage with 50 µl of bacterial suspension (~ 1×10^7 cfu per mouse) and held at 25°C for 24 h, after which time they were sacrificed and their intestines removed. The intestines were homogenised in 5 ml PBS and individual bacteria were quantitated by plate counts of serial dilutions of each homogenate on TSAY plates.

For competition assays, log-phase cultures of wild-type and mutant strains were diluted to ~ 2×10^7 cfu/ml and a suspension containing equal volumes of each strain was prepared. Each mouse was administered 50 µl (~ 5×10^5 of each test strain) of this suspension by intragastric lavage as described above. The precise input ratio was determined retrospectively by plating serial dilutions of the suspension on TSAY (total bacteria) and TSAY containing 50 mg/ml spectinomycin (mutant bacteria). Mice were sacrificed after 24 h, and their intestines were removed and homogenised in PBS. Individual bacteria were quantitated on selective media, as described above. The colonisation index was calculated as the ratio of wild-type to mutant colonies following 24 h incubation.

2.16.2. Removable intestinal tie adult rabbit diarrhoeal (RITARD) model

The RITARD model was performed by Dr. M. J. Albert at the International Center for Diarrhoeal Disease Research, Dhaka, Bangladesh. In brief, *Aeromonas* strains were tested in New Zealand white rabbits (1.25 to 1.65 kg, 7 to 9 weeks old, 3 to 4 weeks post weaning) according to the protocol of Pazzaglia *et al.* (1990). Each strain was tested in nine rabbits, while five rabbits received *E. coli* EC101 as a negative control. Each rabbit was administered with 1×10^{10} cfu in 10 ml BHIB, injected into the jejunum close to the ligament of Treitz. Animals were monitored for seven days for diarrhoeal symptoms and faecal shedding of bacteria. They were sacrificed 8 d post challenge.

Chapter 3

Identification of two distinct families of type IV pili in *Aeromonas* species associated with gastroenteritis

3.1. Introduction

As outlined in section 1.10.2.2, the type IV pili purified from diarrhoeal *Aeromonas* strains comprise a related family of structures, which have been termed Bfp pili. The initial aim of the experiments described in this chapter was to clone the gene(s) encoding the Bfp pilus expressed by *A. veronii* biovar sobria BC88. This would allow construction of an isogenic mutant strain, lacking this pilus, which could be used to establish definitively that this structure plays an important role in enterocyte adhesion and *in vivo* colonisation. Secondly, the availability of the cloned gene(s) would allow their distribution in *Aeromonas* species to be determined, and establish whether this pilus might be useful as a marker for virulent strains.

3.2. Cloning of part of a type IV pilus gene cluster from *Aeromonas veronii* biovar sobria BC88

Several different approaches were employed to clone genes encoding type IV pili from *A. veronii* biovar sobria BC88. These approaches included traditional methods such as transposon mutagenesis, the use of oligonucleotide and DNA probes, and library construction followed by screening with a Bfp-specific antiserum. However, these methods were not successful at cloning the genes encoding the Bfp pilus. Therefore, I will provide a brief overview of these experiments below. The 5' end of a gene encoding the Bfp pilin was ultimately cloned by PCR using degenerate primers based on the N-terminal amino acid sequence of this protein.

Initial attempts to identify the *bfp* gene(s) utilised the transposon Tn*phoA*, which specifically enriches for mutations in genes encoding exported proteins. This approach was unsuccessful due to something intrinsically incompatible between the transposon system chosen and *Aeromonas* species. Following mating of five separate *phoA* strains of *A. veronii* biovar sobria BC88 with *E. coli* SM10lpir (pRT733), kanamycin resistant transconjugants were obtained at a frequency of approximately 1×10^{-5} following five hours mating. However, transconjugants expressing active *phoA* fusions were obtained at a frequency less than 1×10^{-4} per kanamycin-resistant

transconjugant. These observations are consistant with pRT733 being efficiently mobilised into the recipient *Aeromonas* strains, but being stably maintained in these strains. Alternatively, the *phoA* strains may have been pleiotrophic protein export mutants rather than being mutated specifically in the *phoA* gene. To answer this latter possibility, each of the *phoA* mutants were examined by transmission electron microscopy for the ability to produce surface pili. All strains were found to express pili on their surface, indicating that they retained the ability to secrete extracellular proteins. Therefore, it appeared that the suicide vector used to deliver this transposon was at least partially stable in the *Aeromonas* recipient strains. Furthermore, subsequent experiments indicated that *Aeromonas* species may encode multiple copies of the *bfp* subunit gene (Section 3.2.2, below), which would make transposon mutagenesis unsuitable for preparing a Bfp mutant. Consequently, attempts were made to clone and mutate the *bfp* gene(s) directly.

Four DNA probes were then used in an attempt to identify *bfp* gene(s) encoding type IV pili by Southern hybridisation. This approach led to the cloning of a type IV prepilin gene, although, as described below, this gene did not encode the Bfp pilin protein, but led to the demonstration that *Aeromonas* species possess genes encoding a second family of type IV pili.

3.2.1. Design and construction of probes

Four DNA probes were initially used in an attempt to identify the gene encoding the Bfp pilin by Southern hybridisation. These probes are illustrated in Fig. 3.1.

3.2.1.1. Degenerate oligonucleotide probe based on the Bfp N-terminal amino acid sequence (P001)

The availability of the N-terminal amino acid sequence of Bfp from BC88 (Kirov and Sanderson, 1996) provided the opportunity to construct a degenerate probe, which could be used to identify DNA sequences corresponding to the Bfp sequence. This probe was constructed based on amino acids 19 to 25 of the N-

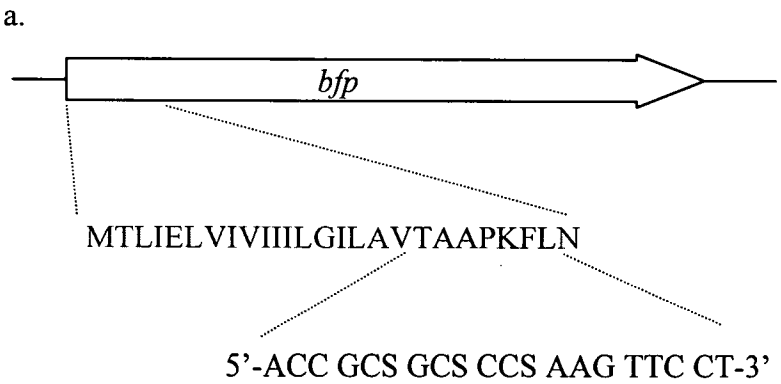
Fig. 3.1. DNA probes used in this study.

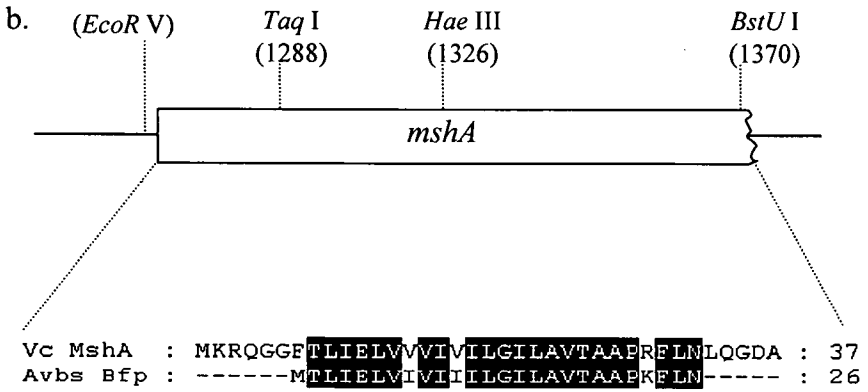
[a] Degenerate oligonucleotide probe P001 based on the N-terminal amino acid sequence of the Bfp pilin protein.

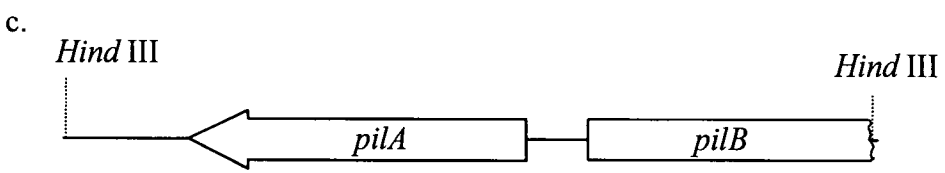
[b] 111 bp probe to the N-terminal 37 amino acids of *Vibrio cholerae* MshA. Below is a sequence alignment of the N-terminal amino acid sequence of MshA (Jonson *et al.*, 1994) and Bfp (Kirov and Sanderson, 1996).

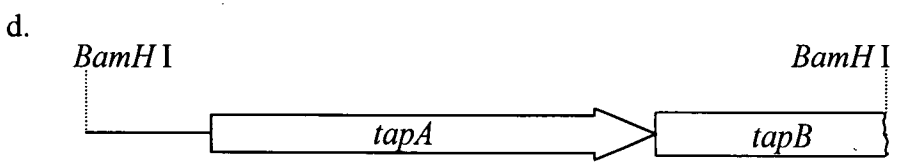
[c] 1.2 kb *Hind* III fragment containing *Pseudomonas aeruginosa* PAO1 *pilA*.

[d] 1.0 kb *BamH* I fragment containing *Aeromonas hydrophila* Ah65 *tapA*.


a.
bfp
MTLIELVIVIIILGILAVTAAPKFLN
5'-ACC GCS GCS CCS AAG TTC CT-3'


b.
(EcoR V)
Taq I
(1288)
Hae III
(1326)
BstU I
(1370)
mshA
Vc MshA : MKRQGGFTLIELVVVIVILGILAVTAAPRFLNLQGDA : 37
Avbs Bfp : ------MTLIELVIVIIILGILAVTAAPKFLN----- : 26


c.
Hind III
Hind III
pilA
pilB


d.
BamH I
BamH I
tapA
tapB

terminal amino acid sequence to avoid possible cross-reaction with proteins that possess type IV pilin-like N-terminal amino acid sequences, and was designed with reference to *Aeromonas* codon usage data (Codon Usage Database, DNA Information and Stock Center, National Institute of Agrobiological Resources, Tsukuba, Japan; http://www.dna.affrc.go.jp/~nakamura). The construction of this probe (P001) is depicted in Fig. 3.1a.

3.2.1.2. *Vibrio cholerae mshA* N-terminal probe

As described in section 1.10.2.2, the N-terminal amino acid sequence of the Bfp pilin proteins exhibit high homology with the *Vibrio cholerae* mannose-sensitive haemagglutinin pilus subunit, MshA. The gene encoding this protein from *V. cholerae* O1 El Tor (strain Phil 6973) has been cloned and sequenced by Jonson *et al.* (1994). Therefore a probe based on the region of the *mshA* encoding the first 37 amino acids of MshA was constructed. In this region, there are only 5 amino acid residue mismatches between the Bfp subunit protein and MshA (see Fig. 3.1b). This probe was constructed by subcloning a 111 bp *EcoR* V/*BstU* I fragment from pGJX111 (Jonson *et al.*, 1994) into the *EcoR* V site of plasmid pZErO-2.1, generating pTB024. The insert of pTB024 was labelled with digoxigenin by PCR using M13 forward and reverse primers.

3.2.1.3. *Pseudomonas aeruginosa pilA* probe

A probe consisting of the entire gene encoding the *Pseudomonas aeruginosa* prepilin, *pilA* and the N-terminal region of *pilB* (Fig. 3.1c), was constructed by labelling the 1.2 kb *Hind* III fragment contained in plasmid pAW102 with digoxigenin, as described in section 3.2.1.2.

3.2.1.4. *Aeromonas hydrophila* Ah65 *tapA* probe

During the course of these studies, the sequence of a type IV pilus biogenesis gene cluster (*tapABCD*) from *A. hydrophila* Ah65 was deposited into the Genbank database (accession number U20255), and later published (Pepe *et al.*, 1996). The predicted amino acid sequence of the cloned pilin gene (*tapA*) differed slightly from the N-terminal sequences of the Bfp pili purified from diarrhoeal isolates. However,

these authors suggested that the discrepancies may reflect difficulties in obtaining reliable N-terminal sequences, particularly for the modified (N-methylated) N-terminal amino acid residues characteristic of mature type IV pilin proteins (Pepe *et al.*, 1996). The *tapA* prepilin gene (contained in plasmid pCP1065; Fig. 3.1d) was labelled with digoxigenin as described in section 3.2.1.2.

3.2.2. Degenerate oligonucleotide probe (P001)

The degenerate oligonucleotide probe P001 was used to probe *BamH* I digests of DNA from BC88, and other *Aeromonas* strains from which Bfp-homologous proteins had been purified. The results are shown in Fig. 3.2.

P001 hybridised with multiple bands from BC88, *A. hydrophila* Ae6, *A. veronii* biovar sobria Ae24 and *A. caviae* CA195, but did not hybridise with DNA from *A. veronii* biovar sobria strains Ae1 and Tap13 under high stringency conditions (65°C, 0.5 × SSC). P001 hybridised with two *BamH* I fragments from BC88, with sizes approximately 5.8 kb and 1.0 kb, respectively.

3.2.2.1. Cloning of the *BamH* I fragments that hybridised with P001

The 5.8 kb and ~1.0 kb *BamH* I fragments that hybridised with P001 were cloned from *BamH* I sub-genomic libraries. *BamH* I digested DNA fragments approximately 4.5 to 7.0 kb were eluted from an agarose gel, ligated into plasmid pUCP19, and transformed into competent *E. coli* JM109. Similarly, *Bam*H I fragments 0.2 to 2.0 kb were eluted, ligated into plasmid pZErO-2.1, and transformed into *E. coli* TOP10F'. Recombinant colonies (116) from the 5.8 kb library and 270 colonies from the 1 kb library were screened with the P001 probe. Single clones from the 5.8 kb library (designated pTB001), and the 1 kb library (designated pTB004), exhibited strong hybridisation with this probe, while two clones from the latter library (pTB005 and pTB006), exhibited slightly weaker hybridisation signals. The ~1 kb clones were all distinct from one another as determined by their different restriction enzyme digestion patterns following digestion with two restriction enzymes, *Sau3A* I and *Msp* I, which have four base pair recognition sequences.

Fig. 3.2. Southern hybridisation of *Aeromonas* strains probed with P001.

Chromosomal DNA was digested with *BamH* I, transferred to nylon membrane and hybridised with P001 under high stringency conditions. DNA size markers (λ DNA digested with *Hind* III, size in kilobases) are indicated to the left.

Lane 1. *A. veronii* biovar sobria BC88

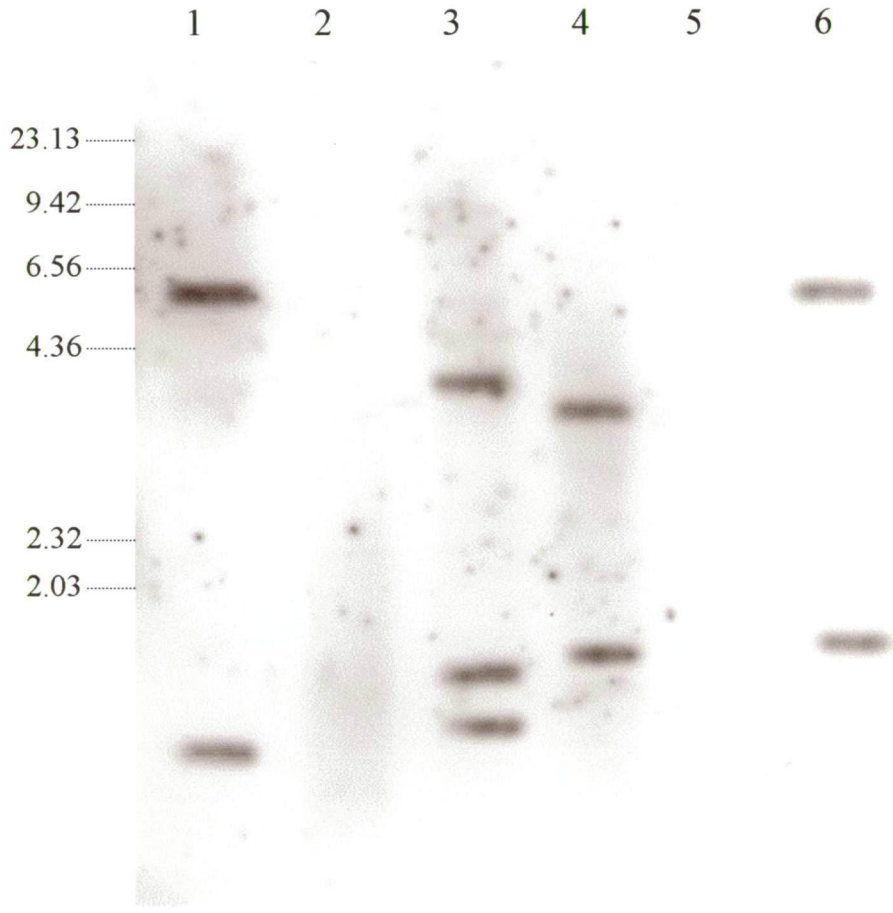
Lane 2. *A. veronii* biovar sobria Ae1

Lane 3. *A. hydrophila* Ae6-W

Lane 4. *A. veronii* biovar sobria Ae24

Lane 5. *A. veronii* biovar sobria TAP13

Lane 6. *A. caviae* CA195


1
2
3
4
5
6
23.13
9.42
6.56
4.36
2.32
2.03

In order to allow subcloning of a smaller fragment of pTB001 for sequence analysis, Southern hybridisation analysis of various restriction enzyme digests was performed. The hybridising sequence was localised to a 1.5 kb *EcoR* I/*Kpn* I restriction enzyme fragment, and this fragment was subcloned into pGEM3zf(+), designated pTB002, for sequence analysis.

3.2.2.2. Sequence analysis

Nucleotide sequences of pTB004, pTB005 and pTB006 were determined for both strands using M13 forward and reverse primers. In all cases, no sequences homologous to the N-terminus of the Bfp subunit protein were identified. Similarly, no sequences corresponding with P001 were found. This indicates that probe P001 was binding non-specifically to DNA from *A veronii* biovar sobria strains BC88 and Ae24, *A. hydrophila* Ae6-W, and *A. caviae* CA195.

Good sequence data could not been obtained for the ~ 1.5 kb insert of pTB002, despite two separate attempts at sequencing this insert. The reason for this remains unclear, although this insert may have had a particularly GC-rich sequence, which is known to produce sequencing problems. As sequencing of this insert proved difficult, the entire 5.8 kb *BamH* I fragment contained in pTB001 was subcloned into the mobilisable Cm^R plasmid pBBR1MCS (Kovach *et al.*, 1994), designated PTB003, and mobilised into *A. veronii biovar* sobria BC96 and *A. caviae* CA195. Both of these strains produce a serologically distinct Bfp pilus, and an intact copy of the Bfp pilin gene contained in plasmid pTB003 would presumably be expressed by these strains. No expression of the Bfp subunit protein was detected by western blot analysis.

The insert of pTB004 was 1239 bp. Sequence analysis identified one open reading frame (ORF), which exhibited limited homology with hypothetical proteins from *E. coli* (32% identity and 47% similarity across 162 amino acids) and *Helicobacter pylori* (39% identity and 54% similarity across 103 amino acids), and with a cardiolipin synthase from *E. coli* (33% identity and 53% similarity across 101 amino acids).

The insert of pTB005 was 692 bp, and contained a 302 bp region that encoded a 100 amino acid sequence which exhibited homology with ATP synthase proteins UncD from *E. coli* (87% identity and 97% similarity to amino acids 347 to 446), and AtpD from *Haemophilus influenzae* (88% identity and 94% similarity).

The insert of pTB006 was 735 bp. The first 103 bp encoded a 33 amino acids sequence which exhibited 96% identity and 100% similarity with the C-terminal region (amino acids 304 to 336) of the L-allo-threonine aldolase from *A. jandaei*, and 40% identity and 61% similarity with a similar enzyme from *Pseudomonas aeruginosa*.

In summary, the degenerate probe P001 was not specific for the *bfp* gene(s) and use of this probe was not pursued further.

3.2.3. *Vibrio cholerae mshA* and *Pseudomonas aeruginosa pilA* probes

Digests of chromosomal DNA from *A. veronii* biovar sobria BC88 were examined by Southern hybridisation with the *V. cholerae mshA* and *P. aeruginosa pilA* probes. Digests of chromosomal DNA from *P. aeruginosa* PAO1 and *V. cholerae* O1 El Tor H1 were includes as positive controls for the appropriate probe. Hybridisation was performed under high and low stringency conditions. No homologous sequences in *A. veronii* biovar sobria BC88 were identified using these probes.

3.2.4. *Aeromonas hydrophila* Ah65 *tapA* probe

Various digests of chromosomal DNA from *A. veronii* biovar sobria BC88 were also examined by Southern hybridisation with the *A. hydrophila* Ah65 *tapA* probe. This probe hybridised with a single, ~1.0 kb *BamH* I fragment (Fig. 3.3, lane 1). Therefore, *BamH* I digested DNA fragments of sizes 0.5 to 2 kb were eluted from an agarose gel, ligated into the plasmid pUCP19 and transformed into *E. coli* strain JM109. Approximately 300 recombinant colonies were screened with the *tapA* probe, and this probe hybridised strongly to one clone, which was designated pTB011.

Fig. 3.3. Southern hybridisation of *A. veronii* biovar sobria BC88 probed with *tapA* cloned from *A. hydrophila* Ah65.

Chromosomal DNA was digested with the restriction enzymes listed below, transferred to nylon membrane and hybridised with digoxigenin-labelled *tapA* under high stringency conditions. DNA size markers (λ DNA digested with *Hind* III, size in kilobases) are indicated to the left.

Lane 1. *BamH* I

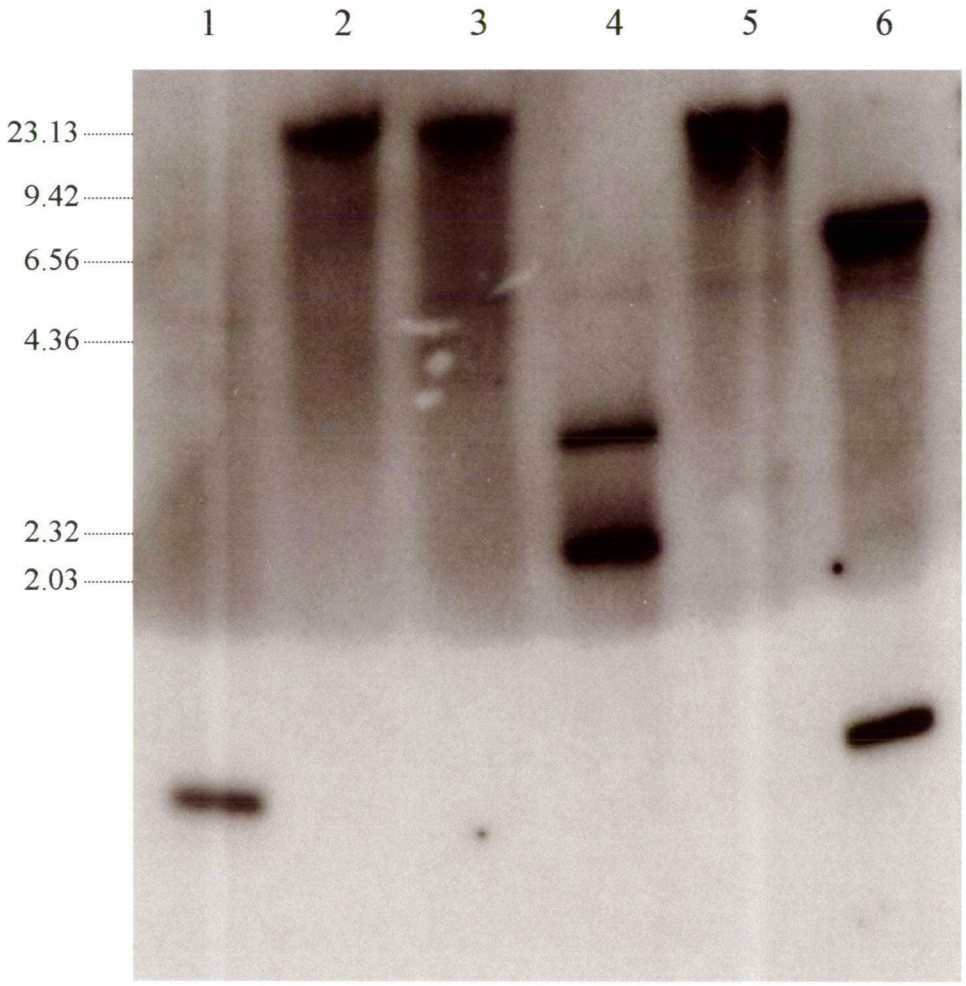
Lane 2. *Hind* III

Lane 3. *EcoR* I

Lane 4. *Pst* I

Lane 5. *Sac* I

Lane 6. *Kpn* I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23.13
9.42
6.56
4.36
2.32
2.03

A restriction map of pTB011 was generated using the enzymes *BamH* I, *Hind* III, *Pst* I, *EcoR* I, *Kpn* I and *Sac* I. The insert contained in pTB011 possessed single *Pst* I and *Kpn* I sites, and these appeared to be in similar positions to those of the *A. hydrophila tapA* clone, pCP1065. Therefore, three restriction fragments (1 kb *BamH* I , pTB012; 0.5 kb *BamH* I-*Pst* I , pTB013; and 0.5 kb *Pst* I-*BamH* I, pTB014) were subcloned into pGEM®-3zf(+) for sequence analysis.

3.2.4.1. Sequence analysis

Nucleotide sequences were determined for pTB012, pTB013, and pTB014, using M13 forward and reverse sequencing primers. Subsequent sequence alignments identified a single complete (453 bp) open reading frame and one partial (321 bp) open reading frame (Fig. 3.4), which exhibited high homology with *tapA* and *tapB*, respectively, from *A. hydrophila* Ah65 (Pepe *et al.*, 1996). These open reading frames were, therefore, also designated *tapA* and *tapB* (submitted to EMBL/Genbank/DDBJ Data Libraries under accession number U81377).

A sequence resembling a consensus sigma 54 (σ54)-dependent (RpoN-dependent) promoter was located approximately 200 bp upstream of *tapA*. The *tapA* and *tapB* ORFs would both be transcribed in the same direction. This orientation is the same as the organisation of the pilus gene clusters of *A. hydrophila* and *V. cholerae*, but was different to the pilus gene cluster possessed by *P. aeruginosa* (Johnson *et al.*, 1986; Pepe *et al.*, 1996; Fullner and Mekalanos, 1999).

3.2.4.2. Amino acid sequence comparisons

The sequence of pTB012 was used to derive amino acid sequences of the *tapA* and partial *tapB* open reading frames. When compared, it was seen that the *tapA* gene from strain BC88 encoded a protein that was clearly different from the purified Bfp pilin (Fig. 3.5). The TapA proteins from *A. veronii* biovar sobria BC88 and *A. hydrophila* Ah65 were more closely related to the "classical" type IV pilin proteins of *Pseudomonas syringae*, *Dichelobacter nodosus*, and *Pseudomonas aeruginosa*, than they were to the *Aeromonas* Bfp pilin proteins (Fig. 3.5).

Fig. 3.4. Genetic map of the 1.0 kb *BamH* I fragment contained in pTB012.

The direction of transcription is indicated by the direction of the arrowheads for the open reading frames. Relevant restriction sites are also shown.

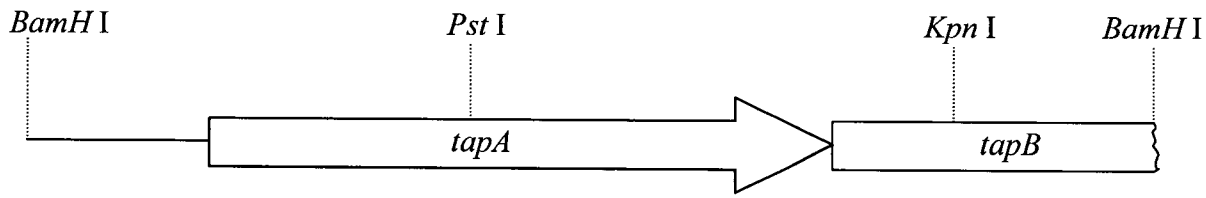

BamH I
Pst I
Kpn I
BamH I
tapA
tapB

Fig. 3.5. Amino acid sequence comparison of *A. veronii* biovar sobria BC88 TapA with type IV prepilins from other bacteria.

Residues conserved in all sequences are shaded black. Conserved residues unique to each group are shaded grey. Sequences of Bfp pilin proteins were generated by N-terminal amino acid sequencing of purified proteins. Sequences of "classical" type IV pilin and Tap pilin proteins were derived from DNA sequences. Species abbreviations and the pilin subunit designations are as follows:

"Classical" type IV pili; Ps, *Pseudomonas syringae* DC3000, PilA (Raineri *et al.*, unpublished); Dn, *Dichelobacter nodosus* 351, FimA (Hoyne *et al.*, 1989); Pa, *Pseudomonas aeruginosa* P1, PilA (Pasloske *et al.*, 1988).

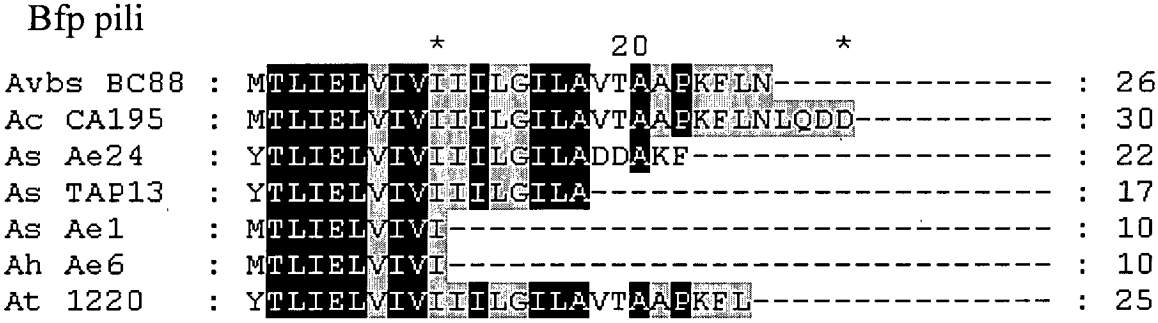
Tap pili; Avbs, *A. veronii* biovar sobria BC88, TapA; Ah, *A. hydrophila* Ah65, TapA (Pepe *et al.*, 1996); As, *A. salmonicida* A449, SfpA (Lutwyche *et al.*, unpublished).

Bfp pili; Avbs BC88, *A. veronii* biovar sobria BC88, Sfp (Kirov and Sanderson, 1996); Ac CA195, *A. caviae* CA195, Cfp (Kirov *et al.*, 1998); As Ae24, '*A. sobria*' Ae24 (Hokama and Iwanaga, 1992); As TAP13, '*A. sobria*' TAP13 (Iwanaga and Hokama, 1992); As Ae1, '*A. sobria*' Ae1 (Hokama and Iwanaga, 1991); Ah Ae6, *A. hydrophila* Ae6 (Hokama *et al.*, 1990); At 1220, *A. trota* 1220 (Nakasone *et al.*, 1996).

"Classical" type IV pili

Tap pili

Bfp pili



The TapA and Bfp pilin proteins also appear to have different molecular weights. Values published for the Bfp pilins have ranged from 19 to 23 kDa, and this protein from *A. veronii* biovar sobria strain BC88 had a molecular weight of 21 kDa (Kirov and Sanderson, 1996). The molecular weight of TapA from this strain (calculated from its derived amino acid sequence) was estimated at ~ 17 kDa, similar to the molecular weight estimated for TapA from *A. hydrophila*. The size of the latter was confirmed by western blotting with *A. hydrophila* TapA antiserum obtained from Dr. M. S. Strom, Northwest Fisheries Science Center, Seattle, U.S.A. (section 2.12; Fig. 3.6). This antiserum also recognised an ~ 17 kDa protein from whole cell lysates of *A. veronii* biovar sobria TAP13. Slight reactivity was observed for an ~ 17 kDa band *A. veronii* biovar sobria Ae24, suggesting that there may be significant immunological cross reactivity between TapA proteins and that this cross reactivity is not confined to species-specific boundaries. Unfortunately, western blots of whole cell lysates of *A. veronii* biovar sobria BC88 grown under the same conditions did not react with this antiserum.

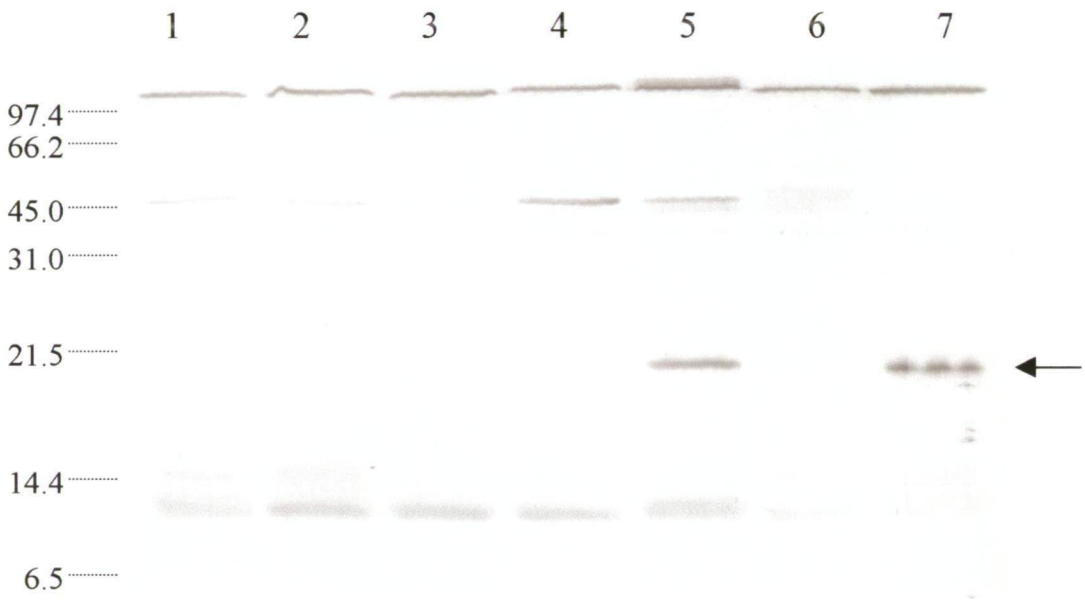
The pilin subunit protein encoded by *tapA* (TapA) consisted of 151 amino acids, nine more than the *A. hydrophila* TapA, and exhibited extensive homology with type IV prepilin proteins from *A. hydrophila* (51% identity, 67% similarity), *A. salmonicida* (55% identity, 66% similarity), *Pseudomonas syringae* (45% identity, 56% similarity), *Dichelobacter nodosus* (38% identity, 53% similarity),and *Pseudomonas aeruginosa* (39% identity, 50% similarity) (Fig. 3.7). Homology was highest in the N-terminal regions as is characteristic of these proteins. The prepilins from *A. veronii* biovar sobria and *Pseudomonas aeruginosa* exhibited 75% identity and 87% similarity in the first 41 amino acids.

The N-terminal region of *tapA* from strain BC88 contained a consensus type IV leader peptidase/N-methyltransferase cleavage site (Fig. 3.7, arrow). The first six amino acids encode the putative leader sequence that is presumably cleaved from this protein. The resulting N-terminal phenylalanine would be methylated to produce the mature pilin (Strom and Lory, 1993; Tennent and Mattick, 1994). The primary amino acid sequence of TapA contains a total of four cysteine residues at positions 56, 65,

Fig. 3.6. Western blot analysis of *Aeromonas* strains with *A. hydrophila* Ah65 TapA antiserum.

Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose where they were reacted with the *A. hydrophila* Ah65 TapA antiserum. This antiserum was prepared against a recombinant TapA protein as described in section 5.5.1.) Molecular weight markers (in kilodaltons) are indicated to the left. The 17 kDa protein corresponding to the TapA protein is indicated to the right with an arrow.

Lane 1. *A. veronii* biovar sobria BC88
Lane 1. *A. veronii* biovar sobria Ae1
Lane 2. *A. hydrophila* Ae6-W
Lane 3. *A. veronii* biovar sobria Ae24
Lane 4. *A. veronii* biovar sobria TAP13
Lane 5. *A. caviae* CA195
Lane 6. *A. hydrophila* Ah65


1
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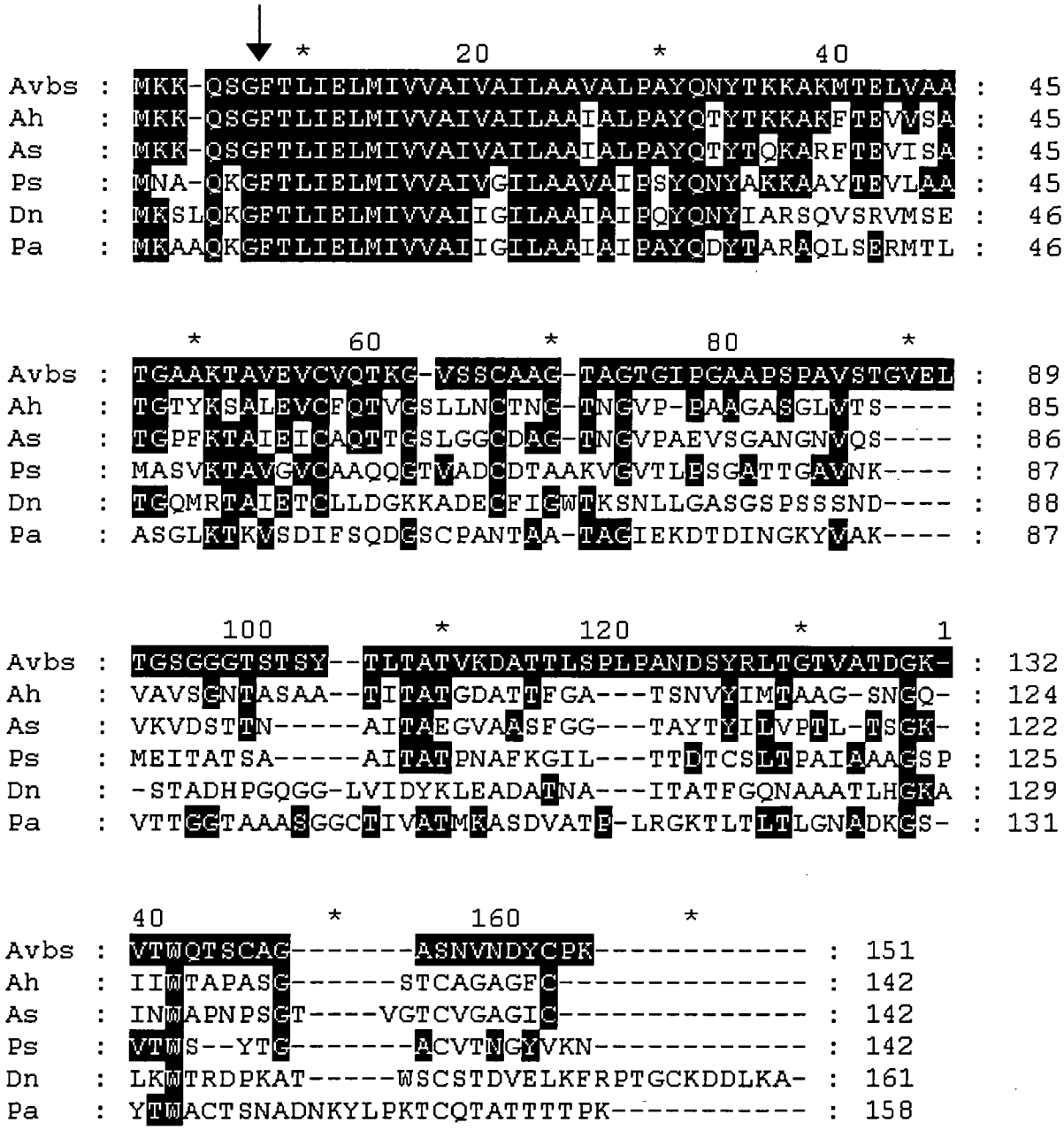
6
7
97.4
66.2
45.0
31.0
21.5
14.4
6.5

Fig. 3.7. Amino acid sequence comparison of *A. veronii* biovar sobria BC88 TapA with type IV prepilins from other bacteria.

The alignment was generated using ClustalW software. Residues conserved with the *A. veronii* biovar sobria BC88 TapA are shaded black. The predicted cleavage site where the pilin is cleaved and N-methylated to generate the mature pilin is indicated with an arrow. Species abbreviations and the pilin subunit designations are as follows:

Avbs, *A. veronii* biovar sobria BC88, TapA; Ah, *A. hydrophila* Ah65, TapA (Pepe *et al.*, 1996); As, *A. salmonicida* A449, SfpA (Lutwyche *et al.*, unpublished); Ps, *Pseudomonas syringae* DC3000, PilA (Raineri *et al.*, unpublished); Dn, *Dichelobacter nodosus* 351, FimA (Hoyne *et al.*, 1989); Pa, *Pseudomonas aeruginosa* P1, PilA (Pasloske *et al.*, 1988).


* 20 * 40
Avbs : MKK-QSGFTLIELMIVVAIVAILAAVALPAYQNYTKKAKMTELVAA : 45
Ah : MKK-QSGFTLIELMIVVAIVAILAAIALPAYQTYTKKAKFTEVVSA : 45
As : MKK-QSGFTLIELMIVVAIVAILAAIALPAYQTYTQKARFTEVISA : 45
Ps : MNA-QKGFTLIELMIVVAIVGILAAVAIPSYQNYAKKAAYTEVLAA : 45
Dn : MKSLQKGFTLIELMIVVAIIGILAAIAIPQYQNYIARSQVSRVMSE : 46
Pa : MKAAQKGFTLIELMIVVAIIGILAAIAIPAYQDYTARAQLSERMTL : 46
* 60 * 80 *
Avbs : TGAAKTAVEVCVQTKG-VSSCAAG-TAGTGIPGAAPSPAVSTGVEL : 89
Ah : TGTYKSALEVCFQTVGSLLNCTNG-TNGVP-PAAGASGLVTS---- : 85
As : TGPFKTAIEICAQTTGSLGGCDAG-TNGVPAEVSGANGNVQS---- : 86
Ps : MASVKTAVGVCAAQQGTVADCDTAAKVGVTLPSGATTGAVNK---- : 87
Dn : TGQMRTAIETCLLDGKKADECFIGWTKSNLLGASGSPSSSND---- : 88
Pa : ASGLKTKVSDIFSQDGSCPANTAA-TAGIEKDTDINGKYVAK---- : 87
100 * 120 * 1
Avbs : TGSGGGTSTSY--TLTATVKDATTLSPLPANDSYRLTGTVATDGK- : 132
Ah : VAVSGNTASAA--TITATGDATTFGA---TSNVYIMTAAG-SNGQ- : 124
As : VKVDSTTN-----AITAEGVAASFGG---TAYTYILVPTL-TSGK- : 122
Ps : MEITATSA-----AITATPNAFKGIL---TTDTCSLTPAIAAAGSP : 125
Dn : -STADHPGQGG-LVIDYKLEADATNA---ITATFGQNAAATLHGKA : 129
Pa : VTTGGTAAASGGCTIVATMKASDVATP-LRGKTLTLTLGNADKGS- : 131
40 * 160 *
Avbs : VTWQTSCAG-------ASNVNDYCPK---------- : 151
Ah : IIWTAPASG------STCAGAGFC---------- : 142
As : INWAPNPSGT----VGTCVGAGIC---------- : 142
Ps : VTWS--YTG-------ACVTNGYVKN---------- : 142
Dn : LKWTRDPKAT-----WSCSTDVELKFRPTGCKDDLKA- : 161
Pa : YTWACTSNADNKYLPKTCQTATTTTPK---------- : 158

139 and 149, suggesting that this protein is likely to form two separate intra-chain disulphide bridges, of similar size and location to those present within the *D. nodosus* class II pilins (Mattick *et al.*, 1991).

The partial sequence of *tapB* cloned from *A. veronii* biovar sobria (representing approximately 20% of *tapB* from strain Ah65) encoded 107 amino acids. This amino acid sequence was virtually identical (98% identity, 98% similarity) to the corresponding region of TapB from *A. hydrophila* and *A. salmonicida* (Fig. 3.8).

3.2.4.3. Conservation of the *tap* gene cluster in *Aeromonas*

The entire *tap* cluster appeared to be well conserved between *A. veronii* biovar sobria BC88 and *A. hydrophila* Ah65. For both strains, *tapA* and the 5' end of *tapB* were located on 1 kb *BamH* I fragments, and both genes were orientated in the same direction and appeared to possess a putative σ54-dependent promoter upstream of *tapA*. To determine whether the rest of the *tap* cluster was also present, Southern hybridisation was employed to detect the presence of *tapD*, which lies at the distal end of the *tap* cluster. Like *A. hydrophila* Ah65, *tapD* was detected on a 2.7 kb *Pst* I restriction fragment under high stringency hybridisation conditions, suggesting that the entire *tap* gene cluster is likely to be encoded on the BC88 genome.

Other strains of *Aeromonas* known to express Bfp pili were obtained from Dr. M. Iwanaga, University of Ryukus, Ryukus, Japan (Hokama *et al.*, 1990; Hokama and Iwanaga, 1991; Hokama and Iwanaga, 1992; Iwanaga and Hokama, 1992). These strains, and *A. caviae* CA195 (Kirov *et al.*, 1998), were also examined by Southern hybridisation for sequences homologous to the *tapA* and *tapD* genes to determine whether these genes may be widespread in *Aeromonas* species. Both probes hybridised to DNA from these strains at high stringency, although unlike strain BC88, some degree of restriction fragment length polymorphism was observed, and this did not appear to be species-related. For one of the five Bfp-positive strains examined, *A. veronii* biovar sobria Ae24, the probes hybridised to restriction fragments of identical size to fragments from *A. hydrophila* Ah65 and *A. veronii* biovar sobria BC88 (Fig 3.9, lane 4).

Fig. 3.8. Amino acid sequence comparison of *A. veronii* biovar sobria BC88 TapB with homologous proteins from other bacteria.

The alignment was generated using ClustalW software. Residues conserved with the *A. veronii* biovar sobria BC88 TapB are shaded black. Species abbreviations and the pilin subunit designations are as follows:

Avbs, *A. veronii* biovar sobria BC88, TapB; Ah, *A. hydrophila* Ah65, TapB (Pepe *et al.*, 1996); As, *A. salmonicida* A450, TapB (Pepe and Strom, unpublished); Pa, *Pseudomonas aeruginosa* PAK, PilB (Nunn *et al.*, 1990).

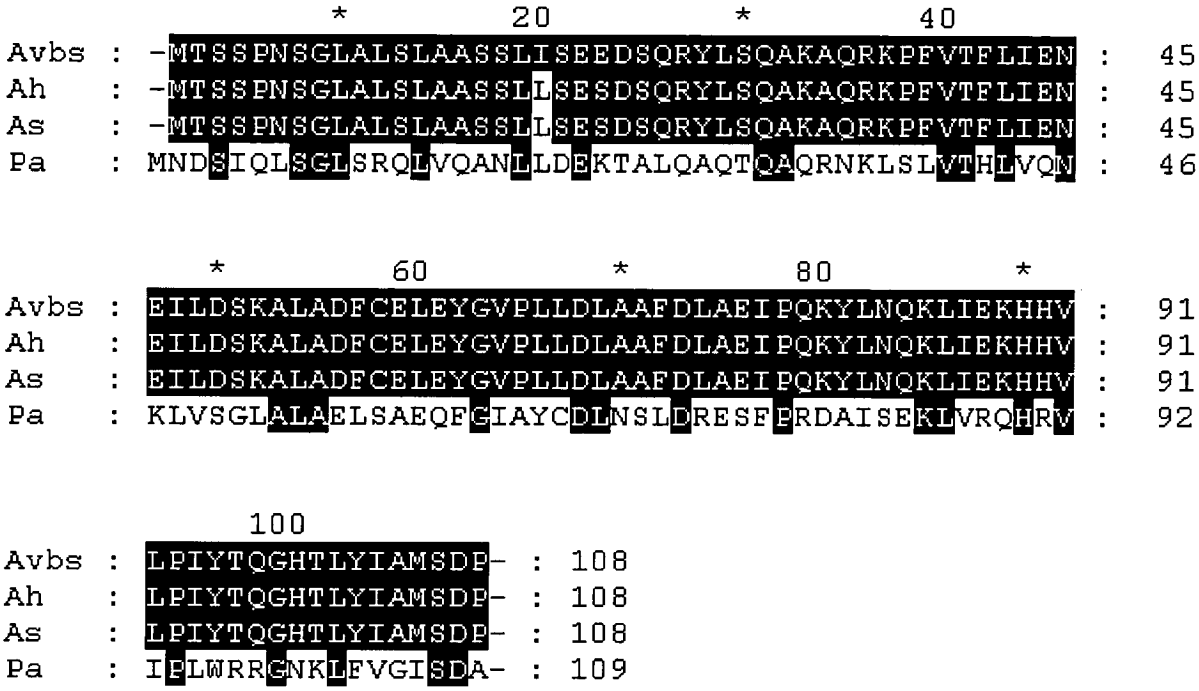

* 20 * 40
Avbs : -MTSSPNSGLALSLAASSLISEEDSQRYLSQAKAQRKPFVTFLIEN : 45
Ah : -MTSSPNSGLALSLAASSLLSESDSQRYLSQAKAQRKPFVTFLIEN : 45
As : -MTSSPNSGLALSLAASSLLSESDSQRYLSQAKAQRKPFVTFLIEN : 45
Pa : MNDSIQLSGLSRQLVQANLLDEKTALQAQTQAQRNKLSLVTHLVQN : 46
* 60 * 80 *
Avbs : EILDSKALADFCELEYGVPLLDLAAFDLAEIPQKYLNQKLIEKHHV : 91
Ah : EILDSKALADFCELEYGVPLLDLAAFDLAEIPQKYLNQKLIEKHHV : 91
As : EILDSKALADFCELEYGVPLLDLAAFDLAEIPQKYLNQKLIEKHHV : 91
Pa : KLVSGLALAELSAEQFGIAYCDLNSLDRESFPRDAISEKLVRQHRV : 92
100
Avbs : LPIYTQGHTLYIAMSDP- : 108
Ah : LPIYTQGHTLYIAMSDP- : 108
As : LPIYTQGHTLYIAMSDP- : 108
Pa : IPLWRRGNKLFVGISDA- : 109

Fig. 3.9. Southern hybridisation of *Aeromonas* strains probed with *A. hydrophila* Ah65 *tapA* and *tapD* gene probes.

a. *BamH* I digests probed with *tapA*. b. *Pst* I digests probed with *tapD*. DNA size markers (λ DNA digested with *Hind* III, size in kilobases) are indicated to the left.

Lane 1. *A. veronii* biovar sobria BC88

Lane 2. *A. veronii* biovar sobria Ae1

Lane 3. *A. hydrophila* Ae6-W

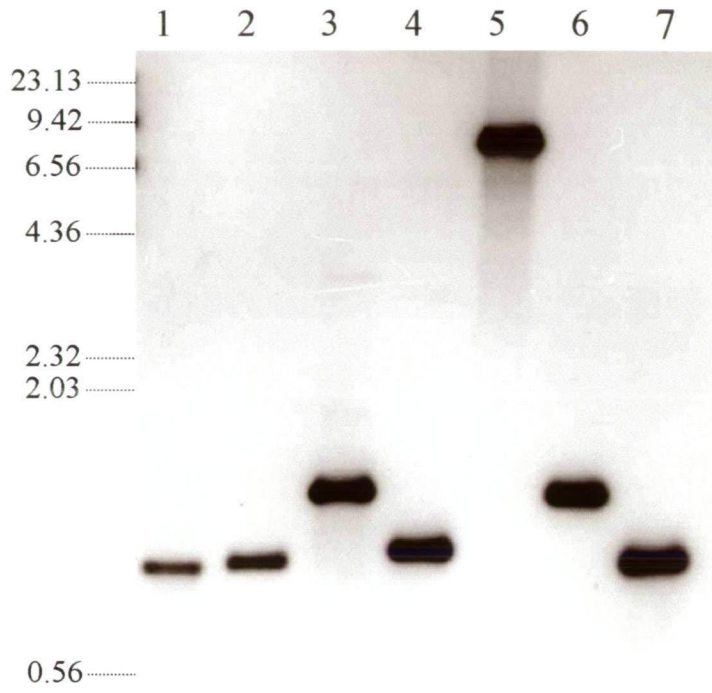
Lane 4. *A. veronii* biovar sobria Ae24

Lane 5. *A. veronii* biovar sobria TAP13

Lane 6. *A. caviae* CA195

Lane 7. *A. hydrophila* Ah65

a.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6
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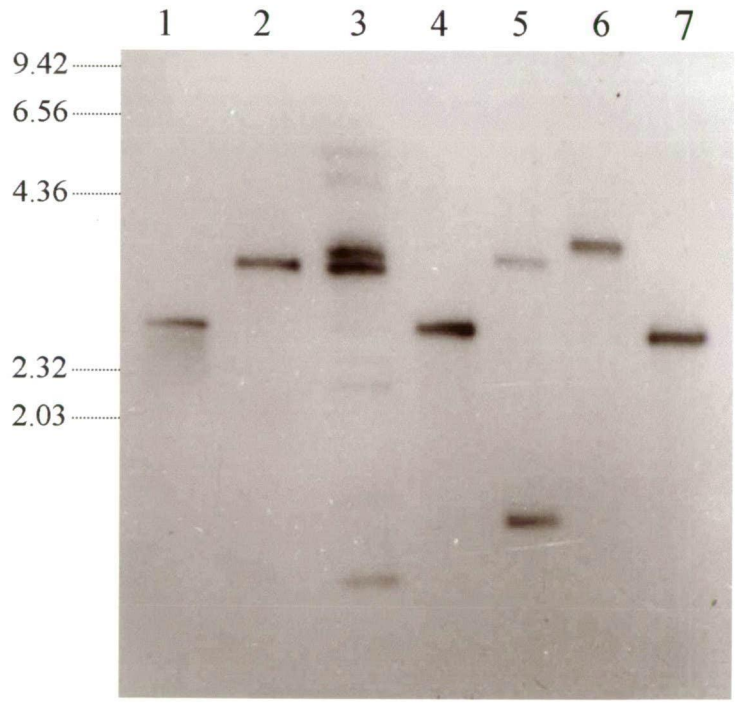
23.13
9.42
6.56
4.36
2.32
2.03
0.56

b.


1
2
3
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5
6
7
9.42
6.56
4.36
2.32
2.03

3.3. Cloning of the N-terminal region of a gene encoding the Bfp pilin protein

3.3.1. Construction of cosmid libraries

Cosmid libraries enable large regions (~ 40 kb) of chromosomal DNA to be cloned, and, therefore, individual cosmid clones are likely to encode full copies of desired gene(s) along with the accessory genes required for expression in a heterologous host. Initially, a cosmid library was constructed from *A. veronii* biovar sobria BC88 DNA with the aim to identify Bfp-expressing clones with Bfp-specific antiserum following transfer of the library to an *Aeromonas* strain expressing a serologically distinct Bfp pilus. Clones containing *bfp* would more likely be detected in an *Aeromonas* strain than in *E. coli*, since all of the genes needed for expression would already be present. Therefore, a cosmid vector capable of transfer into, and stable maintenance in, an *Aeromonas* recipient was constructed.

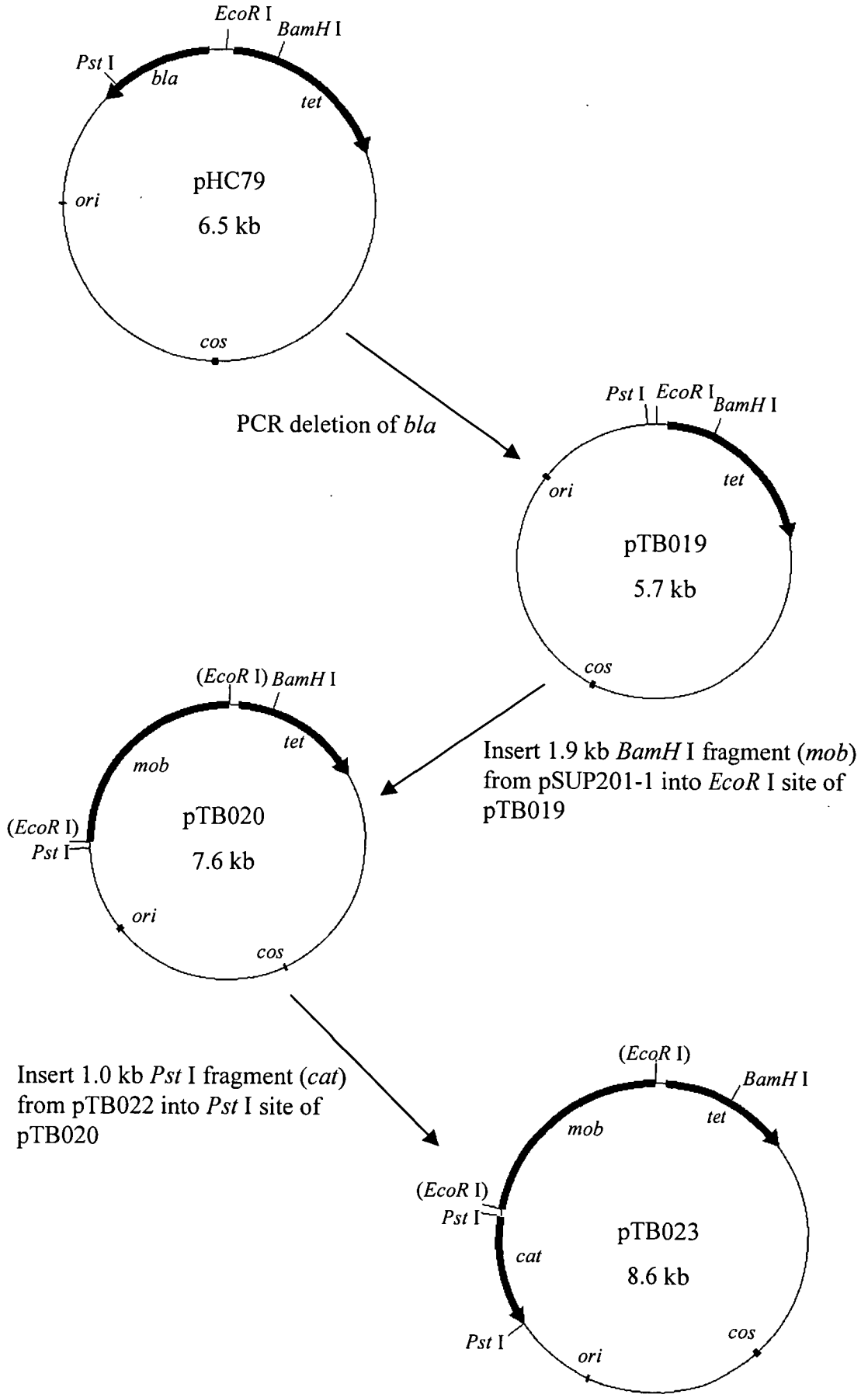
3.3.1.1. Construction of pTB023

The cosmid vector pTB023 is a chloramphenicol (Cml) resistant, mobilisable derivative of pHC79. The construction of pTB023 is depicted in Fig. 3.10. This vector was constructed in essentially the same manner as pPM2101 (Sharma *et al.*, 1989), except that the ampicillin resistance gene (*bla*) was deleted and replaced with a gene encoding chloramphenicol resistance.

The *bla* gene was deleted from pHC79 by long range PCR (Expand™ Long Template PCR System, Boehringer Mannheim) using primers P006 and P007 (Appendix 7). The use of these primers resulted in the introduction of *Pst* I sites at either end of the PCR product, which were used to recircularise the plasmid lacking *bla*, creating pTB019. The *mob* region from pSUP201-1 (Simon *et al.*, 1983), required for mobilisation into recipient bacteria by conjugation, was excised with *Bam*H I, end-filled with the Klenow fragment of DNA polymerase I, and inserted into the end-filled *EcoR* I site of pTB019. The resulting plasmid, designated pTB020, was selected by its ability to be mobilised from *E. coli* S17-1 into *E. coli* SM10. The

Fig. 3.10. Construction of pTB023.

All steps are described in full in section 3.3.1.1.


EcoR I
BamH I
Pst I
bla
tet
pHC79
6.5 kb
ori
cos
PCR deletion of bla
Pst I EcoR I
BamH I
ori
tet
pTB019
5.7 kb
cos
(EcoR I) BamH I
tet
mob
pTB020
7.6 kb
(EcoR I)
Pst I
ori
cos
Insert 1.9 kb BamH I fragment (mob) from pSUP201-1 into EcoR I site of pTB019
Insert 1.0 kb Pst I fragment (cat) from pTB022 into Pst I site of pTB020
(EcoR I)
BamH I
tet
mob
(EcoR I)
Pst I
pTB023
cat
8.6 kb
cos
Pst I
ori

Cml^R vector pTB023 was produced by excising a *cat* gene cassette from pTB022 as a *Pst* I fragment and cloning into the *Pst* I site of pTB020. pTB022 was constructed by excising the *cat* cassette from pBSL121 with *Bam*H I and cloning into the *Bgl* II site of pLitmus29. The Cml^R cassette could then be excised as an ~1 kb *Pst* I fragment that lacked an internal *Bam*H I site.

3.3.1.2. Library construction and mobilisation into *Aeromonas*.

Preparation of vector and insert DNA, packaging and transduction were performed as described in section 2.9. *E. coli* S17-1 was used as a host strain for the library in order to allow transfer to heterologous recipient bacteria.

Construction of a cosmid library in pTB023 produced ~ 1500 cosmid clones of which 672 Cml^R/Tet^S clones were selected for analysis. Initially, 12 cosmids were mobilised into each of the *Aeromonas* strains from which Bfp had been purified (*A. veronii* biovar sobria strains Ae1, Ae24 and TAP13, *A. hydrophila* Ae6 and *A. caviae* CA195) as well as into *A. hydrophila* Ah65. Transfer of all 12 cosmids was only observed when Ah65 was the recipient. Therefore, Ah65 was chosen for large scale screening of the cosmid library.

Individual cosmids mobilised into Ah65 were replica plated onto nitrocellulose membranes and screened with a Bfp antiserum. This antiserum had been absorbed six times to whole cell lysates of Ah65 immobilised onto nitrocellulose membranes (see section 2.13.5). The resulting serum retained activity against a 21 kDa band in BC88 and purified Bfp^{BC88} protein, but exhibited no reactivity with a similar sized band from Ah65. This antiserum was used to screen the cosmid library in Ah65 for clones expressing Bfp. No Bfp-positive clones were identified using this procedure.

In order to detect the *bfp* gene(s) in the cosmid library constructed above, an alternative strategy was used. The N-terminal region of a gene encoding the Bfp pilin protein was cloned, for use as a probe, using degenerate PCR primers based on the published N-terminal sequence of this protein (Kirov and Sanderson, 1996).

3.3.2. Cloning of the 5' end of the *bfp* subunit gene by PCR

The entire N-terminal amino acid sequence of the Bfp pilin was used to derive the corresponding degenerate DNA sequence, and this DNA sequence was used to construct a series of forward and reverse degenerate oligonucleotide primers for PCR amplification of BC88 DNA. In the first instance, all possible combinations of forward and reverse primers were used in 40 cycles of PCR (94°C denaturation, 30 s; 35°C annealing, 30 s; 60°C extension, 4 min). PCR products were obtained for all primer combinations.

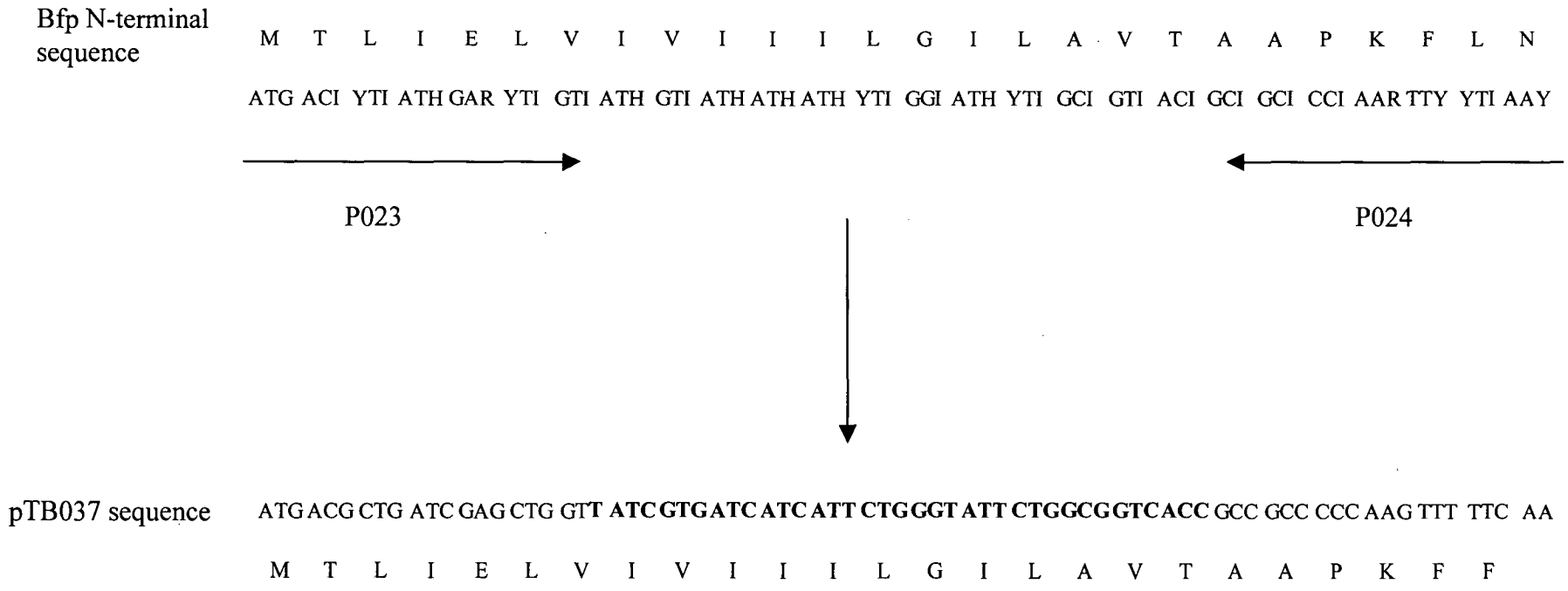
The N-terminal amino acid sequence of the Bfp pilin (31 amino acids) would be encoded by a 93 bp DNA sequence. Depending on the primers used in each reaction, a Bfp-specific PCR product would have an expected size range of 56 - 83 bp. As the use of primers P019 and P022 appeared to give the best results, these primers were resynthesised with the 5' restriction enzyme sites *Hind* III (P023) or *Xba* I (P024). The above PCR reaction was repeated with these primers and the product was cloned into the *Hind* III/*Xba* I sites of pZErO-2.1, generating pTB037, for sequence analysis. The sequence of insert contained in pTB037 is given in Fig 3.11. The translated sequence of the insert contained in pTB037 corresponded almost exactly to the N-terminal sequence of the purified Bfp pilin protein. The only difference was in the coding for a F residue at position 25 in place of a L. However, this sequence was in the area encoded by the degenerate primer P024, so this substitution was not deemed to be significant.

Hence, the above procedure resulted in the cloning of a small region of a gene encoding the Bfp pilin. However, due to time constraints for this thesis, this could not be pursued further.

In summary, the results presented in this chapter have described the cloning of part of a type IV pilus gene cluster from *A. veronii* biovar sobria BC88. The pilin gene (*tapA*) encoded a protein distinct to that expressed by this strain (Bfp). The *tapA* and *tapD* genes were also identified in other strains from which Bfp pili had been purified, demonstrating that *Aeromonas* species associated with gastroenteritis have

Fig. 3.11. Sequence of pTB037.

pTB037 contains a 78 bp insert generated by PCR amplification of *A. veronii* biovar sobria BC88 DNA using primers P023 and P024. The unique sequence data not encoded by P023 and P024 is shown in bold. The predicted amino acid sequence encoded by the insert in pTB037 is also shown. Note that this sequence is identical to the published N-terminal amino acid sequence of the Bfp pilin (Kirov and Sanderson, 1996).


Bfp N-terminal sequence
M T L I E L V I V I I I L G I L A V T A A P K F L N
ATG ACI YTI ATH GAR YTI GTI ATH GTI ATH ATH ATH YTI GGI ATH YTI GCI GTI ACI GCI GCI CCI AAR TTY YTI AAY
P023
P024
pTB037 sequence
ATG ACG CTG ATC GAG CTG GTT ATC GTG ATC ATC ATT CTG GGT ATT CTG GCG GTC ACC GCC GCC CCC AAG TTT TTC AA
M T L I E L V I V I I I L G I L A V T A A P K F F

the potential to express two distinct families of type IV pili. Finally, the cloning of a small region of the *bfp* pilin gene should allow the cloning of the entire *bfp* gene cluster in the near future.

3.4. Discussion

The results in this chapter have demonstrated that *Aeromonas* species associated with gastroenteritis encode two distinct families of type IV pili. The genes encoding Tap pili were cloned from *A. veronii* biovar sobria BC88, a strain from which the Bfp pili had been purified. The predicted amino acid sequence of *tapA* was different from the N-terminal sequence of the Bfp pilin, demonstrating that the *tap* gene cluster could not be encoding the pili purified from this strain. In addition, other strains from which Bfp pili had been purified (one *A. hydrophila*, one *A. caviae*, and three *A. veronii* biovar sobria strains) also possessed the *tap* gene cluster, as determined by Southern hybridisation. The cloning of a small region of the *bfp* gene from *A. veronii* biovar sobria BC88 further demonstrates that this strain possesses genes encoding two distinct families of type IV pili, Bfp and Tap.

The N-terminal amino acid sequences of the TapA and Bfp pilin proteins show that they are both subunits of type IV pili. However, both pilin proteins are characterised by certain amino acid residues unique to each family. In addition, the TapA proteins appear to be smaller than Bfp pilin proteins, as determined by western blotting and comparison of molecular masses predicted from sequence data. However, such size differences could also be accounted for by different post-translational glycosylation patterns (Virji *et al.*, 1993).

The genetic organisation of the *tap* gene clusters from *A. veronii* biovar sobria and *A. hydrophila* are very similar. For both clusters, the prepilin gene was coupled to a gene encoding a nucleotide-binding protein homologous to PilB from *Pseudomonas aeruginosa*. The layout of the *tap* cluster is very similar to gene clusters cloned from *Vibrio vulnificus* (Paranjpye *et al.*, 1998), *V. cholerae* (Fullner and Mekalanos, 1999) and *E. coli* (Whitchurch and Mattick, 1994). This, and the observations that the *tapA* and *tapD* genes from different species of *Aeromonas* were

often present on identically-sized restriction enzyme fragments, and that TapA proteins from different species are occasionally antigenically cross-reactive, suggests that they have been acquired by horizontal gene transfer.

Upstream of *tapA* from both *A. veronii* biovar sobria BC88 and *A. hydrophila* Ah65 was a sequence resembling a σ54-dependent promoter. This type of promoter is responsible for transcription from the *P. aeruginosa pilA* gene, so it is possible that this promoter is also responsible for *tapA* expression by *Aeromonas*. However, the σ54-dependent promoter upstream of the *N. gonorrhoeae pilE* gene is not active because this species encodes a dysfunctional *rpoN* gene, encoding sigma factor 54 (Laskos *et al.*, 1998). The potential σ54 dependent promoter sequence upstream of *tapA* differs somewhat from the consensus sequence for σ54-dependent promoters, which have been derived by comparing the sequences of numerous σ54 dependent promoters from members of the Enterobacteriaceae (Wang and Gralla, 1998). This is not unexpected, however, since *Aeromonas* is significantly diverged phylogenetically from the Enterobacteriaceae. Indeed, σ54-dependent promoters often diverge considerably from the above consensus sequence, even within the Enterobacteriaceae (Wang and Gralla, 1998). It is, therefore, unknown, and impossible to predict, whether this promoter is responsible for the expression of *tapA* in *Aeromonas* without the use of expression assays such as primer extension analysis. It is equally possible that the transcription initiation site lies upstream of the *BamH* I site proximal to *tapA* and would, thus, not have been identified by the sequencing described in section 3.2.5.1.

Bfp pili have not been genetically characterised. Several approaches were investigated in this chapter in an attempt to genetically characterise this structure from *A. veronii* biovar sobria BC88. However, traditional methods such as transposon mutagenesis and the use of DNA probes were not successful. Two probes used in this study, the degenerate oligonucleotide P001 and the N-terminal region of *mshA* from *V. cholerae*, were designed in reference to the N-terminal amino acid sequence of the purified Bfp pilin protein. However, these probes failed to bind to the

corresponding region of the *bfp* subunit gene. This result is likely to reflect differences in codon usage between the *bfp* gene and the *mshA* gene, and the *Aeromonas* codon usage data used in the design of P001. Comparison of the sequence obtained for the cloned N-terminal region of *bfp* with the corresponding region of *mshA* demonstrates this to be the case, with codon mismatches for 9 of the 13 amino acids encoded by the sequenced region. Unfortunately, the degenerate probe sequence could not be compared in this manner since this probe was designed from amino acids 19 to 25 of the Bfp N-terminal amino acid sequence, which resides in the region encoded by the degenerate PCR primer P024 in plasmid pTB037.

The other method used to clone the *bfp* gene(s) involved the screening of a cosmid DNA library with a Bfp-specific antiserum (Kirov and Sanderson, 1996) following mobilisation into *A. hydrophila* Ah65. However, screening of the library in this host background did not identify clones expressing the Bfp pilin, indicating that the *bfp* gene(s) were not efficiently expressed in this strain. Unfortunately, the cosmid library could not be efficiently mobilised into an *Aeromonas* strain known to express an antigenically distinct Bfp pilus, possibly due to the presence of an S-layer or a restriction-modification system in these strains.

The successful cloning of a small region of the *bfp* gene using a degenerate PCR technique should allow the identification of cosmid clones containing this gene. This may be accomplished by screening the cosmid library with this PCR product directly, or following the production of suitable DNA probes by inverse PCR using specific primers designed from the sequenced region. The subsequent sequencing and mutational analysis of this gene will allow the significance of Bfp pili in the pathogenesis of *Aeromonas* gastrointestinal disease to be established definitively.

The role of the Tap pili in *Aeromonas* pathogenesis was completely unknown. Nothing was known about conditions that influence the expression and assembly of Tap pili. Their similarity to the "classical" type IV pilins of *Dichelobacter nodosus*, *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*, all of which are known to mediate attachment to epithelial cells (Strom and Lory, 1993), suggests, however, that Tap pili might also be colonisation factors for *Aeromonas* species. The cloning

of the *tapA* prepilin gene (described in this chapter) allowed the role of this pilus to be investigated further. The distribution of these genes is explored in chapter 4, in order to determine the use of this putative virulence factor as a marker of virulent strains. The expression of Tap pili, and ability of a *tapA* mutant strain of *A. veronii* biovar sobria BC88 to adhere to, and colonise, intestinal tissue is investigated in chapter 5.

Chapter 4

Distribution of the *tap* gene cluster in the genus *Aeromonas*

4.1. Introduction

4.2. Prevalence of the *tap* cluster in hybridisation group reference strains

4.3. Distribution of the *tap* cluster in *Aeromonas* isolates from different sources

4.4. Hybridisation with *tapA* differentiated clinical and environmental isolates of *Aeromonas veronii* biovar sobria

4.5. Sequencing of the *tapA*-proximal region

4.6. Discussion

4.1. Introduction

The aim of the experiments described in this chapter was to examine the distribution of the *tap* gene cluster, as a first step towards evaluating the significance of these genes in *Aeromonas* species. The distribution of the genes located at either end of the *tap* cluster (*tapA* and *tapD*) was determined for the majority of hybridisation group reference strains (HG1 to HG12 and HG17), and clinical and environmental isolates of the three species most commonly associated with human gastrointestinal disease.

4.2. Prevalence of the *tap* cluster in hybridisation group reference strains

As described in section 1.2.3, of the 17 hybridisation groups currently recognised, only three are commonly associated with human gastrointestinal disease, and several have not been isolated from human clinical specimens (see Table 1.1.). Initially, to determine the distribution of the *tap* cluster in *Aeromonas*, the presence of *tapA* and *tapD* was investigated in representative members of each of the most common hybridisation reference groups. These strains included hybridisation groups (HG1 to HG12), and a newly described species, *A. popoffii* HG17.

Digoxigenin-labelled probes were prepared by PCR amplification of *tapA* (contained in plasmid pCP1065) and *tapD* (contained in pCP1121) from *A. hydrophila* Ah65 (Fig. 4.1.), as described in section 2.10.2. Since results described in section 3.2.5.3. suggested that *tapA* was preferentially localised on a 1.0 or 1.2 kb *BamH* I restriction enzyme fragment, and *tapD* on a 2.7 or 3.9 kb *Pst* I fragment, these enzymes were again chosen to prepare the chromosomal DNA digests. Southern hybridisation with the *tapA* and *tapD* probes was performed under conditions of both low and high stringency as described in section 2.11.1. Results are shown in Fig. 4.2.

The *tapA* and *tapD* probes reacted with DNA from all strains examined under low stringency conditions. Fig. 4.2a shows hybridisation with *tapA* at low stringency.

Fig. 4.1. Schematic representation of the *tap* gene cluster of *A. hydrophila* Ah65.

The regions cloned in plasmids pCP1065 and pCP1121 are indicated. Horizontal lines below *tapA* and *tapD* indicate the areas used as probes in hybridisation assays. Numbers in parentheses are the nucleotide residues at the start and end of each probe sequence. Relevant restriction sites are also shown.

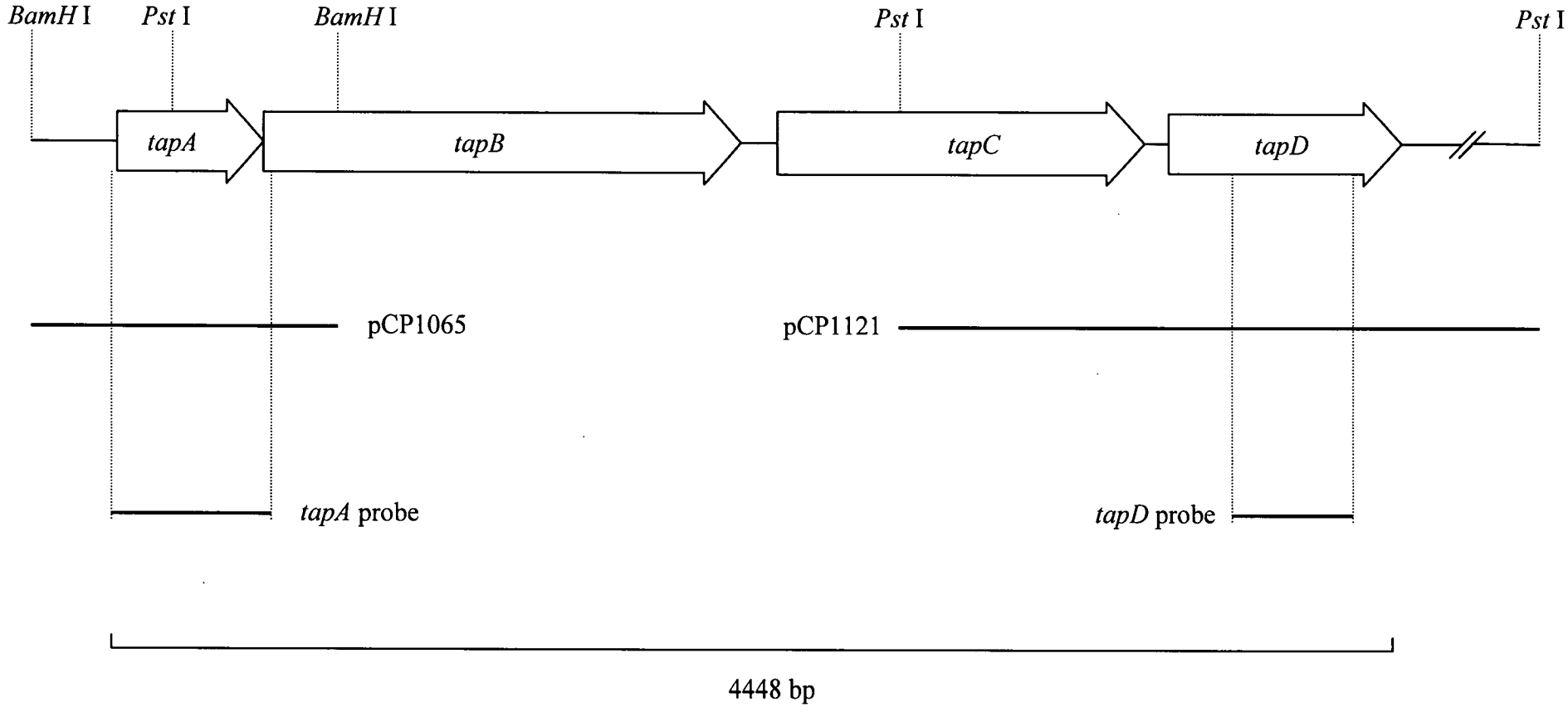
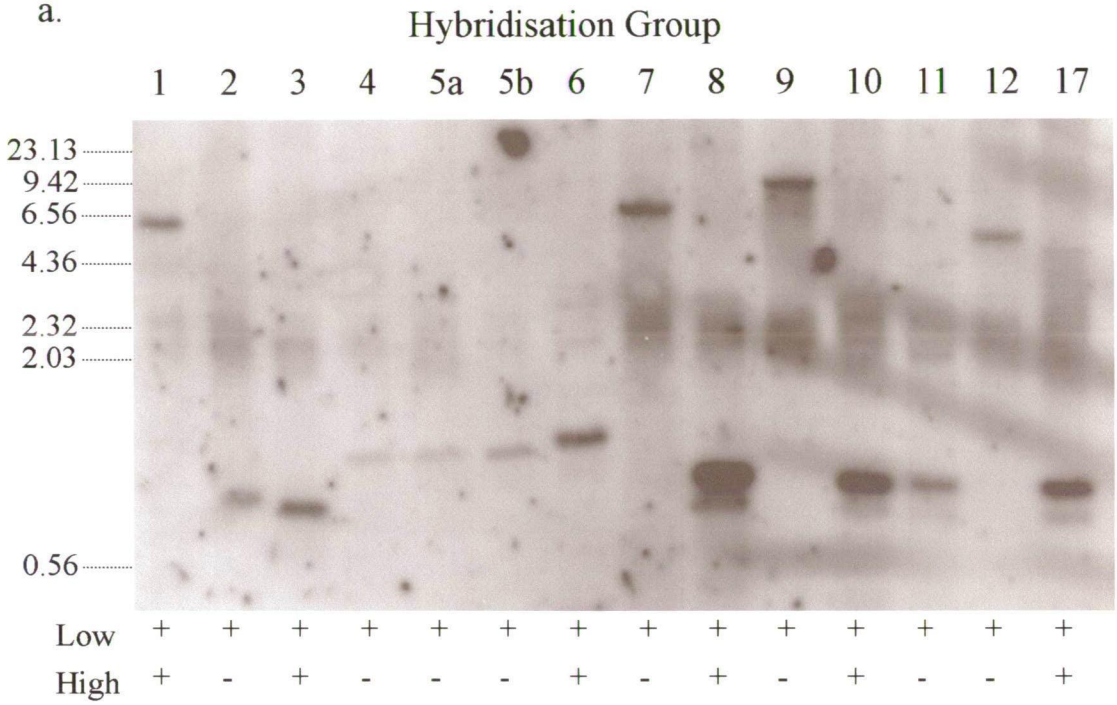

BamH I
Pst I
BamH I
Pst I
Pst I
tapA
tapB
tapC
tapD
pCP1065
pCP1121
tapA probe
tapD probe
4448 bp

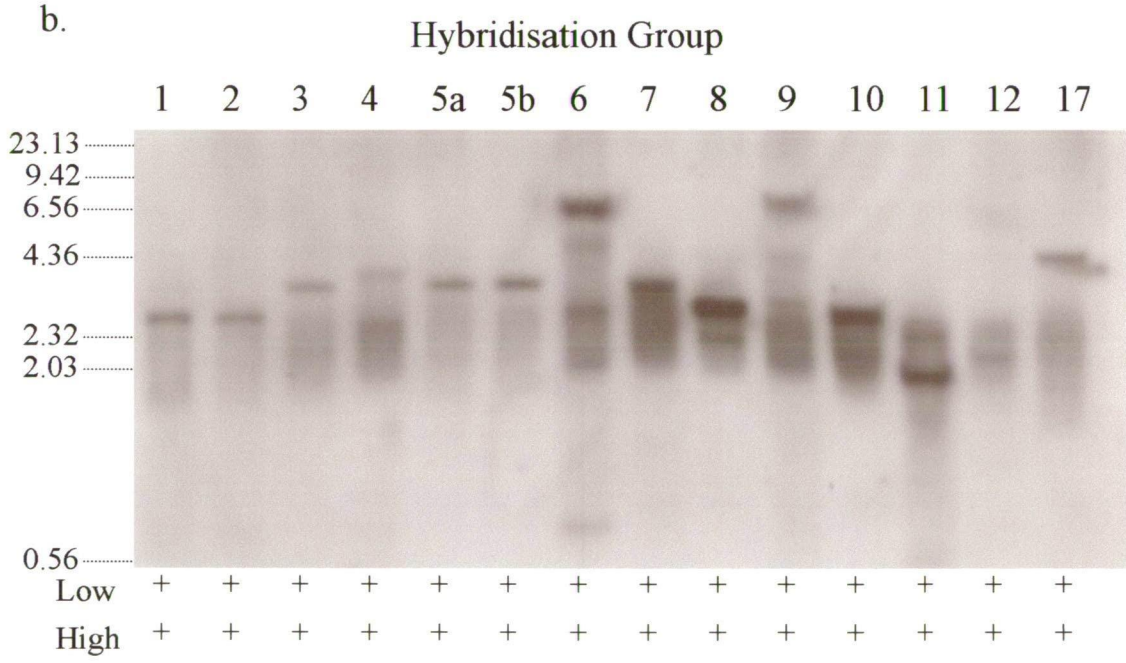
Fig. 4.2. Southern hybridisation of genomic DNA from *Aeromonas* reference strains.

a. *BamH* I digests probed with *tapA* under low stringency conditions. b. *Pst* I digests probed with *tapD* under high stringency conditions. High and low stringency hybridisation results for each strain are also shown below each lane. Molecular weight markers (λ DNA digested with *Hind* III) are indicated to the left. Defined bands were observed with both probes for each strain tested despite some background signal.

a.


Hybridisation Group
1 2 3 4 5a 5b 6 7 8 9 10 11 12 17
23.13
9.42
6.56
4.36
2.32
2.03
0.56
Low + + + + + + + + + + + + + +
High + - + - - - + - + - + - - +

b.


Hybridisation Group
1 2 3 4 5a 5b 6 7 8 9 10 11 12 17
23.13
9.42
6.56
4.36
2.32
2.03
0.56
Low + + + + + + + + + + + + + +
High + + + + + + + + + + + + + +

However, when this experiment was repeated under conditions of high stringency, only 6 of 14 strains showed detectable levels of hybridisation with the *tapA* probe. In contrast, all strains hybridised with *tapD* at high stringency, as shown in Fig. 4.2b.

As described in chapter 3, the *tapA* probe again preferentially hybridised with 1.0 kb (HGs 2, 3, 8, 10, 11 and 17) or 1.2 kb (HGs 4, 5a, 5b and 6) *BamH* I restriction fragments. Similarly, the *tapD* probe preferentially hybridised with 2.7 kb (HGs 1, 2, 8 and 10) or 3.3 kb (HGs 3, 5a, 5b and 7) *Pst* I fragments.

4.3. Distribution of the *tap* cluster in *Aeromonas* isolates from different sources

The results described above indicated that the *tap* cluster might be widely distributed in *Aeromonas* species irrespective of their association with human disease, since all hybridisation groups appeared to possess *tapA* and *tapD* homologous sequences. To investigate this further, strains from our culture collection were also surveyed for the presence of *tapA* and *tapD*. Initially, 71 strains were examined in the same manner as described for the reference strains. Species commonly associated with human diarrhoea (*A. veronii* biovar sobria, n = 22; *A. hydrophila*, n = 24; *A. caviae*, n = 22; and *A. trota* n = 3) were chosen from clinical (diarrhoeal faeces) and environmental sources (n = 42 and n = 29, respectively). These strains were predominantly Australian isolates (n = 57), but were from several widely separated regions (Western Australia, South Australia, Tasmania and Queensland). The remaining fourteen isolates (nine clinical and five environmental) were from Japan, Thailand, the United States and Peru. Results of this screening are summarised in Table 4.1.

Both the *tapA* and *tapD* probes hybridised with DNA from all strains under low stringency conditions, irrespective of their source or geographical origin. However, as for the reference strains, only *tapD* hybridised with DNA from all strains at high stringency. Species-associated differences in the stringency conditions that allowed hybridisation with the *tapA* probe were also observed, and these were consistent with results obtained with the reference strains. DNA from all *A. veronii*

Table 4.1. Occurrence of *tap* genes in *Aeromonas* culture collection strains.

Species[a] / source	No. tested	No. with *tapA*		No. tested	No. with *tapD*
		Low[b]	High[b]		High[b, c]
***A. veronii* biovar sobria**					
Clinical	12	12	12	7	7
Environmental	10	10	10	7	7
A. hydrophila					
Clinical	11	11	11	8	8
Environmental	13	13	13	10	10
A. caviae					
Clinical	16	16	0	10	10
Environmental	6	6	0	5	5
A. trota					
Clinical	3	3	3	3	3
Environmental	-	-	-	-	-
Total	71	71	49	50	50

[a] Species determined according to the biochemical criteria of Popoff and Véron (1976).

[b] Low, low stringency hybridisation; High, high stringency hybridisation.

[c] DNA from all strains hybridised with the *tapD* probe under both low and high stringency conditions.

biovar sobria and *A. hydrophila* isolates hybridised with *tapA* at high stringency, but DNA from the 15 *A. caviae* isolates failed to do so.

Species-associated differences were also observed in the fragment sizes to which *tapA* and *tapD* hybridised, especially for clinical isolates. For clinical isolates of *A. veronii* biovar sobria and *A. hydrophila, tapA* was most often detected on a 1.0 kb *BamH* I fragment and *tapD* on a 2.7 kb *Pst* I fragment. In contrast, for *A. caviae* isolates, these genes were most often found on a 1.2 kb *BamH* I fragment and a 3.9 kb *Pst* I fragment, respectively (Table 4.2). This suggests that there may be two separate lineages of the *tap* cluster, one possessed by *A. veronii* biovar sobria and *A. hydrophila* and another possessed by *A. caviae* strains.

4.4. Hybridisation with *tapA* differentiated clinical and environmental isolates of *Aeromonas veronii* biovar sobria

The wide distribution of the *tap* gene cluster in *Aeromonas* species suggested its ability to be used as a marker of pathogenic strains might be limited, since both clinical and environmental strains appeared to possess these genes. However, hybridisation with the *tapA* probe did appear to differentiate clinical and environmental isolates of *A. veronii* biovar sobria (Fig. 4.3). For 10 of 12 clinical isolates in this initial screening, *tapA* localised on a 1.0 kb *BamH* I fragment, whereas it hybridised to a larger fragment (4.8 – 9.0 kb) for eight of 10 environmental isolates (Fig. 4.3a). Similar differentiation based on the fragment sizes to which *tapD* hybridised were also observed (Fig. 4.3b).

The *tapA*-homologous restriction enzyme fragments (~ 1.0 kb) from clinical *A. veronii* biovar sobria did not appear to be identical, as slight differences in the size of each band were apparent. In an attempt to account for such differences, DNA from each strain was amplified with primers designed to flank the initiation and termination codons of the *tapA* open reading frames (based on sequences from *A. veronii* biovar sobria BC88 and *A. hydrophila* Ah65). PCR was performed using primers P004 and P011 in 25 cycles of denaturation (94°C, 40 s), annealing (55°C,

Table 4.2. Summary of fragment sizes to which *tapA* and *tapD* probes hybridise

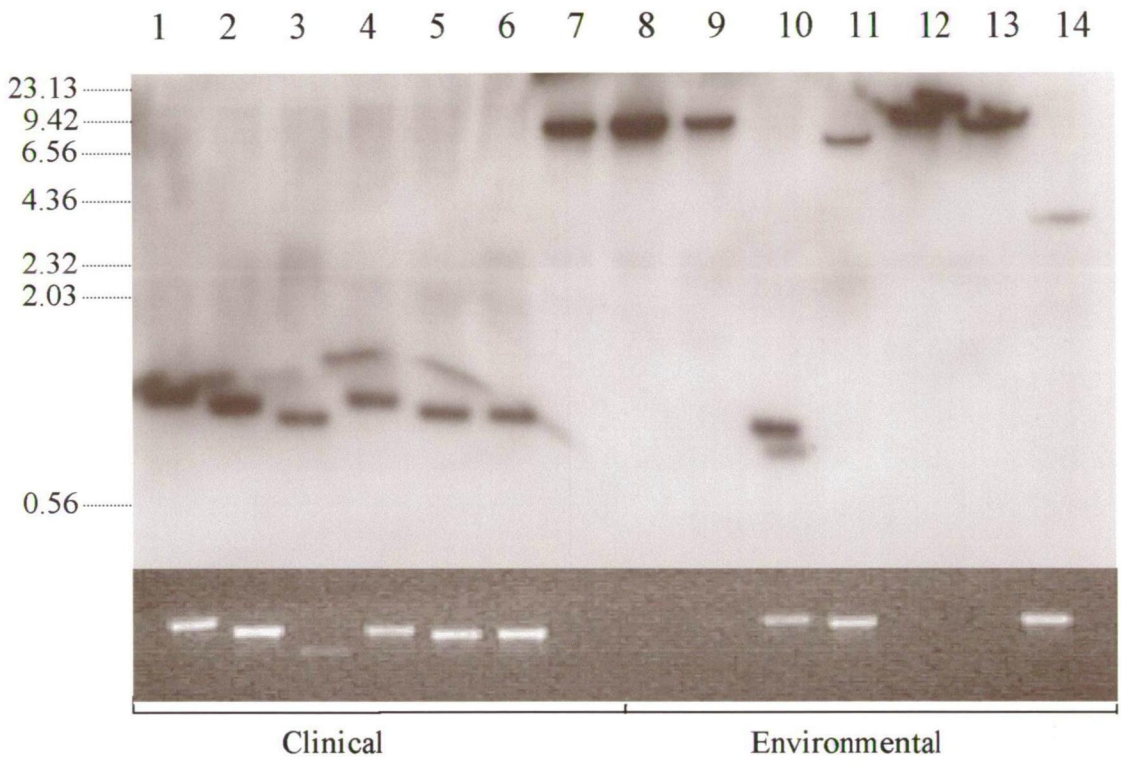
Species/source	*tapA*			*tapD*		
	No. tested	1.0 kb	1.2 kb	No. tested	2.7 kb	3.9 kb
***A. veronii* biovar sobria**						
Clinical	12	10	0	7	5	0
Environmental	10	2[a]	0	7	2	0
A. hydrophila						
Clinical	11	5	3	8	5	0
Environmental	13	5	1	10	5	0
A. caviae						
Clinical	16	3	8	10	0	8
Environmental	6	2	3	5	0	3

[a] For environmental isolates of *A. veronii* biovar sobria, the *tapA* probe hybridised to a 9 kb *BamH* I fragment (see Fig. 4.3.).

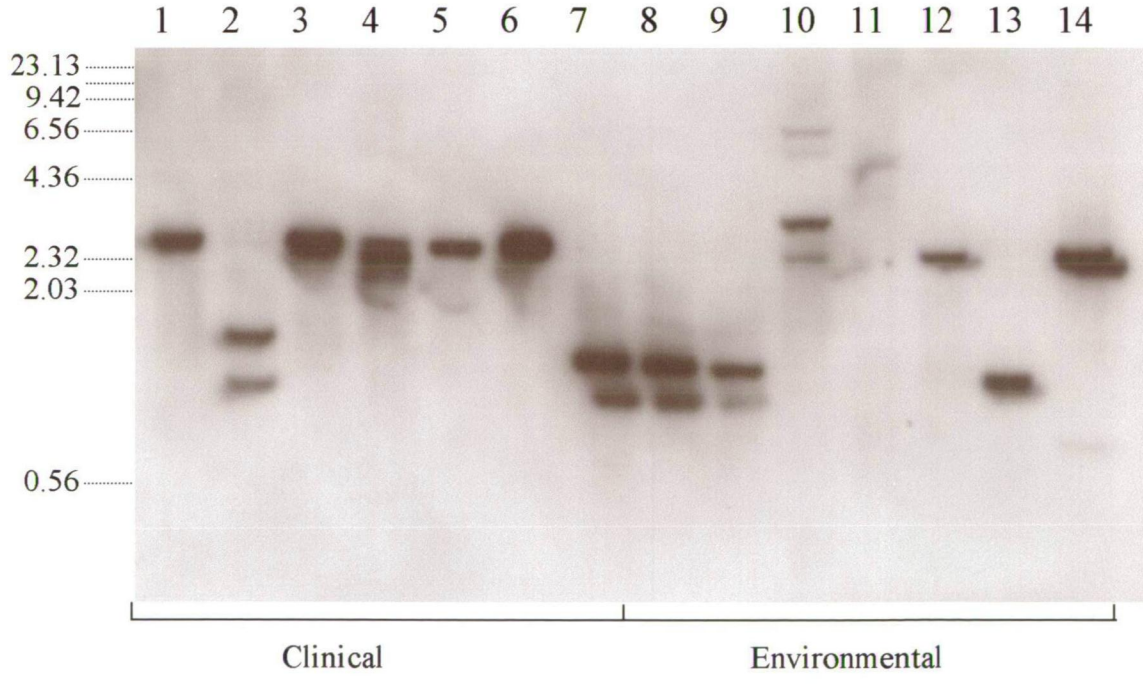
Fig. 4.3. Southern hybridisation of genomic DNA from clinical and environmental isolates of *A. veronii* biovar sobria.

a. *BamH* I digests probed with *tapA*. PCR results using primers flanking the initiation and termination codons of the *tapA* open reading frame (P004 and P011) are indicated below the Southern hybridisation results. b. *Pst* I digests probed with *tapD*. Hybridisation was performed under high stringency conditions. Molecular weight markers (λ DNA digested with *Hind* III) are indicated to the left.

a.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.13
9.42
6.56
4.36
2.32
2.03
0.56
Clinical
Environmental

b.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.13
9.42
6.56
4.36
2.32
2.03
0.56
Clinical
Environmental

40 s) and extension (72°C, 1 min). Results of this experiment are shown below the Southern hybridisation results in Fig. 4.3a.

Comparison of the Southern hybridisation and PCR results demonstrates that the size variations of the ~ 1 kb restriction enzyme fragments correspond to size differences in the *tapA* open reading frames.

The observation that the *tapA* probe appeared to differentiate clinical and environmental isolates of *A. veronii* biovar sobria was of particular interest since this has the potential to be exploited as a marker pathogenic strains of this species. Therefore, a more detailed survey for the presence of *tapA* in isolates of *A. veronii* biovar sobria from a variety of clinical and environmental sources was undertaken, in collaboration with Mr. Gerald Murray (BScHons student, Discipline of Pathology, University of Tasmania). Results of *BamH* I restriction fragment sizes which hybridise with *tapA* are summarised in Table 4.3.

The clinical isolates tested were from diarrhoeal (n=20) and non-diarrhoeal (n=19) stool specimens, as well as six other clinical specimens (e.g. ulcer, blood, wound). Ten animal isolates were also included, which included six anal swabs from koi carp and long neck tortoise. The remaining four animal isolates were from fish and shellfish. A total of 26 environmental *A. veronii* biovar sobria isolates were tested, and these were from various surface water sources. The incidence of *tapA* localised on a 1 kb *BamH* I fragment was higher for the isolates from diarrhoeal stools (85%) when compared to non-diarrhoeal (63%) and environmental (50%) isolates. The incidence in extraintestinal (83%) and animal (90%) isolates was also high, although comparatively few isolates were tested in each of these groups. It was reasoned that the lower incidence in environmental isolates may reflect inaccurate species determination (using biochemical typing results from the laboratory database prior to 1990). However, when examination of environmental isolates was restricted to those which had been classified by genotyping methods, the incidence only rose slightly to 67% (10 of 15), which is similar to the incidence for isolates from non-diarrhoeal stools.

Table 4.3. Summary of *A. veronii* biovar sobria *BamH* I fragment sizes to which the *tapA* probe hybridises

Source	No. tested	*BamH* I fragment size (kb)					
		0.5	0.9-1.1	1.2	2.4	4.0	8.0-9.0
Clinical							
Diarrhoeal stools	20	-	17	-	1	1	1
Non-diarrhoeal stools	19	-	12	2	1	2	2
Extra-intestinal[a]	6	-	5	-	-	-	1
Animal[b]	10	-	9	-	-	-	1
Environmental[c]	26	1	13	-	2	-	10

[a] Extraintestinal isolates were from blood (n=4), a wound (n=1), and an ulcer (n=1).

[b] Animal isolates were from skin (n=4) or anal (n=6) swabs from aquatic organisms.

[c] Environmental isolated were all from fresh surface water.

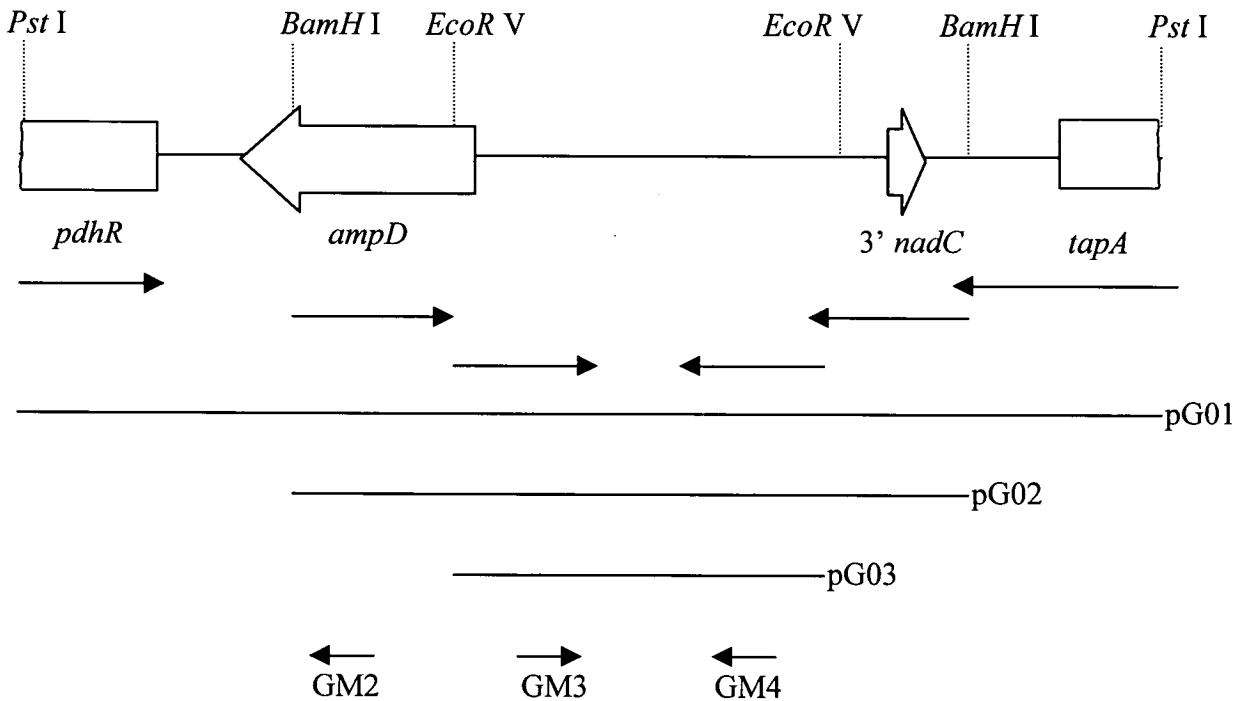
4.5. Sequencing of the *tapA*-proximal region

The observed restriction fragment length polymorphism is consistent with nucleotide sequence differences within the region encompassing *tapA*. Such differences may include the presence of important virulence determinants in clinical strains, variations in genes or promoter sequences responsible for Tap pilus expression, or minor differences within TapA, which may or may not have functional relevance. To investigate these possibilities, the region immediately upstream of *tapA* from strain BC88 was sequenced (This work was done in collaboration with Mr. Gerald Murray, BScHons student, Discipline of Pathology, University of Tasmania. Mr Murray performed all of the experiments involved in the subcloning and sequencing of fragments from pTB032 under the supervision of the candidate. Analysis of the sequence data and subsequent conclusions was performed by the candidate.)

A BC88 genomic library constructed in the cosmid vector pTB023 (construction described in section 3.3.1.) was screened for sequences homologous with the *tapA* and *tapD* probes used in section 4.2.2. Four positive clones were identified and one of these, designated pTB032, was chosen for further analysis. Southern hybridisation of pTB032 following various restriction digests demonstrated that an ~ 3.3 kb *Pst* I fragment would contain ~ 2.8 kb of unsequenced DNA upstream of *tapA*. This region was subcloned as a 3.3 kb *Pst* I fragment from pTB032 into pZErO-2.1, designated pG01. Next, a series of subclones were generated to allow sequencing of the entire 3.3 kb *Pst* I fragment contained in pG01, as depicted in Fig. 4.4. Nucleotide sequences were generated for each of these clones, and gaps were filled by sequencing directly off pG01 using primers as indicated in Fig. 4.4. A graphical representation of the entire 3396 bp *Pst* I fragment contained in pG01 is also given in Fig. 4.4. No open reading frames that would encode other putative virulence factors, or other proteins involved in the expression or assembly of bacterial pili, were identified.

Fig. 4.4. Sequencing of the *tapA*-proximal region from *A. veronii* biovar sobria BC88.

The regions contained in plasmids pG01, pG02 and pGO3 are indicated below the gene map. Arrows below the map represent individual sequencing runs. PCR primers (GM1 to GM4) used to fill sequence gaps are indicated. Relevant restriction sites are also indicated.


Pst I
BamH I
EcoR V
EcoR V
BamH I
Pst I
pdhR
ampD
3' nadC
tapA
pG01
pG02
pG03
GM2
GM3
GM4

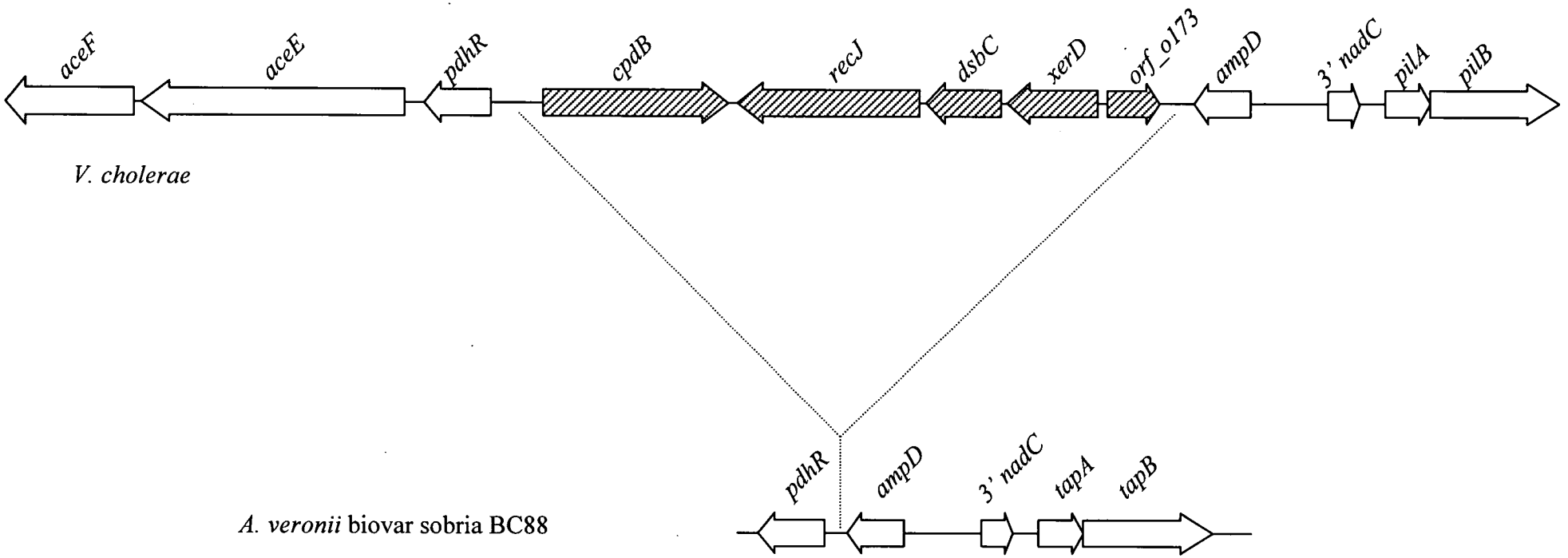
Immediately upstream of *tapA* is a small open reading frame (ORF) that exhibits homology with *nadC* from *Salmonella typhimurium*. However, the protein encoded by this ORF would be truncated and contains only the 3' 67 amino acids of NadC. Upstream of *nadC* is another ORF, transcribed in the opposite direction, which exhibits homology with *ampD* from *E. coli*. Downstream of *ampD*, is a partial ORF exhibiting homology with the 5' end of *pdhR* from *E. coli*. Interestingly this gene organisation is very similar to that published for the *pilA*-proximal region of *V. cholerae*, which also contains *ampD* and a truncated *nadC* (Fullner and Mekalanos, 1999).

To determine whether *pdhR* is also present upstream of *pilA* in this organism, a search of the unfinished *V. cholerae* genome sequence (http://www.tigr.org) was conducted for sequences encoding proteins homologous with the N-terminal region of *tapA*. A BLASTX search with this sequence was then conducted and the resulting protein coding regions compared with those found for strain BC88. A graphical representation of these results is shown in Fig. 4.5. What is immediately apparent following this analysis is that there appears to be an approximately 5 kb region of DNA present in *V. cholerae* that is absent from *A. veronii* biovar sobria BC88. This region encodes 5 separate open reading frames, all of which would encode proteins homologous to proteins that perform housekeeping functions.

Another possibility considered to account for the differences in hybridisation patterns between clinical and environmental isolates of *A. veronii* biovar sobria was the presence of promoter sequences upstream of *tapA*. Therefore, the region spanning the termination codon of *nadC* and the initiation codon of *tapA* was screened for potential promoter sequences. Sigma 54-dependent and σ70-dependent promoters were identified using software from the Department of Biochemistry and Molecular Biology, Pennsylvania State University (http://www.bmb.psu.edu/form1.html) and NNPP software at the Lawrence Berkley Laboratory, University of California (http://www-hgc.lbl.gov/projects/promoter.html), respectively. In this region, one potential σ54-dependent promoter, as described in chapter 3 (section 3.2.5.1.), and

Fig. 4.5. Comparison of the *tapA* proximal region of *A. veronii* biovar sobria BC88 with the *pilA* proximal region of *Vibrio cholerae.*

The 5 kb region containing five open reading frames, which appears to be unique to *V. cholerae*, is indicated.


aceF
aceE
pdhR
cpdB
recJ
dsbC
xerD
orf_o173
ampD
3' nadC
pilA
pilB
V. cholerae
pdhR
ampD
3' nadC
tapA
tapB
A. veronii biovar sobria BC88
1 kb

two potential σ70-dependent promoters were identified (see Fig. 4.6). However, the presence of a putative NifA binding site, required for activation of σ54-dependent promoters, was not identified upstream of the potential σ54-dependent promoter.

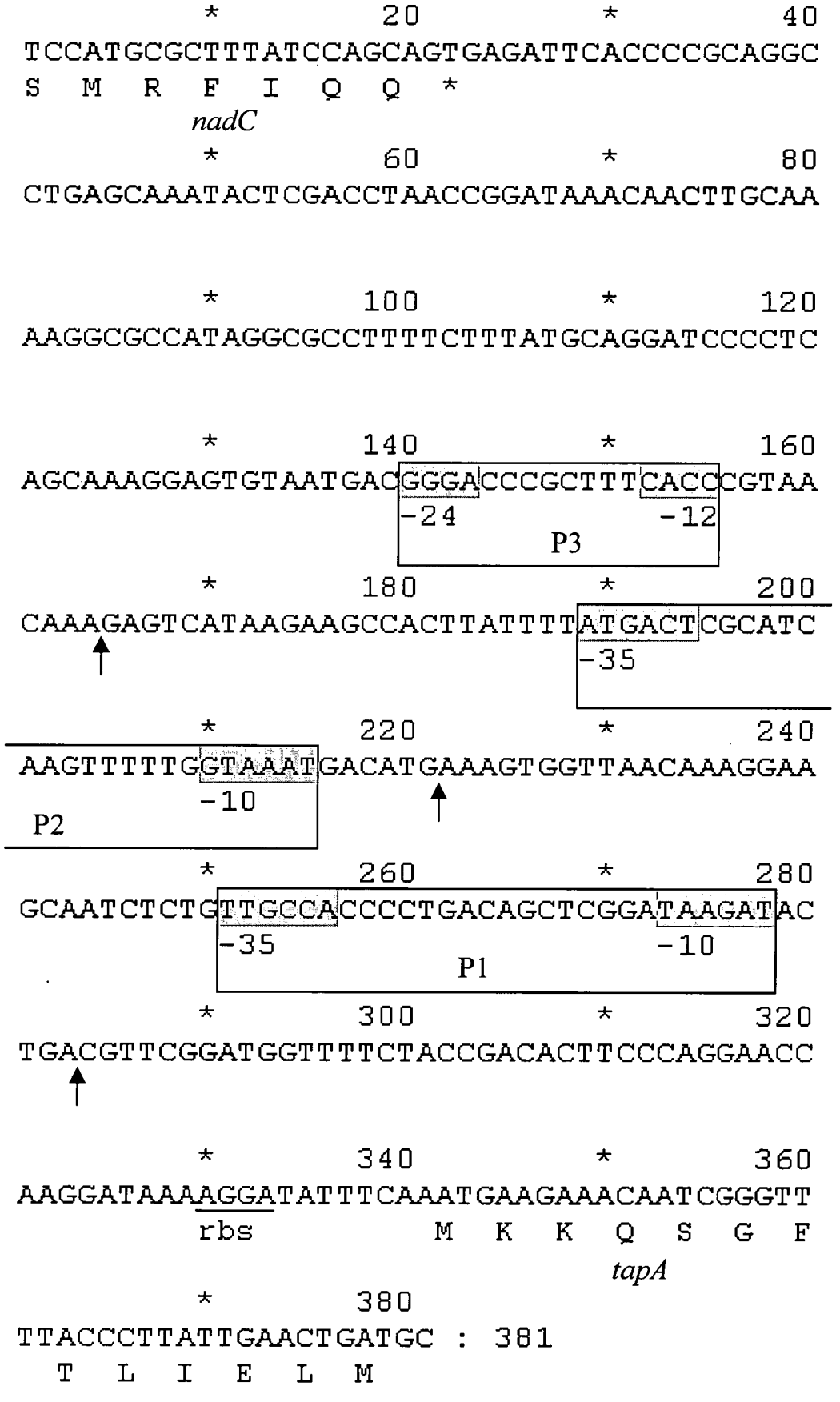
In summary, the results presented in this chapter have demonstrated that the *tap* gene cluster is widely distributed in the genus *Aeromonas*. All strains examined, irrespective of their species or source of isolation, possessed the *tap* cluster. The interesting finding that the *tapA* probe hybridised with an ~ 1 kb *BamH* I fragment from clinical isolates of *A. veronii* biovar sobria more frequently than it hybridised with a similarly-sized *BamH* I fragment from environmental isolates is being investigated further in our laboratory (Mr. G. Murray, BScHons). Sequencing of the *tapA*-proximal region did not identify other genes involved in virulence, pilus assembly, or expression, suggesting that this difference was not due to the presence of upstream genes conferring virulence. Three potential promoter elements were also identified, including one σ54-dependent promoter, which are most often responsible for the expression type IV prepilin genes. Studies are in progress investigating the sequence differences in the *tapA* proximal region from environmental isolates of *A. veronii* biovar sobria.

4.6. **Discussion**

The *Aeromonas tap* gene cluster encodes a putative type IV pilus that exhibits high homology with pilus gene clusters of several Gram-negative bacteria. For many of these organisms, type IV pili are essential virulence factors that mediate adhesion to epithelial cells. As an initial attempt to determine the significance of the *tap* gene cluster in *Aeromonas* species, the presence of the genes located at either end of this cluster was determined for a variety of strains representing species pathogenic and non-pathogenic for humans, and strains isolated from clinical and environmental sources. Results presented in this chapter have established that the *tap* gene cluster is widely distributed in *Aeromonas* species. All hybridisation group reference strains, and clinical and environmental isolates of gastroenteritis-associated species, isolated

Fig. 4.6. Nucleotide sequence of the *nadC-tapA* intergenic region from *A. veronii* biovar sobria BC88 showing putative promoter sequences.

P1 and P2 represent conventional σ70-dependent promoters while P3 represents a σ54-dependent promoter. Predicted transcription start points from each promoter are indicated with arrows below the sequence. A putative ribosomal binding site (AGGA) is underlined.


* 20 * 40
TCCATGCGCTTTATCCAGCAGTGAGATTCACCCCGCAGGC
S M R F I Q Q *
nadC
* 60 * 80
CTGAGCAAATACTCGACCTAACCGGATAAACAACTTGCAA
* 100 * 120
AAGGCGCCATAGGCGCCTTTTCTTTATGCAGGATCCCCTC
* 140 * 160
AGCAAAGGAGTGTAATGACGGGACCCGCTTTCACCCGTAA
-24 -12
P3
* 180 * 200
CAAAGAGTCATAAGAAGCCACTTATTTTATGACTCGCATC
-35
* 220 * 240
AAGTTTTTGGTAAATGACATGAAAGTGGTTAACAAAGGAA
-10
P2
* 260 * 280
GCAATCTCTGTTGCCACCCCTGACAGCTCGGATAAGATAC
-35 -10
P1
* 300 * 320
TGACGTTCGGATGGTTTTCTACCGACACTTCCCAGGAACC
* 340 * 360
AAGGATAAAAGGATATTTCAAATGAAGAAACAATCGGGTT
rbs M K K Q S G F
tapA
* 380
TTACCCTTATTGAACTGATGC : 381
T L I E L M

from various sources, possessed the *tapA* and *tapD* genes. In addition, other investigators have recently reported that this gene cluster is also present in the psychrophilic *Aeromonas* species, *A. salmonicida* (HG3), (Genbank accessions AF059248, AF003962, AF059249 and U95640).

The widespread distribution of the *tap* gene cluster is consistent with the distribution of type IVA pilus genes in other bacteria. For the majority of bacteria for which this type of study has been conducted, the genes encoding pili were found to be widespread in both pathogenic and non-pathogenic species. For example, both virulent and benign isolates of *Dichelobacter nodosus* have been found to encode pili (Rood *et al.*, 1997), and the genes encoding pili from *Legionella pneumophila* were found to be possessed by both the pathogenic (e.g. *L. pneumophila*, *L. longbeachae*) and non-pathogenic (e.g. *L. erythra*, *L. spiritensis*) *Legionella* species (Liles *et al.*, 1998). The exception to this rule are the members of the *Neisseria* genus. For these bacteria, sequences homologous to the pilin subunit gene (*pilE*) have only been found in the pathogenic species *N. meningitidis* and *N. gonorrhoeae* (Aho *et al.*, 1987; Wolff and Stern, 1995). Therefore, the fact that the *tap* cluster is present in both pathogenic and non-pathogenic species does not rule out that these genes may encode an important virulence factor for *Aeromonas* species.

The observed differences in the stringency conditions and the restriction fragment sizes with which the *tapA* and *tapD* probes hybridised may also indicate that there are two separate lineages of the *tap* cluster. The clusters from *A. hydrophila* and *A. veronii* biovar sobria hybridise with a *tapA* probe from *A. hydrophila* Ah65 at high stringency, and most commonly have *tapA* and *tapD* localised on 1.0 kb *BamH* I and 2.7 kb *Pst* I restriction fragments, respectively. In contrast, the *tap* cluster from *A. caviae* strains is characterised by hybridisation with the *A. hydrophila* Ah65 *tapA* gene only under low stringency conditions, and strains of *A. caviae* most commonly have *tapA* and *tapD* localise to 1.2 kb *BamH* I and 3.3 kb *Pst* I restriction fragments, respectively.

The sequence of *tapD* appears to be well conserved, as DNA from all isolates hybridised with a *tapD* probe under high stringency conditions. Such sequence

conservation is not unexpected, however, as the *tapD* gene encodes a protein with multiple enzymatic functions, which is likely to reside in the cytoplasmic membrane. In contrast, the sequences of *tapA* appear to be more variable, since strains of *A. caviae* hybridised with this probe only under low stringency conditions. If Tap pili are assembled on the cell surface, variation in *tapA* would presumably be driven by *in vivo* selection as are the pilin proteins of other Gram-negative pathogens, such as those belonging to the genus *Neisseria* (Seifert, 1996).

Initial screening results indicated that the *tapA* probe may have potential for use as a marker of pathogenic strains. In these initial experiments, the *tapA* probe hybridised with an ~ 1.0 kb *BamH* I fragment from clinical isolates of *A. veronii* biovar sobria more frequently than it hybridised with an ~ 1.0 kb fragment from environmental isolates. The clinical strains examined were not a homogenous group as they came from diverse geographical regions and varied considerably in their exotoxic activities. Therefore, it is unlikely that the conservation in the clinical strains simply represents multiple isolations of a clonal organism.

Further screening demonstrated that this difference was not as distinct as first thought. However, using genetically typed strains, diarrhoeal isolates almost always had *tapA* localised to an ~ 1.0 kb *BamH* I restriction fragment (85% tested) whereas this frequency was lower for environmental isolates (67% isolates tested). This finding needs to be investigated further using genetically typed strains of known source. It is possible that the results from the initial screening experiments may have been skewed by inaccurate species designation. In particular, some of the environmental isolates of *A. veronii* biovar sobria HG8/10 may have been inaccurately classified *A. sobria* HG7 strains. The methods used to speciate the majority of the strains used in this chapter involved several biochemical tests, which have been developed for clinical isolates, and environmental isolates do not always give reliable results when grown at 37°C. In order to clarify these possibilities, the environmental *A. veronii* biovar sobria strains used in this chapter have been sent to Dr M. Altwegg (University of Zurich, Zurich, Switzerland), to be genotyped. Unfortunately, the results of this testing are not yet available.

The observed restriction fragment length polymorphism between clinical and environmental isolates of *A. veronii* biovar sobria is consistent with nucleotide sequence differences within the region encompassing *tapA*. Such differences may have included the presence of important virulence determinants in clinical strains, or variations in genes or promoter sequences responsible for Tap pilus expression.

Sequencing upstream of *tapA* from strain BC88 revealed that this region is very similar to the *pilA*-proximal region of *V. cholerae*. However, based on this sequence data, it seems unlikely that differences upstream of *tapA* between clinical and environmental *A. veronii* biovar sobria could have accounted for the differences in hybridisation results. No ORFs upstream of *tapA* from strain BC88 resembled genes encoding virulence factors from other bacteria, or genes involved in the regulation of virulence factor expression. However, two genes identified did exhibit homology with genes encoding proteins involved in the regulation of expression of other gene systems. The *ampD* gene encodes a negative regulator of AmpC beta-lactamase expression responsible for cephalosporin resistance in the Enterobacteriaceae, while *pdhR* encodes a transcriptional repressor of the pyruvate dehydrogenase complex. All identified ORFs would encode full length proteins with the exception of *nadC*, which encodes a quinolinate phosphoribosyl transferase involved in NAD biosynthesis. Again, the nature of this gene is identical to the *nadC*-homologous ORF from *V. cholerae*. The *nadC* genes from these two organisms are truncated, and only the C-terminal 67 amino acids would be expressed by *A. veronii* biovar sobria BC88.

Analysis of DNA sequence between *nadC* and *tapA* revealed the presence of one potential σ54-dependent promoter and two potential σ70-dependent promoters. The majority of type IV prepilin genes described to date are transcribed from σ54-dependent promoters. However, while both of the σ70-dependent promoters conformed reasonably well to the consensus sequence, giving scores of 0.99 (P1) and 0.91 (P2), the sequence of the putative σ54-dependent promoter upstream of *tapA* ($GGN_{10}AC$) did not conform exactly to the consensus sequence for this class of promoter ($GGN_{10}GC$). Furthermore, a consensus sequence for the NifA binding site

(required for activation from σ54-dependent promoters) was not found within the region 75 to 125 bp upstream of this potential σ54-dependent promoter. It is interesting to note, however, that specific mutations have been identified that allow transcription from σ54-dependent promoters in the absence of upstream regulatory sequences (Wang *et al.*, 1997). One class of these mutants possessed a dysfunctional –12 promoter recognition sequence. It is, therefore, possible that the non-consensus –12 sequence in *A. veronii* biovar sobria BC88 allows transcription of *tapA* in the absence of an upstream NifA binding site.

In summary, the *tap* cluster is widely distributed in the genus *Aeromonas*. This suggests that the *tap* gene cluster encodes a structure important for some aspect of the biology of these bacteria. Experiments described in chapter 5 will investigate whether Tap pili mediate adhesion to, and colonisation of, the gastrointestinal tract and factors influencing their expression.

Chapter 5

The role of Tap pili in the adhesion and colonisation of intestinal tissue by *Aeromonas veronii* biovar sobria

5.1. Introduction

The aim of this chapter was to determine whether Tap pili mediate binding to host tissues and whether these structures are involved in intestinal colonisation by *Aeromonas* species. This was accomplished by investigating the ability of wild-type (BC88) and *tapA* mutant (BC88*tapA*::Ω) strains to adhere to intestinal tissue cell lines, and assessing these strains in two animal models of gastrointestinal tract infection. Expression of Tap pili by BC88 was also investigated as part of these studies, and this necessitated the preparation of an antiserum against TapA from *A. veronii* biovar sobria BC88.

5.2. Construction and *in vitro* characterisation of a *Aeromonas veronii* biovar sobria BC88*tapA*::Ω mutant strain

A. veronii biovar sobria BC88*tapA*::Ω was constructed by Dr. M. S. Strom, NWFSC, Seattle, U.S.A. This strain was prepared by replacing the wild type copy of *tapA* with a copy that had been interrupted by insertion of the omega (Ω) interposon, encoding spectinomycin resistance. A 1.0 kb *BamH* I fragment (containing *tapA*) was isolated from pTB012 and inserted into the *Bgl* II site of the *sacB* suicide vector pEP185.2, producing pMS012. The Ω interposon (from plasmid pUC19Ω) was then inserted as a 2.1 kb *Sma* I fragment into the end-filled *Pst* I site of pMS012, producing pMS012Ω. Plasmid pMS012Ω was subsequently transformed into *E. coli* S17-1*λpir* and mobilised into *A. veronii* biovar sobria BC88 by conjugation, and transconjugants were selected on BHIA containing ampicillin and spectinomycin. Plasmid pEP185.2 requires the *λpir* gene product to replicate, and is therefore unable to replicate in *Aeromonas*. Selection of double recombinant strains, which possessed the *tapA*::Ω mutation with the suicide vector resolved from the chromosome, was confirmed by Southern hybridisation, using a digoxigenin-labelled 1.0 kb *BamH* I fragment (*tapA*) from pTB012 as a probe.

The omega interposon creates a mutation with strong polar transcription and translation termination signals (Frey and Krisch, 1985). The *tapA*::Ω mutation lies

upstream of *tapB*, *-C*, and *–D*. The *tapD* gene product has been shown to be involved in extracellular protein secretion, including the secretion of at least one virulence factor, aerolysin. A mutation in *tapD* causes pleiotrophic secretion defects presumably due to a lack of processing of prepilin-like components of the type II secretion pathway (ExeG, -H, -I, and –J). Therefore, for the subsequent experiments involving BC88*tapA*::Ω (*tapA* mutant), it was necessary to establish whether the *tapA*::Ω mutation had polar downstream effects on the expression of *tapD*. This was accomplished by assaying extracellular toxic activities, since haemolytic, cytotoxic and enterotoxic activities are all mediated, at least in part, by aerolysin. These toxin assays were performed by Mr. A. Kelleher (BScHons 1998, Discipline of Pathology, University of Tasmania). The results are summarised in Table 5.1.

BC88*tapA*::Ω (*tapA* mutant) produced wild-type levels of haemolytic, cytotoxic and enterotoxic activities. Therefore, the extracellular protein secretion of *A. veronii* biovar sobria BC88 did not appear to be affected by the *tapA*::Ω mutation.

5.3. *In vitro* adhesion of BC88 and BC88*tapA*::Ω

In an initial attempt to determine whether Tap pili are required for adhesion of *Aeromonas* bacteria to human tissues, BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) were assessed for their abilities to adhere to human intestinal tissue and to HEp-2 epithelial and Henle 407 intestinal cell lines.

5.3.1. Adhesion to human intestinal tissue

The ability of BC88 and BC88*tapA*::Ω to adhere to human intestinal tissue was assessed by measuring the adherence of *A. veronii* biovar sobria BC88 and BC88*tapA*::Ω strains to intestinal tissues obtained from routine biopsies at the Royal Hobart Hospital (Tasmania, Australia), followed by examination by light microscopy. These experiments were performed by Ms. A. Stimming (BMedSc, Discipline of Pathology, University of Tasmania).

Table 5.1. Toxin production by *A. veronii* biovar sobria strains BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant)

Strain	Haemolytic titre[a]	Cytotoxic titre[b]	Enterotoxic activity[c]
BC88	1024[d]	128[d]	0.087
BC88*tapA*::Ω	1024[d]	128[d]	0.085

[a] Haemolytic activity expressed as the highest dilution of cell free culture supernatants causing lysis to rabbit erythrocytes.

[b] Cytotoxic activity expressed as the highest dilution of cell-free culture supernatants causing 50% lysis to Vero cells.

[c] Enterotoxic activity expressed as the ratio of intestinal weight to remaining body weight following administration of 100 µl cell free culture supernatant. A value above 0.075 was considered positive.

[d] The comparatively low cytotoxic titre is due to the methodology of the cytotoxin assay used.

Both BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) appeared to adhere at similar levels to the microvillus brush border of the enterocytes, suggesting that the *tapA*::Ω mutation did not effect the ability of strain BC88 to adhere to human intestinal tissue. However, this assay was not performed so as to allow quantitative measurements of adhesion. Minor differences in adhesion would, therefore, not have been observed.

5.3.2. Adhesion to HEp-2 and Henle 407 cell lines

Quantitative adhesion to HEp-2 and Henle 407 (INT407) cell lines was assessed according to the protocol of Carrello *et al.* (1988). For these experiments, strains were grown under standard *in vitro* growth conditions (BHIB, 37°C, static), as described by Carrello *et al.* (1988). The results of these experiments are summarised in Table 5.2.

No significant difference in the adhesion of BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) was observed to either HEp-2 (p=0.759) and Henle 407 (p=0.129) cells. For these experiments, bacteria were grown under standard *in vitro* culture conditions. It has already been established in our laboratory that Tap pili either constitute a minor pilus population under these conditions, or that they are not assembled on the bacterial surface (Kirov and Sanderson, 1996). Hence, it was important to evaluate the colonisation of the intestinal tract *in vivo* by BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant), where it is possible that expression of Tap pili is induced.

5.4. *In vivo* behaviour of BC88*tapA*::Ω

To establish whether the *tapA*::Ω mutation had any affect on the ability of strain BC88 to colonise the intestinal tract *in vivo*, BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) were assessed in infant mice (colonisation and competition experiments) and rabbits using the removable intestinal tie adult rabbit diarrhoeal (RITARD) model.

Table 5.2. Adhesion to epithelial and intestinal cell lines by *A. veronii* biovar sobria BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant).

	Cell adherence[a]	
Strain	**HEp-2**	**Henle 407**
BC88	10.1 ± 0.7	13.3 ± 2.1
tapA::Ω	11.1 ± 1.6	16.2 ± 1.3
	p = 0.759	p = 0.129

[a] Adherence expressed as the mean number of bacteria per cell ± standard deviation.

5.4.1. Colonisation of infant mice

The infant mouse model of bacterial colonisation has been well established for use to examine the virulence and ability to colonise the intestine by strains of *Vibrio cholerae* (Attridge *et al.*, 1993; Attridge *et al.*, 1996; Fullner and Mekalanos, 1999). This model has also been used to measure the virulence of *Aeromonas hydrophila* (Wong *et al.*, 1996) using the LD_{50} assay described for *V. cholerae* (Chaicumpa and Rowley, 1972). Results from the report by Wong *et al.* (1996) also indicated that *Aeromonas* bacteria may colonise the infant mouse intestinal tract at sub-lethal doses.

Since the infant mouse model had not been used to assess colonisation by *A. veronii* biovar sobria, initial experiments were conducted to establish whether BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) were capable of persisting in the infant mouse intestinal tract. These experiments involved the administration of each strain separately to groups of six BALB/c infant mice. At 24 h post infection, mice were sacrificed and bacteria were enumerated by plate counts. The results are summarised in Table 5.3.

Both strains appeared to colonise at similar levels. The wild-type strain was retrieved at a level of $3.1 \times 10^7 \pm 4.4 \times 10^7$ (range: 4×10^6 to 9.6×10^7), compared to a level of $2.6 \times 10^7 \pm 2.0 \times 10^7$ (range: 4×10^6 to 4.8×10^7) for BC88*tapA*::Ω (*tapA* mutant). A high level of variation was observed for intestinal recoveries of each organism from individual mice and, consequently, no statistical difference in adhesion was observed. Therefore, to compare more accurately the ability of these strains to colonise the intestine, an alternative method was adopted. This involved administration of BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) simultaneously to individual mice (competition assay). This method is well established for assessing mutants defective in the production of putative colonisation factors of *V. cholerae* (Attridge *et al.*, 1993; 1996). BC88 and BC88*tapA*::Ω were administered in competition to groups of six or seven infant mice according to the protocols of Attridge *et al.* (1993) and Fullner and Mekalanos (Fullner and Mekalanos, 1999).

Table 5.3. Infant mouse colonisation by *A. veronii* biovar sobria BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant)[a]

Strain	Inoculum[b]	Recovery[c,d]	
BC88	1.4×10^7	1.6×10^6	$3.1 \times 10^7 \pm 4.4 \times 10^7$
		9.6×10^7	
		6.0×10^7	
		4.0×10^6	
		4.0×10^6	
		2.0×10^7	
BC88*tapA*::Ω	2.1×10^7	2.8×10^7	$2.6 \times 10^7 \pm 2.0 \times 10^7$
		4.8×10^7	
		4.0×10^6	
		8.0×10^6	
		4.4×10^7	
		2.4×10^7	

[a] Six mice were included in each test group.

[b] The inoculum was determined retrospectively by plate counts.

[c] The recovery was calculated as the total number of bacteria recovered from intestinal tissue after 24 h ± standard deviation.

[d] The recovery of strains BC88 and BC88*tapA*::Ω were not statistically different (students t-test, p = 0.78548).

Comparative inoculation of BC88 and BC88*tapA*::Ω strains was repeated twice, and the results are indicated in Fig. 5.1.

Fig. 5.1 shows the ratios of BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) bacteria recovered from intestinal homogenates of individual mice (output ratio, represented as individual symbols), for each of two experiments. The input ratio for each experiment is indicated below each test group. For each experiment, the output ratios were very similar to the ratios present in the challenge inoculum, indicating that the *tapA*::Ω mutation did not decrease the ability of BC88 to colonise the infant mouse intestinal tract.

5.4.2. Removable intestinal tie adult rabbit diarrhoeal (RITARD) model

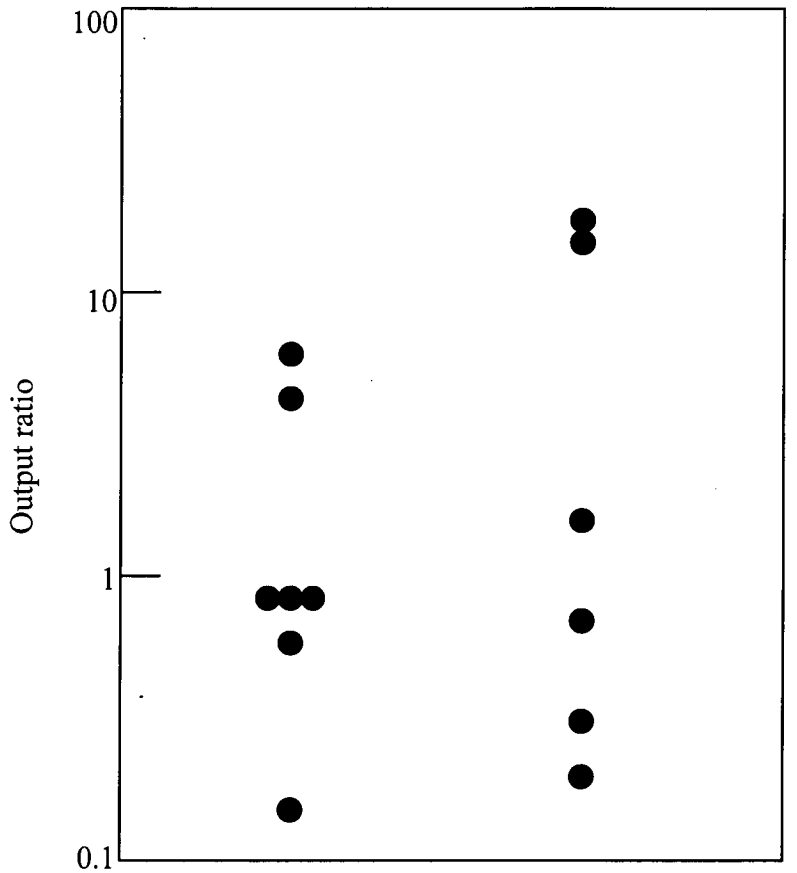
The BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) strains were tested in the removable intestinal tie adult rabbit diarrhoeal (RITARD) model, as described by Pazzaglia *et al.* (1990). These experiments were performed by Dr. M. J. Albert (International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh).

Nine rabbits each were challenged with BC88 and BC88*tapA*::Ω, and five rabbits were challenged with a control organism, *E. coli* EC101. Each animal was administered the appropriate bacterial strain at a dose of 10^{10} organisms. Rabbits were observed for eight days for diarrhoeal symptoms and shedding of the test organism. Results are summarised in Table 5.4.

No diarrhoeal symptoms were observed when the negative control strain, *E. coli* EC101, was administered to five rabbits in this experiment, or to 12 rabbits used as similar controls in other experiments. The BC88 wild-type strain caused diarrhoeal symptoms lasting for one to three days in six of nine rabbits. Hence, *A. veronii* biovar sobria BC88 is able to cause a mild, self-limiting gastrointestinal illness in rabbits. The BC88*tapA*::Ω (*tapA* mutant) strain produced diarrhoeal symptoms in four of nine rabbits. This difference cannot be considered significant due to the small number of test animals used in this experiment. The fact that four

Fig. 5.1. Colonisation of infant mice by BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant).

Individual mice were fed mixed suspensions of wild-type and mutant bacteria. Input ratios are indicated below each test group (n=2). Each symbol represents the wild-type:mutant output ratio for bacteria recovered from the intestines of individual mice.



Experiment no.	1	2
Input ratio	0.9	0.75

Table 5.4. Ability of *A. veronii* biovar sobria BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) to cause disease in the RITARD model

Strain	No. rabbits tested	No. shedding *Aeromonas*[a]	No. with diarrhoea
BC88	9	7	6
BC88*tapA*::Ω	9	8	4
E. coli EC101	5 (12)[b]	0	0

[a] All rabbits were monitored for eight days. Both BC88 and BC88*tapA*::Ω were shed for periods ranging from one to three days.

[b] Five rabbits were included as controls in this experiment; 12 control animals were from previous experiments conducted at the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh (M. J. Albert, personal communication).

rabbits administered BC88*tapA*::Ω (*tapA* mutant) exhibited diarrhoeal symptoms indicates that possession of Tap pili is not essential for this process.

Rabbits administered the wild-type and mutant strains were also assessed for faecal shedding of each test strain. Both strains were shed for periods ranging from one to five days, by seven of nine rabbits (BC88), or eight of nine rabbits (BC88*tapA*::Ω). The results obtained also indicate that the *tapA*::Ω mutation does not attenuate the virulence of *A. veronii* biovar sobria BC88 in this animal model.

Hence, *in vitro* and *in vivo* studies suggest that Tap pili do not mediate adherence to epithelial and intestinal cell surfaces, and that they are not essential for colonisation of the intestinal tract. However, it is unclear whether the *tap* gene cluster encodes a pilus that is expressed and assembled on the bacterial cell surface. From the *in vitro* studies conducted in our laboratory, these pili are either not expressed, or constitute a minor pilus under standard *in vitro* culture conditions. Therefore, experiments were performed to investigate the assembly of Tap pili by *A. veronii* biovar sobria BC88.

5.5. Expression and assembly of Tap pili by *Aeromonas veronii* biovar sobria BC88

To investigate whether Tap pili are expressed, an antiserum against the protein encoded by *tapA* from *A. veronii* biovar sobria BC88 was produced. Since Tap pili have never been purified from the surface of *Aeromonas* species, a recombinant TapA protein was produced and used to raise a polyclonal rabbit antiserum, which could then used to assess TapA expression and assembly on the bacterial cell surface.

To prepare a TapA antiserum, rabbits were immunised with a recombinant TapA protein, hereafter, r-TapA, produced by overexpression of *tapA* in *E. coli* BL21(DE3). To facilitate the identification and purification of r-TapA, this protein was fused to a His•tag sequence, which can be purified by affinity chromatography to divalent cations (see section 2.12).

5.5.1. Construction of a *tapA*^{BC88} His•tag expression construct

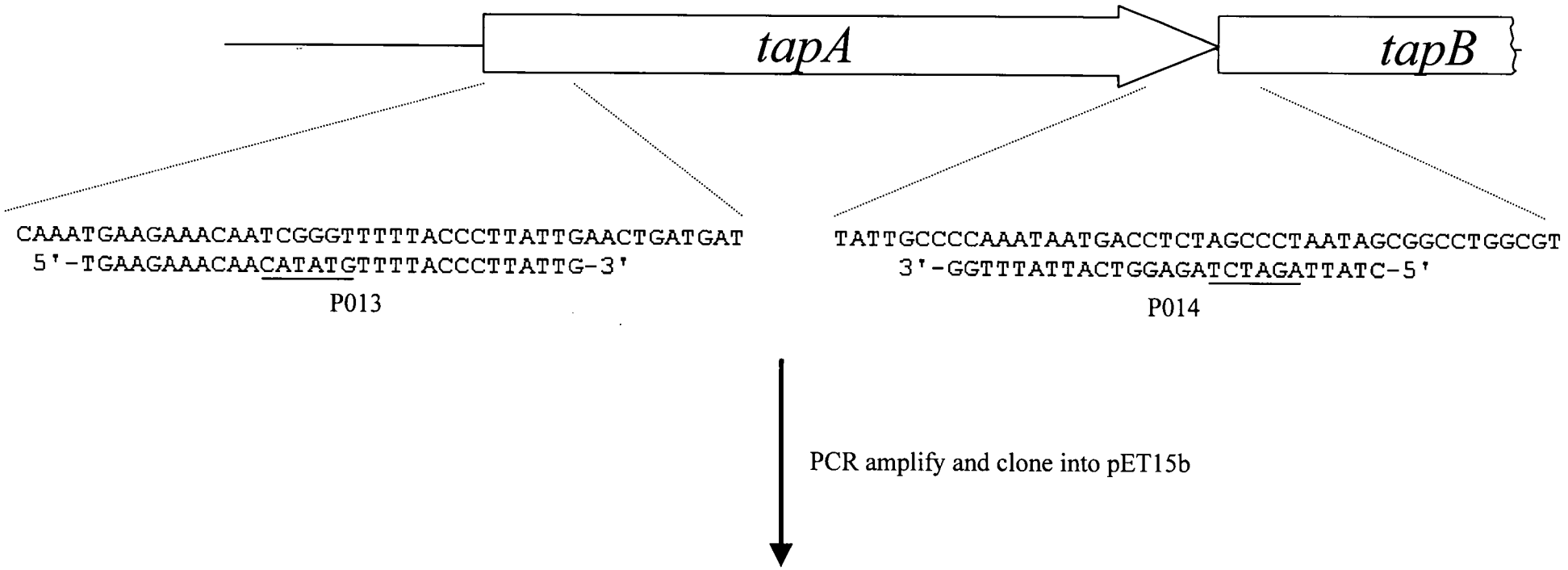
Initially, the entire *tapA* gene (including the type IV leader sequence) was incorporated into the r-TapA expression construct. The *tapA* open reading frame from *A. veronii* biovar sobria BC88 was amplified using primers P004 and P005, which contain engineered sites *BamH* I and *EcoR* V sites, respectively, to allow subsequent cloning into the expression vector pQE32 (Appendix 7). The resulting PCR product was digested with *BamH* I and *EcoR* V and cloned into pQE32. This construct, designated pTB025, was transformed into *E. coli* TOP10F' and cultures were induced with IPTG as described in section 2.12.1. However, upon induction of r-TapA expression from this construct, the cells appeared to lyse. This may have occurred as a result of toxicity associated with the type IV leader peptide. The toxicity of *Neisseria gonorrhoeae* PilE, following expression in *E. coli*, has been shown to be associated with the type IV leader peptide (Fyfe *et al.*, 1993).

Therefore, an alternative strategy was adopted, which involved producing a fusion protein that lacked the type IV leader peptide, followed by expression using the pET expression system. This system had been used successfully to prepare an antiserum against the C-terminal region of the *A. hydrophila* Ah65 TapA protein, and to demonstrate that this protein is expressed (Dr. M. S. Strom, personal communication). The pET system utilises an extremely strong expression system involving the T7 RNA polymerase. This system can be kept transcriptionally silent and, upon induction, produces the highest transcription levels of any commercially available expression system. The revised cloning strategy for production of the TapA-His•tag fusion protein construct is indicated in Fig. 5.2.

The *tapA* open reading frame from *A. veronii* biovar sobria BC88 was amplified using primers P013 and P014 (Appendix 7). P013 was designed so as to incorporate a *Nde* I site after the preTapA leader sequence, in order to remove this sequence following digestion with *Nde* I. The PCR product produced by this method was digested with *Nde* I and *Bgl* II, and cloned into the *Nde* I and *BamH* I sites of pET15b to produce pTB028. Expression from this construct would produce a fusion

Fig. 5.2. Cloning strategy for the construction of pTB028.

The DNA sequences at the 5' and 3' ends of *tapA* used for the construction of primers P013 and P014, respectively, are shown. Restriction sites incorporated to facilitate cloning are underlined. pTB028 was constructed by PCR amplification of *tapA* with P013 and P014 and digesting the resulting PCR product with *Nde* I and *Bgl* II. The resulting DNA fragment was cloned into the *Nde* I and *BamH* I sites of pET15b, generating pTB028. The sequence of the r-TapA fusion protein is also indicated. The vector-derived His•tag sequence is in bold font.



r-TapA **MGSSHHHHHHSSGLVPRGSHM**FTLIELMIVVAIVAILAAV.....CAGASNVNDYCPK

protein containing an N-terminal His•tag sequence, as shown in Fig. 5.2. The predicted sequence of this protein was confirmed by sequencing of the pTB028 expression region, using a primer to the T7 promoter located upstream of *tapA* (Appendix 7).

5.5.2. Identification of clones which express r-TapA

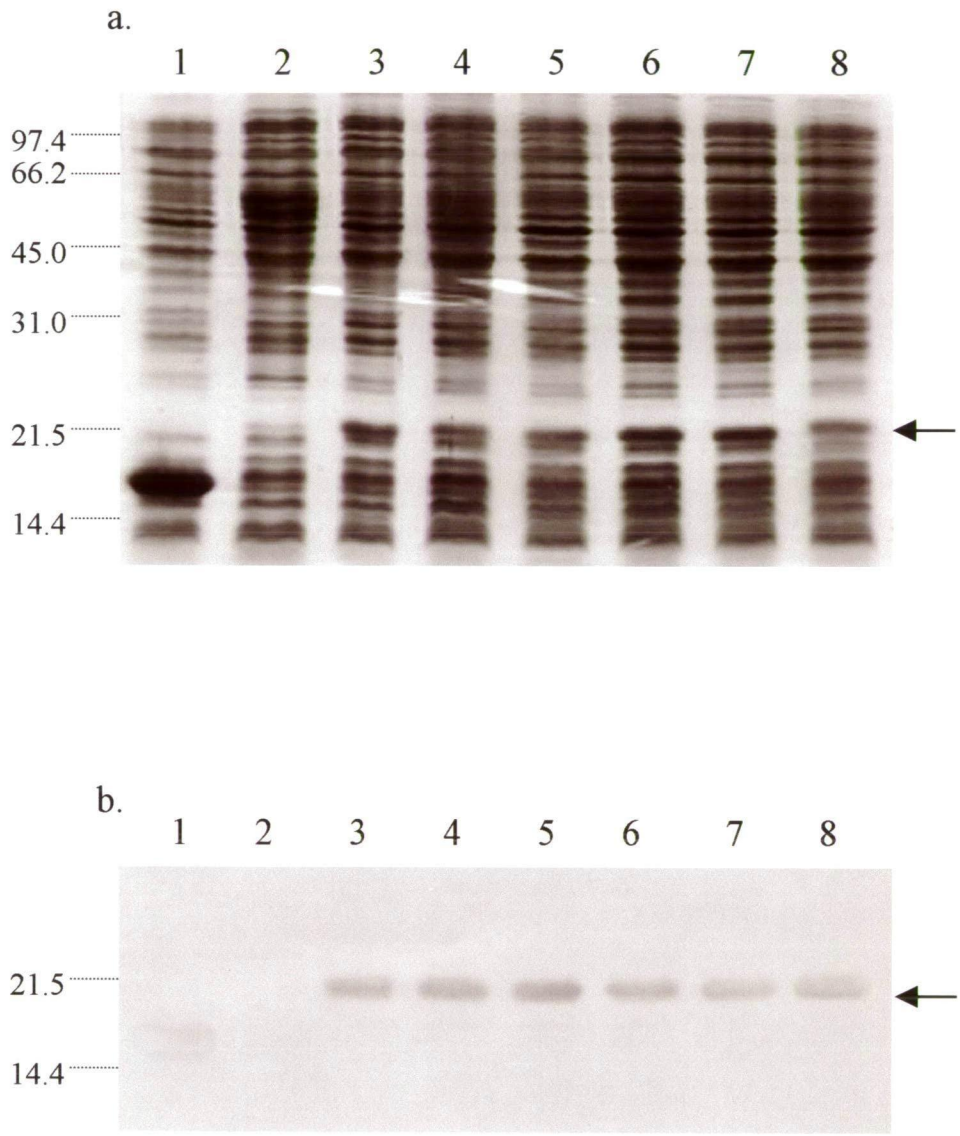
Plasmid pTB028 was isolated and transformed into *E. coli* BL21(DE3), a strain that possesses the gene encoding T7 RNA polymerase under control of a *lac* promoter. Expression of T7 RNA polymerase occurs following addition of 1 mM IPTG to the growth medium and this, in turn, results in the expression of the TapA-His•tag fusion protein from the T7 promoter. Six BL21 clones were grown and induced as described in section 2.13.1, and whole cell lysates were analysed for r-TapA expression by SDS-PAGE. The results are shown in Fig. 5.3a.

In Fig. 5.3a, lane 1 contains plasmid pCP1183 in *E. coli* BL21(DE3). This plasmid contains the region of *tapA* from *A. hydrophila* Ah65 encoding the C-terminal 126 amino acids, fused to the His•tag sequence in pET15b (obtained from Dr. M. Strom), and was used as a positive control in all expression and purification studies described in this section. Lane 2 contains plasmid pET15b in *E. coli* BL21(DE3). This strain was used as a negative control in all expression experiments. Lanes 3 to 8 contain plasmid pTB028 in *E. coli* BL21(DE3). All pTB028 clones produced a 21 kDa protein following induction, as indicated by an arrow. This protein was absent in the negative control strain (lane 2). The molecular weight of this protein corresponds exactly to the predicted molecular weight of the r-TapA protein, as judged from the amino acid sequence. The identity of the 21 kDa protein was confirmed by western blot analysis with a tetra•His antibody (Qiagen, Hilden, Germany). Results are shown in Fig. 5.3b. This antibody is specific for four successive histidine residues, which are present within the His•tag sequence. However, the level of expression was lower than that of the positive control plasmid pCP1183.

Fig. 5.3. Identification of r-TapA expression clones.

a. SDS-PAGE analysis. b. Western blot analysis. His•tag proteins are detected with a tetra•His monoclonal antibody specific for four successive histidine residues. Molecular weight markers (size in kilodaltons) are indicated to the left. The 21 kDa protein (r-TapA) is indicated to the right with an arrow.

Lane 1. BL21(DE3) containing pCP1183, positive control
Lane 2. BL21(DE3) containing pET15b, negative control
Lane 3. Individual BL21(DE3) clones containing pTB028


a.
1
2
3
4
5
6
7
8
97.4
66.2
45.0
31.0
21.5
14.4
b.
1
2
3
4
5
6
7
8
21.5
14.4

5.5.3. Optimisation of expression

The levels of r-TapA expression were fairly low, as judged by SDS-PAGE analysis. Therefore, two factors known to influence the levels of protein expression in the pET system were investigated to determine whether the relative levels of r-TapA production could be increased. These factors were the stability of the expression construct, and assessment of r-TapA expression in different *E. coli* host strains.

Plasmid pTB028 was found not to be unstable in BL21(DE3) in the presence of 100 μg/ml carbenicillin. The use of three different host strains, *E. coli* strains BL21(DE3), BL21(DE3) (pLysS), and JM109(DE3) were also tested. Plasmid pLysS encodes T7 lysozyme, which is a repressor of residual T7 RNA polymerase-mediated expression and is often used to minimise baseline expression of toxic proteins in *E. coli*. The highest levels of expression were observed when *E. coli* BL21(DE3) was the host strain. Therefore, this strain was used for all subsequent experiments.

5.5.4. Reduction of background protein expression

Despite the experiments described above, the level of r-TapA expression in *E. coli* BL21(DE3) remained low. The r-TapA protein was estimated to be expressed at ~ 1 to 5% of the total cell protein in *E. coli* BL21(DE3), compared with ~ 30% for the positive control plasmid pCP1183. Since the experiments in section 5.3.1.3. failed to increase the amount of r-TapA produced, attempts were made to reduce the amount of background host cell protein production by inhibiting the host RNA polymerase with the antibiotic rifampicin. T7 RNA polymerase is unique in that its activity is not inhibited by rifampicin. Consequently, this antibiotic has been used to inhibit host cell protein production during T7 RNA polymerase protein expression (Tabor and Richardson, 1985). Expression of the T7 RNA polymerase enzyme in *E. coli* BL21(DE3) is under the control of the *E. coli* RNA polymerase enzyme, which is inhibited by rifampicin. Hence, a time course of expression was conducted where

200 μg/ml of rifampicin was added at various intervals following induction with IPTG. The results of this experiment are shown in Fig. 5.4.

After induction, a 21 kDa protein was observed in all cultures containing pTB028. However, the levels of background protein expression were much lower in those cultures to which rifampicin had been added. The relative level of r-TapA expression was highest when rifampicin was added 30 min after induction with IPTG. This condition was, therefore, used in all subsequent experiments.

5.5.5. Purification of r-TapA

The levels of r-TapA produced following the induction conditions described above were approximately five times lower than the protein levels observed following induction from the positive control plasmid pCP1183. Therefore, culture volumes were scaled up ten-fold to ensure that suitable amounts of this protein would be available for purification. Processing of the cell pellets following induction indicated that the r-TapA protein was contained within insoluble inclusion bodies. Consequently, purification was carried out under denaturing conditions (6 M guanidine hydrochloride).

The His•tag purification procedure relies on the affinity of the amino acid histidine to divalent cations. The His•tag sequence binds reversibly to nickel ions immobilised on a NTA resin (Qiagen, Hilden, Germany) and, after unbound proteins are removed by washing, the target protein can be eluted by competition with the histidine analogue, imidazole. Attempts to purify the r-TapA protein using this approach, however, were unsuccessful due to the extremely insoluble nature of this protein when expressed in *E. coli*. The majority of r-TapA protein was not able to be dissolved following overnight incubation in 8 M urea or 6 M guanidine hydrochloride.

The insoluble nature of r-TapA was eventually exploited to remove the majority of contaminating host proteins, and obtain a relatively pure protein preparation. This procedure involved incubating the insoluble fraction in increasing concentrations of urea (2, 4, 6, and 8M) using a slight modification of the protocol

Fig. 5.4. Reduction of background protein expression from r-TapA expression clones.

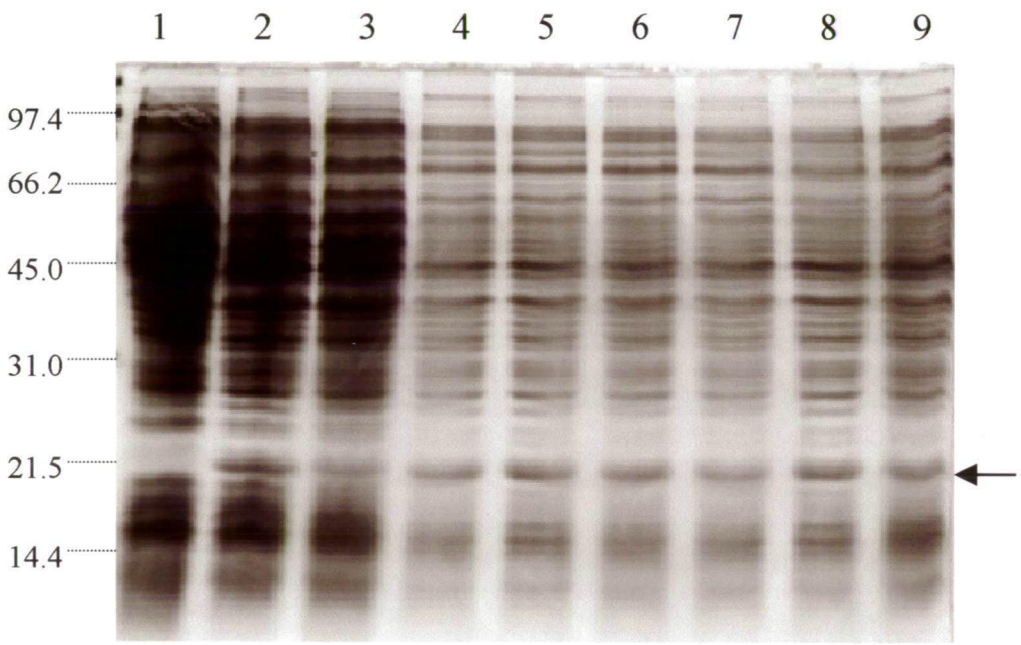
Following induction with IPTG, 200 μg/ml rifampicin was added to inhibit host protein synthesis at the time points indicated and each culture was subjected to SDS-PAGE. Molecular weight markers (size in kilodaltons) are indicated to the left. The 21 kDa r-TapA protein is indicated to the right with an arrow. Note the reduced levels of background proteins in lanes 4-9.

Lane 1. BL21(DE3) pTB028 uninduced

Lane 2. BL21(DE3) pTB028 induced

Lane 3. BL21(DE3) pTB028 induced, rifampicin added after 4 h induction

Lane 4. BL21(DE3) pTB028 induced, rifampicin added after 15, 30, 60, 90, 120, and 180 min induction, respectively


1
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3
4
5
6
7
8
9
97.4
66.2
45.0
31.0
21.5
14.4

described by Reischl (1998) (section 2.13). The results of this experiment are shown in Fig. 5.5.

In Fig. 5.5, lane 1 contains the soluble protein fraction. Lanes 2 to 5 contain fractions successively solubilised in 2, 4, 6 and 8 M urea, and lane 6 contains the insoluble fraction. A 21 kDa protein band was apparent in all lanes, and the concentration of this protein was highest in the 8M urea-insoluble fraction. This protein was isolated by SDS-PAGE and used to immunise New Zealand white rabbits, according to the protocol outlined in section 2.13.4.

In summary, the pET expression system was used to produce a relatively pure preparation of a recombinant TapA protein. New Zealand white rabbits were immunised with this protein. For the initial challenge, 100 μg of r-TapA was emulsified in Freund's complete adjuvant and administered to each of three rabbits subcutaneously (sc). Subsequent challenges (100 μg r-TapA emulsified in Freund's incomplete adjuvant) were administered sc at days 21, 35 and 49. Test bleeds were obtained at days 28, 42 and 56, and assessed for reactivity against whole cell lysates of *A. veronii* biovar sobria BC88 and *E. coli* BL21(DE3) (pTB028).

5.5.6. Antiserum characterisation

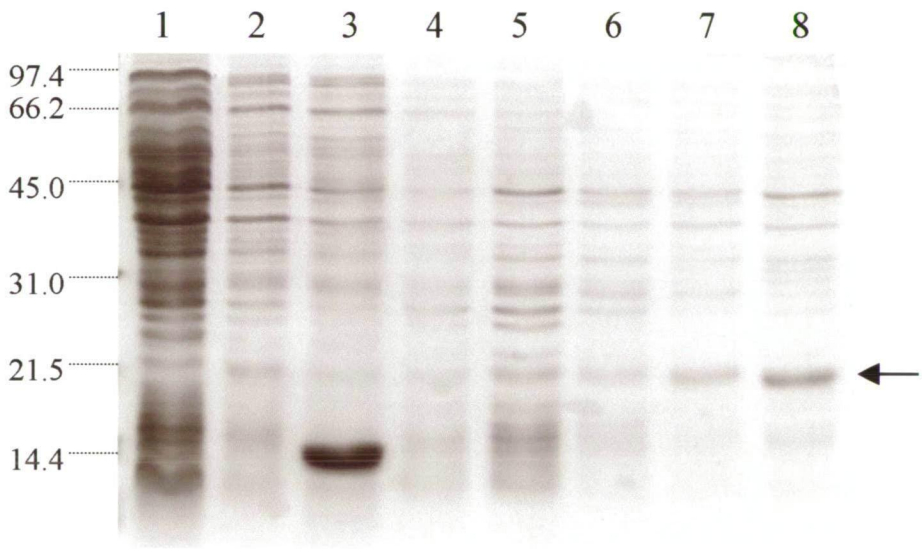
Test bleeds (~ 5 ml; section 5.5.5) were obtained and tested for reactivity by western blotting. Although these bleeds indicated that the rabbits were responding with a good immune response, the unabsorbed antiserum exhibited high reactivity against a wide range of antigens from both strains tested. Consequently, specific reactivity against TapA was not readily observed. Therefore, it was decided to absorb each antiserum against an acetone powder of BC88*tapA*::Ω as described in section 2.13.5. Results of western blots, using this absorbed antiserum, against whole cell lysates of *A. veronii* biovar sobria BC88 and BC88*tapA*::Ω are shown in Fig. 5.6.

Of the three rabbits immunised with r-TapA, one produced a good level of reactivity against TapA. This antiserum reacted with a strong protein band from BC88, ~ 20.5 kDa is size, which is absent in the BC88*tapA*::Ω lane (Fig. 5.6). This

Fig. 5.5. Semi-purification of r-TapA by removal of contaminating proteins.

SDS-PAGE analysis of partially-purified r-TapA fractions. Contaminating proteins were removed following successive solubilisation in urea by a modification of the procedure of Reischl (1998). Molecular weight markers (size in kilodaltons) are indicated to the left. The 21 kDa r-TapA protein is indicated to the right with an arrow.

Lane 1. BL21(DE3) pTB028 uninduced
Lane 2. BL21(DE3) pTB028 induced
Lane 3. soluble fraction
Lane 4. 2 M urea-soluble fraction
Lane 5. 4 M urea-soluble fraction
Lane 6. 6 M urea-soluble fraction
Lane 7. 8 M urea-soluble fraction
Lane 8. 8 M urea-insoluble fraction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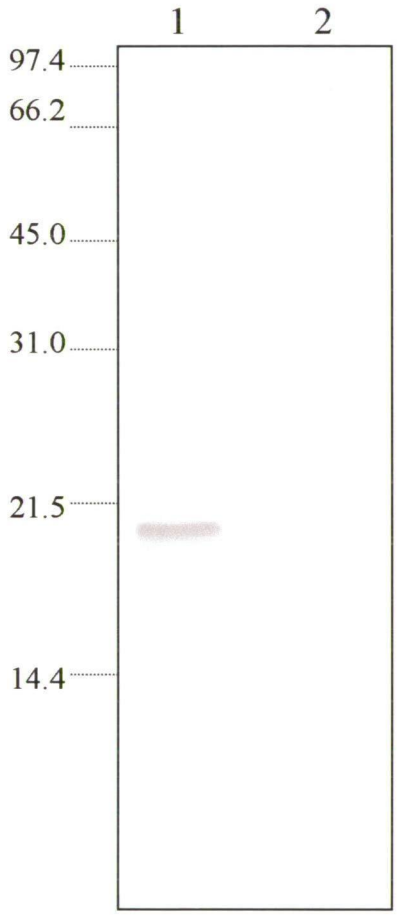
8
97.4
66.2
45.0
31.0
21.5
14.4

Fig. 5.6. Western blot analysis of whole cell lysates of BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) with the r-TapA antiserum.

The r-TapA antiserum was absorbed four times against an acetone powder of BC88*tapA*::Ω (*tapA* mutant). Note a 21.5 kDa protein present in BC88 (lane 1) and absent in BC88*tapA*::Ω (lane 2). The observed laddering effect of this protein may be due to altered glycosylation states, or some chemical property of the TapA amino acid sequence.


1
2
97.4
66.2
45.0
31.0
21.5
14.4

demonstrates both that the TapA antiserum is specific for TapA, and that *tapA* is expressed by *A. veronii* biovar sobria BC88. However, the size of the TapA protein (20.5 kDa) was higher than that predicted by the gene sequence (17 kDa). This discrepancy may have been caused by some chemical property of the TapA primary amino acid sequence, causing some effect on charge or folding. Alternatively, the slower apparent migration of TapA may have been caused by alternate glycosylation states of this protein. The primary amino acid sequence of TapA does contain two possible *N*-linked glycosylation sites at residues 32 and 118. These possibilities may also account for the slight laddering effect of this protein, which is visible in Fig. 5.6, lane 1.

5.5.7. Electron microscopic examination of Tap pilus assembly

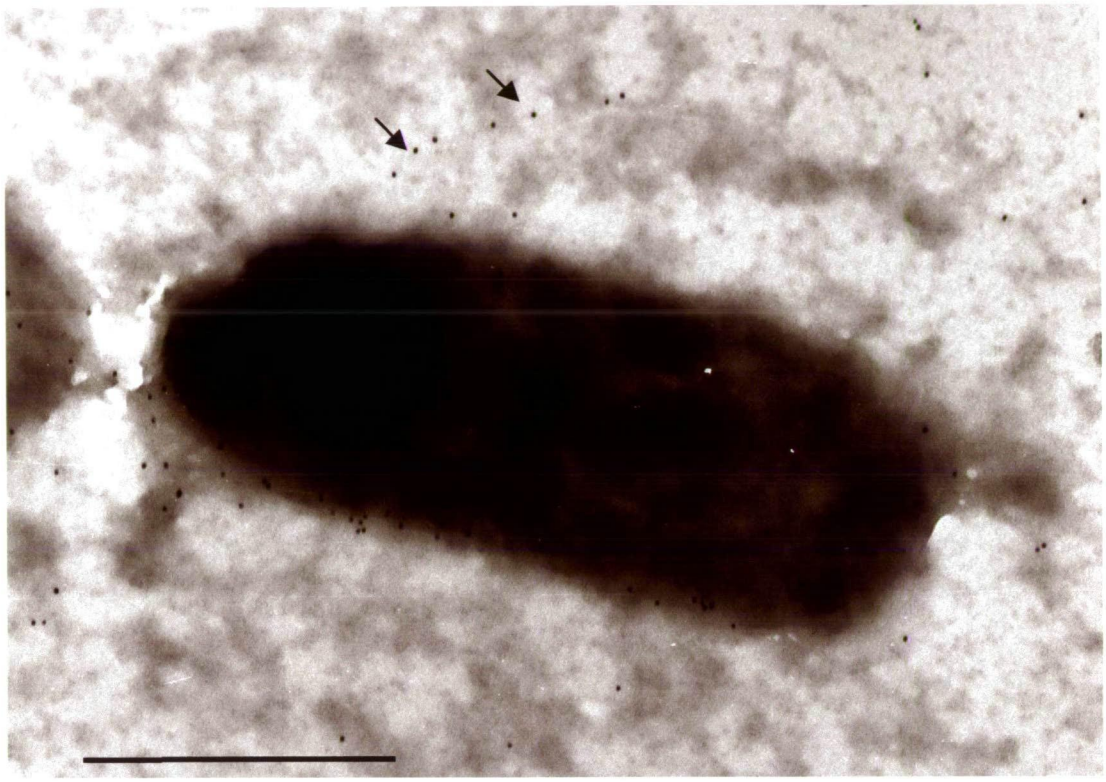
Previous studies in our laboratory have demonstrated that almost all pili expressed on the surface of *A. veronii* biovar sobria BC88 when grown under standard *in vitro* culture conditions (TSBY, 22°C, 48 h) are Bfp pili (Kirov and Sanderson, 1996). However, Kirov and Sanderson (1996) also documented conditions (TSAY, 22°C, 48 h) where a significant proportion of the pilus structures were distinct from Bfp pili. Therefore, *A. veronii* biovar sobria BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) were grown under the different conditions examined above, and examined by immune electron microscopy using the TapA antiserum. A variety of dilutions of this antiserum were assessed (1:10, 1:100 and 1:1000), and a 1:100 dilution of Bfp antiserum (Kirov and Sanderson, 1996) served as a positive control. The results of this experiment are shown in Fig. 5.7.

Fig. 5.7 shows immunogold electron microscopic examination of *A. veronii* biovar sobria BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant), grown on TSAY at 22°C for 48 h, stained with a 1:10 dilution of the TapA antiserum. No labelling of pilus structures was detected with this antiserum. The Bfp antiserum, however, labelled pili on the surface of both BC88 and BC88*tapA*::Ω.

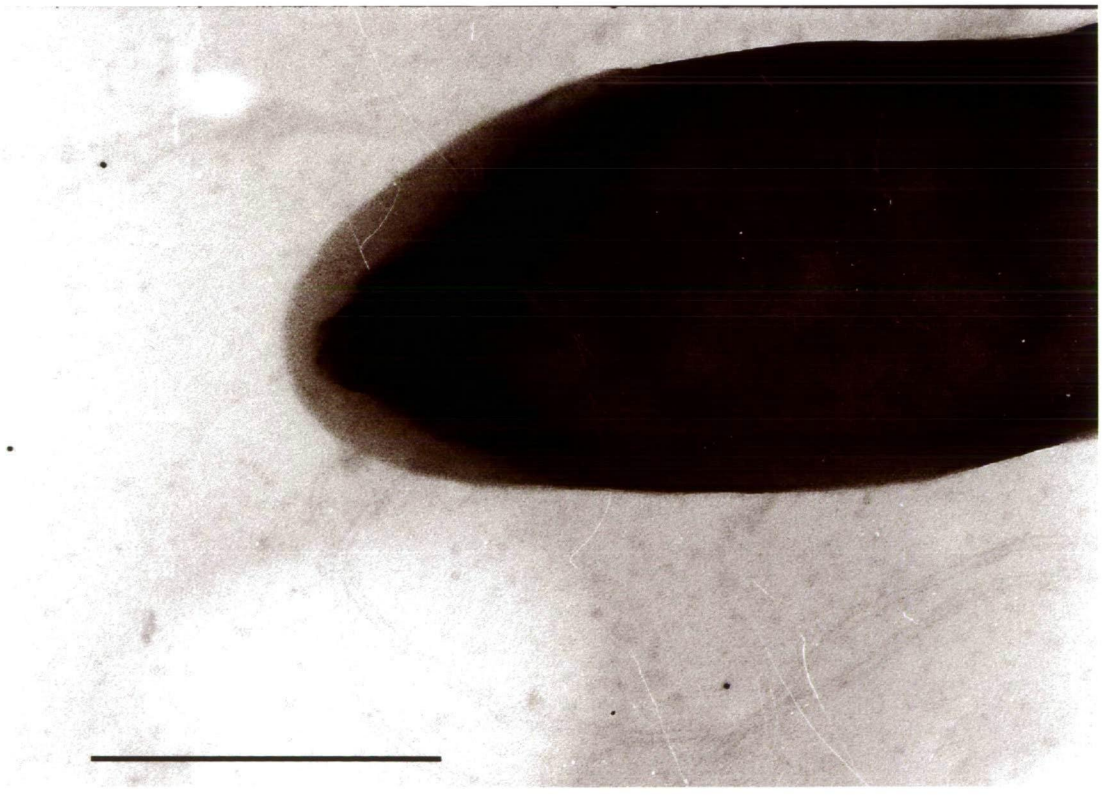
Fig. 5.7. Immunogold electron microscopic analysis of Tap pilus assembly by *A. veronii* biovar sobria BC88.

A. veronii biovar sobria BC88 (wild-type) was grown on TSAY for 48 h at 22°C and stained with the appropriate antiserum preparation. a. 1:100 dilution of Bfp antiserum. Arrows indicate immonogold labelling of a Bfp pilus. b. 1:10 dilution of r-TapA antiserum. Bar represents 0.5 μm. Note the absence of staining with the r-TapA antiserum.

a.



b.



5.6. Discussion

The experiments outlined in this chapter have described the construction of an isogenic *tapA* mutant strain of *A. veronii* biovar sobria BC88, and the evaluation of this strain using *in vitro* models of adhesion and *in vivo* models of intestinal colonisation.

Adhesion to cell lines has been shown to correlate with enteropathogenicity for *Aeromonas* species, and this is thought to reflect a strains ability to colonise the intestinal tract (Kirov *et al.*, 1995a). The *tapA*::Ω mutation did not attenuate *A. veronii* biovar sobria BC88 in any of the *in vitro* adherence models tested. BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) adhered to human intestinal tissue, HEp-2 epithelial cells, and Henle 407 intestinal cells at similar levels and the differences in adhesion were not statistically significant. However, given that Tap pili do not appear to be assembled under *in vitro* culture conditions, this observation is not unexpected. *A. veronii* biovar sobria BC88 has been the subject of intensive investigations in our laboratory with respect to its filamentous surface structures. Immunoelectron microscopy has revealed that the vast majority of pili expressed by this strain are label with Bfp antiserum (Kirov and Sanderson, 1996). However, a small proportion (< 2%) of the bacterial proportion produced an immunologically distinct pilus, which was also present in very low numbers (< 3 per cell on average) (Kirov and Sanderson, 1996). Whether these unlabelled pili are Tap pili remains to be determined. If so, Tap pili would constitute a minor pilus when bacteria are grown under standard *in vitro* culture conditions.

The widespread conservation of the *tap* gene cluster suggests that Tap pili play some role in the biology of *Aeromonas* bacteria, such as in the adhesion to inorganic substrates and the formation of biofilms in environmental niches. Tap pili may also be involved in adhesion to cells, despite the observations discussed above, where their low level of expression *in vitro*, and the presence of expressed Bfp, masks the effect of the *tapA*::Ω mutation. Another possibility is that the Tap pilus, while not important for adhesion to epithelial or intestinal cells, is a colonisation factor by

mediating bacterium-bacterium adhesion and microcolony formation. This property has been attributed to type IV pili, but primarily to those of the type IVB pili represented by the *Vibrio cholerae* TCP, enteropathogenic *E. coli* BFP, and enterotoxigenic *E. coli* Longus pilus.

The effect of the *tapA*::Ω mutation was, therefore, assessed in two *in vivo* models of intestinal infection to further investigate its possible role as a colonisation factor. The rabbit (RITARD) model used is one of two models of *Aeromonas* gastrointestinal disease reported to mimic diarrhoeal symptoms (Pazzaglia *et al.*, 1990). The RITARD model is a complex model involving surgical administration of the test strain directly into the intestines of test animals and, as such, makes the testing of large numbers of animals difficult and expensive. Hence the relatively few animals able to be investigated in these studies.

The infant mouse model is often used to investigate the colonisation factors of *Vibrio cholerae* (Attridge *et al.*, 1993; Attridge *et al.*, 1996; Fullner and Mekalanos, 1999). This model has also been applied to the measurement the virulence of *Aeromonas* species by determining a 50% lethal dose (LD_{50}) as described by Wong *et al.* (1996). Wong *et al.* (1996) demonstrated that a strain of *A. hydrophila* (strain A6) was able to persist in the infant mouse intestine for considerable periods of time following administration at a level corresponding to 0.4 LD_{50} units. Based on this report, it was decided to investigate the colonisation of BC88 (wild type) and BC88*tapA*::Ω (*tapA* mutant) using this infant mouse model. However, due to the availability of BALB/c mice, these were substituted for the Swiss-Webster mice used by Wong *et al.* (1996) and Attridge *et al.* (1993; 1996).

Initial experiments with infant mice involved the administration of BC88 (wild type) and BC88*tapA*::Ω (*tapA* mutant) strains separately to individual mice. These experiments were primarily designed to determine whether *A. veronii* biovar sobria BC88 could colonise BALB/c mice at similar levels as *A. hydrophila* A6 in Swiss-Webster mice. However, it was also designed so that major defects in the colonisation of the intestinal tract by BC88*tapA*::Ω strain would be detected. Results

indicated that BC88 could persist in BALB/c mice for periods greater than 24 h, since the output levels of both BC88 and BC88*tapA*::Ω strains 24 h post-inoculation was similar to their input levels. This experiment, therefore, did not detect any differences between the recovery of BC88 (wild-type) and BC88*tapA*::Ω (*tapA* mutant) from the mouse intestinal tract.

An infant mouse competition assay was used in order to make a better comparison of the ability of BC88 (wild type) and BC88*tapA*::Ω (*tapA* mutant) to colonise intestinal tissue. Again, this approach indicated that the *tapA*::Ω mutation did not attenuate colonisation of the intestinal tract by *A. veronii* biovar sobria BC88. The results obtained for this assay supported the results observed for the RITARD model, in that Tap pili are either not expressed in sufficient levels, or do not function as intestinal colonisation factors.

The *tap* genes represent a conserved gene cluster whose components are necessary for extracellular protein secretion. The putative prepilin gene, *tapA*, encodes a full length protein of 151 amino acids which contains all the features currently recognised to be essential for assembly into surface pili. Furthermore, the *tapA* genes from both *A. hydrophila* Ah65 and *A. veronii* biovar sobria BC88 are expressed as determined by western blot analysis using antisera raised against recombinant TapA proteins. However, Tap pili do not appear to be assembled in large numbers on the surface of either *A. veronii* biovar sobria BC88 (section 5.5.7) or *A. hydrophila* Ah65 (Dr. M. S. Strom, personal communication), at least under standard *in vitro* culture conditions. It seems unlikely that Tap pili are not assembled by *Aeromonas* species, since it is likely that expression of TapA would be selected against if this protein was of no use to the cell.

The assembly of Tap pili on the bacterial surface may be regulated by the expression, or phase variation, of an accessory gene required for assembly, as previous studies in our laboratory demonstrated that non-Bfp pili are expressed when the *in vitro* growth conditions are altered. However, despite growing BC88 under these latter conditions, no Tap pili could be identified.

It cannot be discounted, however, that the TapA antiserum does not recognise TapA in its native conformation, and that the Bfp-distinct pili described above are in fact Tap pili. The TapA antiserum was prepared using a denatured recombinant protein purified by SDS-PAGE, and it is possible that the epitopes recognised by this serum are unnatural or sequestered in the assembled pilus structures. Furthermore, it is possible that the His•tag sequence, or the absence of disulphide bonds, in the recombinant proteins may have altered their folding when compared with the native proteins. It would be useful to check the reactivity of this antiserum against native TapA by enzyme-linked immunosorbant assay, or by western blotting following native polyacrylamide gel electrophoresis.

In summary, the results presented in this chapter have explored the function and expression the prepilin gene, *tapA*, encoded by the *tap* gene cluster. The evidence thus far suggests that this gene does not encode a pilus as significant in adhesion and colonisation as the Bfp pilus. In this respect, it appears that the *tap* gene cluster is similar in both structure (chapter 4) and function (chapter 5) to the *pil* gene cluster recently identified in *Vibrio cholerae*, which also had no role in adhesion or colonisation of infant mice.

Chapter 6

Concluding discussion

6.1. Introduction

6.2. *Aeromonas* species associated with gastrointestinal disease possess two distinct families of type IV pili

6.3. Distribution of the *tap* gene cluster

6.4. Expression of Tap pili

6.5. Function of Tap pili

6.6. Further studies

6.7. Conclusion

6.1. Introduction

Bacteria belonging to the genus *Aeromonas* are ubiquitous in the aquatic environment and have been linked to serious invasive diseases of both humans and animals. Although the subject of some controversy, some strains of *Aeromonas* bacteria are also believed to be primary gastrointestinal pathogens of humans. There is, however, no method currently available to identify these enteropathogenic strains. Although accurate species determination of an isolate is important, this identification alone is not sufficient. It is likely that only a subset of the three most common diarrhoea-associated species, *A. hydrophila* HG1, *A. caviae* HG4 and *A. veronii* biovar sobria HG8/10 possess the combinations of virulence factors that allow them to cause disease. Much effort has centred on elucidating the varied virulence mechanisms possessed by *Aeromonas* bacteria in an effort to identify characteristics that may be useful for the identification of pathogenic strains.

For other enteropathogenic bacteria, type IV pili are often the primary bacterial structures mediating colonisation of intestinal tissues (Attridge *et al.*, 1996; Donnenberg *et al.*, 1997). At the commencement of the studies described in this thesis, very little was known about *Aeromonas* intestinal colonisation factors. Type IV pili had been purified from several diarrhoea-associated strains, and there was some indirect evidence that these structures mediated bacterial adhesion to enterocytes. The overall aim of this thesis, therefore, was to expand studies in this area by studying the genetics and function of *Aeromonas* type IV pili.

6.2. *Aeromonas* species associated with gastrointestinal disease possess two distinct families of type IV pili

The initial aim of this thesis was to clone the genes encoding the bundle-forming pilus (Bfp) expressed by a diarrhoea-associated *Aeromonas* strain (BC88). To accomplish this, a number of probes were used to identify genes encoding pili by Southern hybridisation. One probe was a type IV prepilin gene, *tapA*, recently cloned from a strain of *A. hydrophila* (Pepe *et al.*, 1996). The use of this probe resulted in

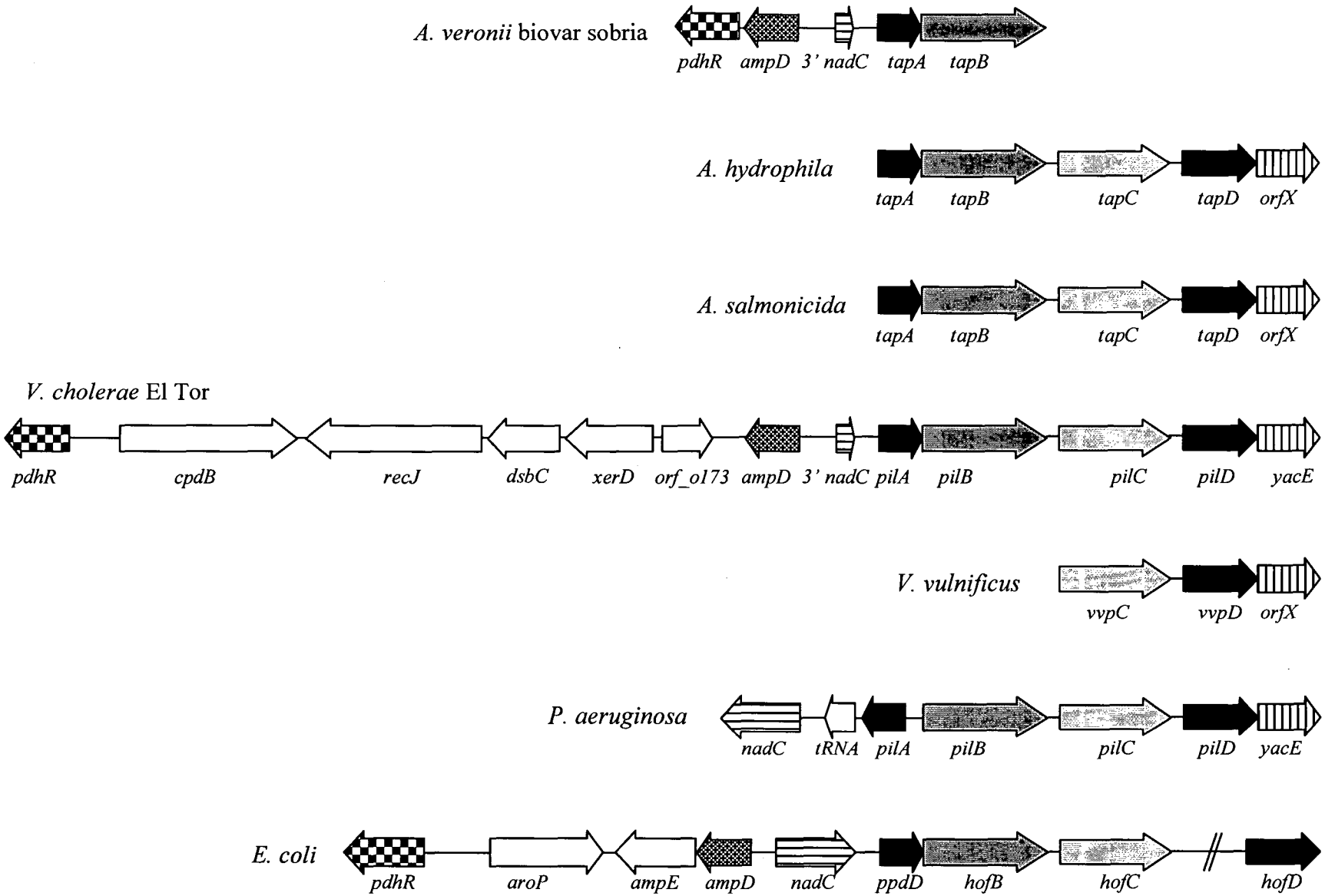
the cloning of genes encoding a second family of type IV pili from *A. veronii* biovar sobria BC88. The cloning of the *tap* gene cluster from this strain, which was known to express Bfp pili (Kirov and Sanderson, 1996), clearly established that there are two families of type IV pili in *Aeromonas* species. This finding helped to resolve confusion in the literature between the *tap* cluster and the purified Bfp pili, and raised questions regarding the function(s) of these two families of pili. The cloning of the *tap* gene cluster from BC88 also allowed the production of an isogenic *tapA* mutant strain, which was used to analyse the function of this family of pili at the genetic level.

The four genes that comprise the *tap* cluster are all transcribed in the same direction. However, these genes do not appear to form an operon. The intergenic space between *tapB* and *tapC* is quite large (Pepe *et al.*, 1996), and a polar mutation constructed in *tapA* had no effect on protein secretion, an affect that would have been clearly visible if *tapD* was affected by this mutation. Expression of *tapA* is probably driven from one of the three promoters (one σ54 and two σ70) identified upstream of this gene in *A. veronii* biovar sobria BC88. For the majority of type IV pilus systems (e.g. *P. aeruginosa* and *D. nodosus*), expression of the pilin gene is initiated from a single σ54-dependent promoter (Johnson *et al.*, 1986; Thony and Hennecke, 1989). However, for strains of *N. gonorrhoeae*, a σ70-dependent promoter appears to mediate *pilE* expression (Fyfe *et al.*, 1993). Primer extension analysis could be used to map the transcription start site and determine which of these potential promoter sequences is responsible for *tapA* expression.

The organisation of the *tap* cluster, and the flanking DNA regions, bears striking similarity to the clusters from *V. vulnificus*, *V. cholerae*, and *E. coli* (Fig. 6.1). The presence and orientation of genes upstream of *tapA* suggests that the *tap* cluster may have arisen by lateral transfer. This theory is supported by the fact that sequence similarities in type IV pilin proteins do not fit into taxospecies classification (Spangenberg *et al.*, 1997).

Fig. 6.1. Organisation of representative pilus gene clusters from various bacteria.

The organisation of each gene cluster was assembled from sequences submitted to the Genbank database as follows: *A. hydrophila* Ah65 (U20255), *A. salmonicida* A450 (AF059248 and AF059249), *V. vulnificus* M06-24 (AF070934), *E. coli* K12 (AE000120, L28105, and D26562), and *P. aeruginosa* PAK (M32066 and M14849). Similar genes are shaded in a similar manner. Genes not shaded do not exhibit homology with any of the other genes shown.


A. veronii biovar sobria
pdhR ampD 3' nadC tapA tapB
A. hydrophila
tapA tapB tapC tapD orfX
A. salmonicida
tapA tapB tapC tapD orfX
V. cholerae El Tor
pdhR cpdB recJ dsbC xerD orf_o173 ampD 3' nadC pilA pilB pilC pilD yacE
V. vulnificus
vvpC vvpD orfX
P. aeruginosa
nadC tRNA pilA pilB pilC pilD yacE
E. coli
pdhR aroP ampE ampD nadC ppdD hofB hofC hofD

6.3. Distribution of the *tap* gene cluster

The *tap* gene cluster was found to be widely distributed in *Aeromonas* isolates representing species both pathogenic and non-pathogenic to humans, and in isolates from clinical and environmental sources. This finding possibly argues against a role for these pili in virulence. However, type IV pilus genes from other pathogenic bacteria may, or may not, be present in non-pathogenic isolates. In *D. nodosus* (Rood *et al.*, 1997) and *Legionella* species (Liles *et al.*, 1998), genes encoding type IV pili are widely distributed among pathogenic and non-pathogenic isolates, whereas in *Neisseria* species (Aho *et al.*, 1987; Wolff and Stern, 1995), *V. cholerae* (Karaolis *et al.*, 1998), and *E. coli* (Sohel *et al.*, 1996) they are limited to pathogenic strains. Therefore, the widespread distribution of the *tap* cluster does not discount the possibility that these genes encode a pilus important in the pathogenesis of *Aeromonas* infections.

Differences in the hybridisation results of the different species examined suggests that there may be two lineages of the *tap* gene cluster. The *tap* clusters from *A. hydrophila* and *A. veronii* biovar sobria appear to be more closely related to each other than they are to the *tap* cluster from *A. caviae*. This situation is reminiscent of that of *Dichelobacter nodosus*. Strains of *D. nodosus* possess either Class I or Class II pili. These classes are defined by both sequence conservation within the pilin subunit, and gene organisation with respect to other pilus biogenesis proteins (Hobbs *et al.*, 1991; Mattick *et al.*, 1991). Although it is likely that the TapA proteins from all *Aeromonas* species exhibit significant sequence divergence outside species boundaries, the organisation of the *tap* cluster may also vary between these two groups. This idea could be confirmed by cloning and sequencing the *tap* cluster from *A. caviae*, since, to my knowledge, no investigations of this type have yet been conducted. (There are no sequences in the Genbank database corresponding to the *tap* cluster from *A. caviae*.)

Despite the above results, which suggested that the *tap* gene cluster would not prove useful as a marker of pathogenic strains, preliminary results did indicate that

hybridisation with a *tapA* probe may differentiate clinical and environmental isolates of one species, *A. veronii* biovar sobria. For clinical isolates of this species, the *tapA* gene tended to be present on an ~ 1 kb *BamH* I fragment more frequently than it was for environmental isolates. However, subsequent analysis using genetically-typed strains indicated that the distinction between clinical and environmental isolates was not as great as first thought. Approximately 85% of diarrhoeal strains had *tapA* localised to an ~ 1 kb *BamH* I fragment, while in non-diarrhoeal and environmental isolates, this frequency was around 67%. Hence, although the *tapA* probe has identified two distinct populations of *A. veronii* biovar sobria strains (*tapA* on an ~ 1 kb *BamH* I fragment, and *tapA* on a larger fragment), the significance of this observation, and ability to characterise pathogenic *A. veronii* biovar sobria is unclear at present and is being further studied in our laboratory (Mr. Gerald Murray, BScHons 1999).

6.4. Expression of Tap pili

Experiments described in this thesis suggest that Tap pili are likely to be assembled into surface pili, since all of the genes that comprise the *tap* cluster encode functional proteins. The *tapA* genes from both *A. veronii* biovar sobria BC88 (this study) and *A. hydrophila* Ah65 (Dr. M. S. Strom, personal communication) were shown to be expressed using polyclonal rabbit antisera. Moreover, *tapB*, *-C*, and *–D* from Ah65 have been shown to complement corresponding *pil* mutations in *P. aeruginosa* (Pepe *et al.*, 1996). However, immune electron microscopic analysis could not demonstrate Tap pili directly on the surface of BC88, indicating that these pili are either expressed at very low levels under standard *in vitro* growth conditions, or they are not expressed under these conditions.

Conditions that favour the assembly of Tap pili on the surface of *Aeromonas* bacteria remain to be defined. As expression of TapA was detected under standard *in vitro* culture conditions, the regulation of Tap pilus expression is most likely mediated by expression of another protein. This may occur at the level of transcription, or by phase variation as is the case for *Neisseria gonorrhoeae*.

Expression of pili by *N. gonorrhoeae* is subject to phase variation of PilC, a protein required for pilus assembly and/or translocation across the outer membrane. Expression of PilC is controlled at the translational level by frameshift mutations in a run of G residues positioned in the region encoding the signal peptide. This variation in PilC expression results in pilus phase variation independent of the pilus subunit (PilE) (Jonsson *et al.*, 1991).

If expression of Tap pili is regulated at the level of transcription, the growth conditions under which these pili are assembled remain to be defined. Growth conditions, shown in a previous study (Kirov and Sanderson, 1996) to result in the expression of surface pili distinct to Bfp, did not appear to influence the expression of Tap pili on the bacterial surface. Therefore, it is likely that another defined *in vivo* or environmental growth condition is required. Electron microscopic examination of bacteria recovered from biofilms, or following intestinal passage, may identify these conditions.

Wild-type and *tapA* mutant strains were examined for their ability to colonise the gastrointestinal tract in two animal models, where conditions might be expected to favour expression of Tap pili if they are indeed important intestinal colonisation factors. If Tap pili were assembled on the bacterial surface under these conditions, however, results indicated that they are unlikely to be the primary structures mediating intestinal colonisation. It is also possible that assembly of Tap pili is down-regulated during *in vivo* passage, since electron microscopic analysis of clinical and environmental strains of *A. veronii* biovar sobria have demonstrated that environmental strains possess far more filamentous surface structures when compared to clinical isolates (Kirov *et al.*, 1995b).

It is interesting to note that assembled pili encoded by the *tap*-homologous gene clusters from *Vibrio* species and *E. coli*, described above, have not been detected. As these clusters all have a similar genetic organisation, it is possible that the assembly of their encoded gene products into pili is mediated by a common mechanism. Hence, further studies aimed at elucidating the conditions necessary for

assembly of Tap pili may shed light on the assembly and role in virulence of similar structures encoded by *V. cholerae*, *V. vulnificus*, and possibly *E. coli*.

6.5. Function of Tap pili

As mentioned in section 6.4, experiments conducted in this thesis indicate that Tap pili are not essential for gastrointestinal colonisation by *Aeromonas* species. Mutation of *tapA* did not affect the ability of *A. veronii* biovar sobria BC88 to adhere to cell lines or to intestinal tissue *in vitro*, or to colonise the intestinal tract of mice or rabbits. However, there are several other possibilities that could account for these observations

Firstly, the experiments described in this thesis were conducted with a wild-type and an isogenic *tapA* mutant strain of *A. veronii* biovar sobria BC88, both of which expressed functional Bfp pili. Recent experiments in our laboratory have demonstrated that Bfp pili mediate adhesion of BC88 to enterocytes and intestinal tissue (Kirov *et al.*, 1999). Therefore, it is possible that the presence of Bfp pili on BC88*tapA*::Ω (*tapA* mutant) could mask the effect of the *tapA*::Ω mutation. Alternatively, the Bfp and Tap pili may have redundant roles in adhesion and colonisation. When the gene encoding the Bfp pilin is cloned and mutated, these possibilities will be able to be explored more fully.

Another possibility is that the low levels, or complete lack, of assembly of Tap pili by *A. veronii* biovar sobria BC88 make the *tapA*::Ω mutation hard to characterise. Although *A. veronii* biovar sobria BC88 is a clinical strain isolated from a child with severe dysentery, this strain was isolated in 1980 and has been subjected to numerous subcultures. Many pathogenic bacteria lose the ability to express essential virulence factors following *in vitro* subculture. It is possible that this could account for why Tap pili are poorly expressed. Passage through the intestinal tract of test animals is known to increase toxin production by *Aeromonas* species (Singh and Sanyal, 1992b). *In vivo* passage of BC88 may be useful to investigate this possibility. Alternatively, investigations using recent clinical or environmental isolates of

Aeromonas species that have been subjected to minimal subculture could resolve these issues.

Overall, results from this thesis indicate that Tap pili are not as important as the Bfp pili for intestinal colonisation by *Aeromonas* bacteria. However, all of the genes that comprise the *tap* gene cluster encode functional proteins and are widely conserved in both pathogenic and non-pathogenic *Aeromonas* species. The reason for this wide conservation is not known. Recently, it has been shown that the *V. cholerae* MSHA pilus is involved in adhesion to inorganic substrates (Watnick *et al.*, 1999). Although MSHA pili exhibit greater homology to the *Aeromonas* Bfp pili, a similar role for Tap pili in environmental adhesion cannot be discounted.

Another less likely possibility is that the *tap* gene cluster is conserved because of the role that *tapD* plays in the secretion of extracellular proteins. Hence, the remaining genes, *tapABC*, are retained simply because of their physical linkage to *tapD*. This theory, however, does not account for why *tapABC* encode functional proteins, or why expression of *tapA* has been maintained. If *tapA* plays no role in the biology of *Aeromonas* it would be expected that, at the very least, expression of this gene would cease due to the accumulation of random mutations. Expression of TapA would be maintained if this protein is directly involved in protein secretion. This idea is based on recent observations by Lu *et al.* (1997). In *P aeruginosa*, the TapA-homologue, PilA, is the major subunit protein of the *P. aeruginosa* pili. However, PilA also forms part of the membrane structure responsible for extracellular protein secretion. PilA was shown to interact with another pilin-like protein, XcpT, and a defined mutation in *pilA* caused pleiotrophic defects in the secretion of elastase, exotoxin A and β-lactamase. It is unlikely, however, that TapA performs this function since mutation of *tapA* had no effect on the haemolytic, cytotoxic or enterotoxic activities of *A. veronii* biovar sobria. These phenotypes would be disrupted if TapA is involved in extracellular secretion of proteins.

A final possibility is that TapA is secreted to divert an immune response, in a similar manner to the secretion of pilin proteins by pathogenic *Neisseria* species (Haas *et al.*, 1987). It could be reasoned that this possibility is unlikely since

Aeromonas bacteria, unlike *N. meningitidis* and *N. gonorrhoeae*, are not obligate pathogens. *Aeromonas* bacteria are primarily water-borne organisms, so such a defined virulence mechanism would not be required.

6.6. Further studies

In conclusion, with respect to the *tap* gene cluster, there are several outstanding issues that need to be resolved. These are the subject of ongoing work in our laboratory.

Firstly, additional investigations are required into the apparent difference between clinical and environmental isolates of *A. veronii* biovar sobria in the *BamH* I restriction fragment size to which *tapA* hybridises. For this study, strains typed by genetic methods are being used. The clinical strains should preferably be diarrhoeal isolates that have been isolated as the sole potential pathogen so as to maximise the chance that they represent pathogenic strains. Similarly, environmental isolates should be isolated from true environmental sources such as fresh surface water and soil sources.

Studies that involve the shearing of filamentous surfaces from the cell surface have been used to demonstrate that Tap pili are assembled on the surface of *A. hydrophila* Ah65 (Dr. M. S. Strom, personal communication). However, their low level of expression complicates the investigation of these structures as adhesive organelles. This could be overcome by heterologous expression of Tap pili in *P. aeruginosa*, as has been done for the pili of *Neisseria gonorrhoeae*, *Moraxella bovis*, and *Dichelobacter nodosus* (Elleman *et al.*, 1986; Elleman and Peterson, 1987; Mattick *et al.*, 1987; Beard *et al.*, 1990; Hoyne *et al.*, 1992). *P. aeruginosa* strains with and without Tap pili on the cell surface could then be analysed using *in vitro* adhesion assays and *in vivo* colonisation models, assuming that methods allowing the expression of *tapA in vivo* can be established. The construction of these strains would also allow the role of Tap pili as adhesins in environmental settings to be established.

With respect to further investigations of intestinal colonisation by *Aeromonas*, it is important that the genetic locus encoding the Bfp pilus is cloned, mutated and

characterised. A number of the traditional methods used to clone genes encoding type IV pili from other bacteria were investigated in this thesis. Initial studies involving the use of various DNA probes were not successful, presumably because the codon usage of the *bfp* gene(s) was not similar enough to the general *Aeromonas* codon usage (used in the degenerate oligonucleotide probe), or the codon usage of the *V. cholerae mshA* gene. The subsequent construction and screening of a cosmid DNA library for clones containing the *bfp* gene(s) was unsuccessful because these genes were not expressed in the host background, despite being transferred to an *Aeromonas* recipient. Unfortunately, the cosmid library could not be transferred to an *Aeromonas* strain expressing an antigenically-distinct Bfp pilus due to the low conjugation frequencies into these strains.

Experiments outlined in chapter 3 did result in the cloning, by PCR, of a small region of a gene encoding the *bfp* pilin. The sequence of this small region has been used to design nested PCR primers, and it is hoped that these can be used to amplify the *bfp*-flanking DNA from a plasmid DNA library by inverse PCR. This approach should allow the identification of cosmid DNA clones containing the *bfp* gene(s), and the cloning and sequencing of these genes as well as analysis of flanking genes that may be involved in Bfp pilus expression and assembly. This will ultimately lead to the construction of strains lacking Bfp pili, and the investigation of these strains in *in vitro* adhesion and *in vivo* colonisation experiments. These studies are essential, as it appears that Bfp pili are structures that mediate intestinal adhesion and colonisation by *Aeromonas* bacteria.

6.7. Conclusion

In conclusion, this thesis has made a number of significant advances in our understanding of *Aeromonas* filamentous adhesins. *Aeromonas* species have been shown to encode two distinct families of type IV pili, Bfp and Tap. The type IV pili expressed on the surface of diarrhoeal isolates (Bfp pili) are not encoded by the *tap* gene cluster. Bfp pili are expressed under *in vitro* growth conditions, and progress was made on their genetic characterisation. The genes encoding Tap pili have been

cloned from a second species of *Aeromonas*, *A. veronii* biovar sobria. Experiments described in this thesis demonstrated that the *tap* gene cluster is widely conserved, and is very similar to gene clusters from pathogenic *Vibrio* species. Tap pili do not appear to be essential for colonisation of the gastrointestinal tract, although Tap pili were not demonstrated on the surface of *A. veronii* biovar sobria BC88 grown under standard culture conditions. However, the fact that all of the genes that comprise the *tap* cluster encode functional proteins, and that the subunit protein, TapA, is expressed, suggests that Tap pili are likely to play an important role in the biology of *Aeromonas* bacteria. Further work is required to investigate the role of this pilus in environmental settings, such as in biofilm development, and factors required for expression of these pili on the bacterial surface.

Appendices

Appendix 1.1. *Aeromonas veronii* biovar sobria isolates used in this thesis

Strain	Isolation	Typing method	Source
Clinical isolates			
123c	stool	ribotyping[c]	Dr. M.J. Figueras
129c	stool	ribotyping[c]	Dr. M.J. Figueras
147c	stool	ribotyping[c]	Dr. M.J. Figueras
15c	stool	ribotyping[c]	Dr. M.J. Figueras
187c	blood	ribotyping[c]	Dr. M.J. Figueras
189c	stool	ribotyping[c]	Dr. M.J. Figueras
206c	stool	ribotyping[c]	Dr. M.J. Figueras
20c	ulcer	ribotyping[c]	Dr. M.J. Figueras
215c	stool	ribotyping[c]	Dr. M.J. Figueras
234c	stool	ribotyping[c]	Dr. M.J. Figueras
55c	stool	ribotyping[c]	Dr. M.J. Figueras
74c	stool	ribotyping[c]	Dr. M.J. Figueras
78c	stool	ribotyping[c]	Dr. M.J. Figueras
79c	stool	ribotyping[c]	Dr. M.J. Figueras
93c	stool	ribotyping[c]	Dr. M.J. Figueras
Ae1	human diarrhoeal	biotyping[a]	Dr. M. Iwanaga
Ae24	human diarrhoeal	biotyping[a]	Dr. M. Iwanaga
AM10111	human diarrhoeal	biotyping[d]	Dr. M.J. Albert
AM10978	human diarrhoeal	biotyping[d]	Dr. M.J. Albert
AM14078	human diarrhoeal	biotyping[d]	Dr. M.J. Albert
AM18155	human diarrhoeal	biotyping[d]	Dr. M.J. Albert
AM18476	human diarrhoeal	biotyping[d]	Dr. M.J. Albert
AM8998	human diarrhoeal	biotyping[d]	Dr. M.J. Albert
BC101	human diarrhoeal	biotyping[a]	Dr. B.J. Chang
BC126	human diarrhoeal	biotyping[a]	Dr. B.J. Chang
BC88	human diarrhoeal	ribotyping[b]	Dr. B.J. Chang
BC96	human diarrhoeal	ribotyping[g]	Dr. B.J. Chang
CA110	human diarrhoeal	ribotyping[g]	A/Prof. S.M. Kirov
CA112	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA118a	clinical	biotyping[a]	A/Prof. S.M. Kirov
CA164	stool	biotyping[a]	A/Prof. S.M. Kirov
CA167	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA17	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA18	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA199	stool	biotyping[a]	A/Prof. S.M. Kirov
CA205.1	clinical	biotyping[a]	A/Prof. S.M. Kirov
CA212.1	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA217	stool	biotyping[a]	A/Prof. S.M. Kirov
CA25	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA26	clinical	biotyping[a]	A/Prof. S.M. Kirov
CA29	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
NZ107	stool	biotyping[a]	A/Prof. S.M. Kirov
NZ2110	stool	biotyping[a]	A/Prof. S.M. Kirov
NZ403	stool	biotyping[a]	A/Prof. S.M. Kirov
TAP13	human diarrhoeal	biotyping[a]	Dr. M. Iwanaga
UTS7	human	biotyping[f]	Dr. J. Oakey

Strain	Isolation	Typing method	Source/Reference
Animal isolates			
111f	shellfish	ribotyping[c]	Dr. M.J. Figueras
AK176	live fish *Rutilus rutilus*	DNA-DNA hybridisation[e]	Dr. A. Kaznowski
UTS13	koi carp anal swab	biotyping[f]	Dr. J. Oakey
UTS16	koi carp anal swab	biotyping[f]	Dr. J. Oakey
UTS17	koi carp anal swab	biotyping[f]	Dr. J. Oakey
UTS19	koi carp anal swab	biotyping[f]	Dr. J. Oakey
UTS21	bull rout skin swab	biotyping[f]	Dr. J. Oakey
UTS21b	bull rout	biotyping[f]	Dr. J. Oakey
UTS25	long neck tortoise anal	biotyping[f]	Dr. J. Oakey
UTS29	long neck tortoise anal	biotyping[f]	Dr. J. Oakey
Environmental isolates			
124p	fountain	ribotyping[c]	Dr. M.J. Figueras
126	lake	ribotyping[c]	Dr. M.J. Figueras
128	river	ribotyping[c]	Dr. M.J. Figueras
347	river	ribotyping[c]	Dr. M.J. Figueras
443	lake	ribotyping[c]	Dr. M.J. Figueras
63	lake	ribotyping[c]	Dr. M.J. Figueras
AK12	water	DNA-DNA hybridisation[e]	Dr. A. Kaznowski
AK279	sewage	DNA-DNA hybridisation[e]	Dr. A. Kaznowski
AK413	water	DNA-DNA hybridisation[e]	Dr. A. Kaznowski
AK59	water	DNA-DNA hybridisation[e]	Dr. A. Kaznowski
AS101	water	biotyping[a]	A/Prof. S.M. Kirov
AS398	water	biotyping[a]	A/Prof. S.M. Kirov
EA34	water	ribotyping[g]	A/Prof. S.M. Kirov
EA39	water	biotyping[a]	A/Prof. S.M. Kirov
EA42	water	biotyping[a]	A/Prof. S.M. Kirov
EA49	water	biotyping[a]	A/Prof. S.M. Kirov
EA50	water	biotyping[a]	A/Prof. S.M. Kirov
EA57	water	biotyping[a]	A/Prof. S.M. Kirov
EA60	water	biotyping[a]	A/Prof. S.M. Kirov
P13	water	biotyping[a]	A/Prof. S.M. Kirov
P14	water	biotyping[a]	A/Prof. S.M. Kirov
P18	water	biotyping[a]	A/Prof. S.M. Kirov
R207	water	biotyping[a]	A/Prof. S.M. Kirov
S129	effluent	ribotyping[g]	A/Prof. S.M. Kirov
S132	river	ribotyping[g]	A/Prof. S.M. Kirov
S16	water	ribotyping[g]	A/Prof. S.M. Kirov

[a] Biotyping performed as described by Kirov *et al.* (1986)

[b] Ribotyping performed as described by Martinetti Lucchini and Altwegg (1992)

[c] Ribotyping performed as described by Borrell *et al.* (1997)

[d] Biotyping performed as described by Carnahan *et al.* (1991b)

[e] DNA-DNA hybridisation performed as described by Kaznowski (1998)

[f] Biotyping performed as described by Oakey *et al.* (1996)

[g] Ribotyping performed as described by Dorsch *et al.* (1994)

Appendix 1.2. *Aeromonas hydrophila* isolates used in this thesis

Strain	Isolation	Typing method	Source/Reference
Clinical isolates			
Ae6	human diarrhoeal	biotyping[a]	Dr. M. Iwanaga
Ah26	human diarrhoeal	biotyping[a]	Dr. A. Ho
BC56	human diarrhoeal	biotyping[a]	Dr. B. J. Chang
CA1	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA111	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA114	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA12	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA162	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA9	stool	biotyping[a]	A/Prof. S.M. Kirov
P8	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
Q1	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
Animal isolates			
Ah65	Rainbow trout	ribotyping[b]	Dr. M. S. Strom
Environmental			
FA129	poultrycarcass	biotyping[a]	A/Prof. S.M. Kirov
FA131	poultrycarcass	biotyping[a]	A/Prof. S.M. Kirov
FA136	poultrycarcass	biotyping[a]	A/Prof. S.M. Kirov
FA145	poultrycarcass	biotyping[a]	A/Prof. S.M. Kirov
EA32	river water	biotyping[a]	A/Prof. S.M. Kirov
EA46	water	biotyping[a]	A/Prof. S.M. Kirov
EA51	water	biotyping[a]	A/Prof. S.M. Kirov
EA94	water	biotyping[a]	A/Prof. S.M. Kirov
EA95	water	biotyping[a]	A/Prof. S.M. Kirov

[a] Biotyping performed as described by Kirov *et al.* (1986)

[b] Ribotyping performed as described by Martinetti Lucchini and Altwegg (1992)

Appendix 1.3. *Aeromonas caviae* isolates used in this thesis

Strain	Isolation	Typing method	Source/Reference
Clinical isolates			
CA163	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA182	stool	biotyping[a]	A/Prof. S.M. Kirov
CA183	stool	biotyping[a]	A/Prof. S.M. Kirov
CA185	stool	biotyping[a]	A/Prof. S.M. Kirov
CA186	stool	biotyping[a]	A/Prof. S.M. Kirov
CA195	human diarrhoeal	ribotyping[b]	A/Prof. S.M. Kirov
CA196	stool	biotyping[a]	A/Prof. S.M. Kirov
CA198	stool	biotyping[a]	A/Prof. S.M. Kirov
CA202	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA203	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA207	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
CA210	stool	biotyping[a]	A/Prof. S.M. Kirov
CA214	human diarrhoeal	biotyping[a]	A/Prof. S.M. Kirov
Environmental			
DH31	raw milk	biotyping[a]	A/Prof. S.M. Kirov
DH61	raw milk	biotyping[a]	A/Prof. S.M. Kirov
S5	river	biotyping[a]	A/Prof. S.M. Kirov
S9	river	biotyping[a]	A/Prof. S.M. Kirov
S30	effluent	biotyping[a]	A/Prof. S.M. Kirov

[a] Biotyping performed as described by Kirov *et al.* (1986)

[b] Ribotyping performed as described by Martinetti Lucchini and Altwegg (1992)

Appendix 1.4. *Aeromonas* reference strains and miscellaneous strains used in this thesis

Strain	Isolation	Characteristics	Source/Reference
Reference strains			
A306	ATCC 7966	HG1	Dr. M Altwegg
A307	CDC 9533-76	HG2	Dr. M Altwegg
A308	CDC 1434-84	HG3	Dr. M Altwegg
A309	ATCC 15468	HG4	Dr. M Altwegg
A310	CDC 0862-83	HG5A	Dr. M Altwegg
A912	ATCC 23212	HG5B	Dr. M Altwegg
A311	ATCC 23309	HG6	Dr. M Altwegg
A312	CIP 7433	HG7	Dr. M Altwegg
A313	CDC 0437-80	HG8	Dr. M Altwegg
A314	CDC 0787-80	HG9	Dr. M Altwegg
A901	ATCC 35624	HG10	Dr. M Altwegg
A902	CDC 1306-83	HG11	Dr. M Altwegg
A903	ATCC 43700	HG12	Dr. M Altwegg
A1792	LMG 17541	HG17	Dr. M Altwegg
Other strains			
Pz1	human diarrhoeal	*A. trota* HG14	Dr. G Pazzaglia
Pz2	human diarrhoeal	*A. trota* HG14	Dr. G Pazzaglia
Pz3	water	*A. trota* HG14	Dr. G Pazzaglia

Appendix 1.5. *Escherichia coli* strains used in this thesis

Strain	Properties	Source/Reference
JM109	F' {*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15} *endA*1 *recA*1 *hsdR*17 (r_K^-, m_K^+) *supE*44 *thi*-1 *gyrA*96 *relA*1 Δ(*lac-proAB*)	Promega, Madison, U.S.A.; Yanisch-Perron *et al.* (1985)
TOP10F'	F' {*lacI*^{q} Tn10(Tet^R)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX*74 *deoR recA*1 *araD*139 Δ(*ara-leu*)7697 *galU galK rpsL*(Str^R) *endA*1 *nupG*	Invitrogen, San Diego, U.S.A.
S17-1	*supE*44 *hsdR endA*1 *recA thi pro* RP4-2-Tc::Mu	Simon *et al.* (1983)
S17-1λpir	*supE*44 *hsdR endA*1 *recA thi pro* RP4-2-Tc::Mu-kan::Tn7 (*λpir*)	de Lorenzo and Timmis (1994)
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu	Simon *et al.* (1983)
BL21(DE3)	F- *ompT hsdS* B(r_B^-, m_B^-) *gal dcm* (DE3)	Novagen, Madison, U.S.A.; Studier and Moffatt (1986)
JM109(DE3)	F' {*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15} *endA*1 *recA*1 *hsdR*17 (r_K^-, m_K^+) *supE*44 *thi*-1 *gyrA*96 *relA*1 Δ(*lac-proAB*) (DE3)	Promega, Madison, U.S.A.
EC101	K12	M. J. Albert
DH5α	F$^-$ ϕ80*lacZ*ΔM15 *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 (r_K^-, m_K^+) *supE*44 *relA*1 *deoR* Δ(*lacZYA-argF*)U169	Gibco BRL, Gaithersburg, U.S.A.

Appendix 2. Plasmids used in this thesis

Strain	Properties	Source/Reference
Cloning vectors		
pUCP19	*E. coli-Pseudomonas* shuttle vector, Amp^R	Schweizer (1991)
pGEM-3z(f)+	General purpose cloning vector, Amp^R	Promega, Madison, U.S.A.
pLitmus29	General purpose cloning vector, Amp^R	New England Biolabs, Beverly, U.S.A.
pZErO-2.1	General purpose cloning vector, Kan^R	Invitrogen, San Diego, U.S.A.
pHC79	Cosmid cloning vector, Amp^R, Tet^R	Hohn and Collins, 1980
pQE32	His•tag fusion protein expression vector, Amp^R	Qiagen, Hilden, Germany
pET15b	His•tag fusion protein expression vector, Amp^R	Novagen, Madison, U.S.A.
pLysS	Encodes T7 lysozyme. Used to inhibit T7 RNA polymerase	Novagen, Madison, U.S.A.; Studier and Moffatt (1986)
pBBR1MCS	Broad-host-range cloning vector, Cml^R	Kovach *et al.* (1994)
Recombinant plasmids		
pAW102	1.2 kb *Hind* III fragment (*pilA*) from *Pseudomonas aeruginosa* PAO1 cloned into pBluescript SK+	R. A. Alm
pBSL121	Chloramphenicol (*cat*) gene cassette flanked with pBluescript polylinker sequences	Alexeyev *et al.* (1995)
pCP1085	1.0 kb *BamH* I fragment (*tapA*) from *A. hydrophila* Ah65 cloned into pBluescript II SK-	Pepe *et al.* (1996)
pCP1089	2.7 kb *Pst* I fragment (*tapD*) from *A. hydrophila* Ah65 cloned into pBluescript II SK-	Pepe *et al.* (1996)
pCP1183	*tapA* from *A. hydrophila* Ah65 cloned into pET15b	M. S. Strom
pG01	3.3 kb *Pst* I fragment from pTB032 containing the *tapA*-proximal region from *A. veronii* biovar sobria BC88 cloned into pZErO-2.1	This thesis
pG02	1.8 kb *BamH* I fragment from pG01 cloned into pZErO-2.1	This thesis
pG03	1.0 kb *EcoR* V fragment from pG01 cloned into pZErO-2.1	This thesis
pGJX111	2.6 kb *Sal* I/*EcoR* I fragment (*mshA*) from *Vibrio cholerae* O1 Phil6973 cloned into pWKS130	Jonson *et al.* (1994)
pUC19Ω	Omega interposon (Ω) cloned into pUC19	Koga *et al.* (1993)
pTB001	5.8 kb *BamH* I fragment from *A. veronii* biovar sobria BC88 identified by hybridisation with P001 cloned into pGEM3zf(+)	This thesis
pTB002	1.5 kb *EcoR* I / *Kpn* I fragment from pTB001 cloned into pGEM3zf(+)	This thesis

Strain	Properties	Source/Reference
pTB003	5.8 kb *BamH* I fragment from pTB001 cloned into pBBR1MCS	This thesis
pTB004	1.2 kb *BamH* I fragment from *A. veronii* biovar sobria BC88 identified by hybridisation with P001 cloned into pZErO-2.1	This thesis
pTB005	0.69 kb *BamH* I fragment from *A. veronii* biovar sobria BC88 identified by hybridisation with P001 cloned into pZErO-2.1	This thesis
pTB006	0.74 kb *BamH* I fragment from *A. veronii* biovar sobria BC88 identified by hybridisation with P001 cloned into pZErO-2.1	This thesis
pTB011	1.0 kb *BamH* I fragment from *A. veronii* biovar sobria BC88 identified by hybridisation with *tapA* from *A. hydrophila* Ah65 cloned into pUCP19	This thesis
pTB012	1.0 kb *BamH* I fragment from pTB011 cloned into pGEM-3zf(+)	This thesis
pTB013	0.5 kb *BamH* I / *Pst* I fragment from pTB011 cloned into pGEM-3zf(+)	This thesis
pTB014	1.0 kb *Pst* I / *BamH* I fragment from pTB011 cloned into pGEM-3zf(+)	This thesis
pTB019	*bla*-deleted version of pHC79 created by PCR with primers P006 and P007	This thesis
pTB020	1.9 kb *BamH* I fragment (*mob*) from pSUP201-1 cloned into the *EcoR* I site of pTB019	This thesis
pTB022	1.0 kb *BamH* I fragment from pBSL121 (*cat*) cloned into the *Bgl* II site of pLitmus29	This thesis
pTB023	1.0 kb *Pst* I fragment from pTB022 (*cat*) cloned into the *Pst* I site of pTB020	This thesis
pTB024	111 bp *EcoR* V / *BstU* I fragment from pGJX111 (*mshA* N-terminal region) cloned into the *EcoR* V site of pZErO-2.1	This thesis
pTB025	0.45 kb fragment from *A. veronii* biovar sobria BC88 (*tapA*) generated by PCR with primers P004 and P005, digested with *BamH* I / *EcoR* V and cloned into pQE32	This thesis
pTB028	0.44 kb fragment from *A. veronii* biovar sobria BC88 (*tapA*) generated by PCR with primers P013 and P014, digested with *Nde* I / *Bgl* II and cloned into the *Nde* I / *BamH* I sites of pET15b	This thesis
pTB032	pTB023 cosmid clone identified by hybridisation with *tapA* and *tapD* from *A. veronii* biovar sobria BC88	This thesis
pTB037	78 bp fragment from *A. veronii* biovar sobria BC88 (*bfp*) generated by PCR with primers P023 and P024, digested with *Hind* III and *Xba* I and cloned into pZErO-2.1	This thesis

Appendix 3. Bacterial and tissue culture media

3.1. Bacterial culture media

Unless otherwise stated, all media were sterilised by autoclaving at 121°C for 15 min.

3.1.1. Minimal maintenance medium (MMM)

Bacteriological agar (Oxoid L11)	5.0 g
NaCl	5.0 g
Bacteriological peptone (Oxoid L37)	2.5 g
Neutralised bacteriological peptone (Oxoid L34)	2.5 g
$Na_2HPO_4.12H2O$	2.8 g
KH_2PO_4	1.4 g
milliQ water to	1000 ml

Dissolve by heating and adjust pH to 6.7. Dispense into 3 ml aliquots and sterilise by autoclaving.

3.1.2. Glycerol freezing medium

Bacteriological peptone (Oxoid L37)	1.0 g
NaCl	0.5 g
Glycerol	10.0 ml
milliQ water to	100 ml

Sterilise by autoclaving and dispense 1.5 ml aliquots into cryogenic vials.

3.1.3. Tryptone soya broth + 0.6% yeast extract (TSBY)

Tryptone soya broth (Oxoid CM129)	30.0 g
Yeast extract (Oxoid L21)	6.0 g
milliQ water to	1000 ml

Sterilise by autoclaving and store at room temperature.

3.1.4. Tryptone soya agar + 0.6% yeast extract (TSAY)

Tryptone soya broth (Oxoid CM129)	30.0 g
Yeast extract (Oxoid L21)	6.0 g
Bacteriological agar (Oxoid L11)	15.0 g
milliQ water to	1000 ml

Sterilise by autoclaving and store at room temperature.

3.1.5. Luria-Bertani (LB) broth

Tryptone (Oxoid L42)	10.0 g
Yeast extract (Oxoid L21)	5.0 g
NaCl	10.0 g
milliQ water to	1000 ml

Sterilise by autoclaving and store at room temperature.

3.1.6. Luria-Bertani (LB) agar

Tryptone (Oxoid L42)	10.0 g
Yeast extract (Oxoid L21)	5.0 g
NaCl	10.0 g
Bacteriological agar (Oxoid L11)	15.0 g
milliQ water to	1000 ml

Sterilise by autoclaving and store at room temperature.

3.1.7. Terrific broth (TB)

Tryptone (Oxoid L42)	12.0 g
Yeast extract (Oxoid L21)	24.0 g
Glycerol	4.0 ml
milliQ water to	900 ml

Sterilise by autoclaving. Allow the solution to cool and add 100 ml of sterile TB salts solution. Store at room temperature.

3.1.8. TB salts solution

KH_2PO_4	2.31 g
K_2HPO_4	12.54 g
milliQ water to	100 ml

Sterilise by autoclaving and store at room temperature.

3.1.9. SOC medium

Tryptone (Oxoid L42)	20.0 g
Yeast extract (Oxoid L21)	5.0 g
NaCl	0.5 g
1 M KCl	2.5 ml
milliQ water to	1000 ml

Adjust pH to 7.0 and sterilise by autoclaving. Allow the solution to cool and add 10 ml sterile 1 M $MgCl_2$ and 20 ml sterile 18% glucose. Store at room temperature.

3.1.10. Brain heart infusion broth (BHIB)

Brain heart infusion broth (Oxoid CM225)	37.0 g
milliQ water to	1000 ml

Sterilise by autoclaving.

3.2. Cell culture media

3.2.1. Minimal essential medium (Eagle) with non-essential amino acids and Earl's salts (MEM)

MEM powder (ICN 10-121-22)	9.61 g
$NaHCO_3$	1.1 g
milliQ water to	1000 ml

Filter sterilise and incubate for one week at 37°C to ensure sterility. Add 5 to 10% foetal calf serum (FCS) as required. Store at 4°C.

Appendix 4. Antibiotics

Antibiotic	Stock solution	Final concentration
ampicillin	50 mg/ml in water	100 μg/ml
chloramphenicol	25 mg/ml in ethanol	25 μg/ml
kanamycin	50 mg/ml in water	50 μg/ml
spectinomycin	50 mg/ml in water	50 μg/ml
carbenicillin	50 mg/ml in water	100 μg/ml
tetracycline	12.5 mg/ml in 1:1 water/ethanol	12.5 μg/ml
rifampicin	200 mg/ml in methanol, made fresh	200 μg/ml

Appendix 5. Enzymes and immunochemicals

Enzyme	Supplier
Restriction enzymes	
BamH I	MBI Fermentas, Vilnius, Lithuania
Bgl II	MBI Fermentas, Vilnius, Lithuania
BstU I	New England Biolabs, Beverly, U.S.A.
EcoR I	MBI Fermentas, Vilnius, Lithuania
EcoR V	MBI Fermentas, Vilnius, Lithuania
Hind III	MBI Fermentas, Vilnius, Lithuania
Kpn I	MBI Fermentas, Vilnius, Lithuania
Msp I	MBI Fermentas, Vilnius, Lithuania
Nde I	MBI Fermentas, Vilnius, Lithuania
Pst I	MBI Fermentas, Vilnius, Lithuania
Sac I	MBI Fermentas, Vilnius, Lithuania
Sau3A I	MBI Fermentas, Vilnius, Lithuania
Xba I	MBI Fermentas, Vilnius, Lithuania
Modifying enzymes	
DNA polymerase I large (Klenow) fragment	Promega, Madison, U.S.A.
HotStarTaq DNA polymerase	Qiagen, Hilden, Germany
RNase A	Boehringer Mannheim, Castle Hill, Australia
T4 DNA ligase	Gibco, Gaithersburg, U.S.A.
Immunoconjugates	
Goat anti-rabbit IgG-10 nm gold	BioCell, Cardiff, U.K.
Goat anti-rabbit IgG-alkaline phosphatase	Dako, Carpinteria, U.S.A.
Rabbit anti-mouse IgG-alkaline phosphatase	Dako, Carpinteria, U.S.A.
Sheep anti-digoxygenin-alkaline phosphatase Fab	Boehringer Mannheim, Castle Hill, Australia
tetra•His antibody (mouse monoclonal)	Qiagen, Hilden, Germany

Appendix 6. Buffers and solutions

6.1. DNA extraction

6.1.1. 100 × TE buffer

Tris base	12.1 g
0.5 M EDTA	20 ml
milliQ water to	100 ml

Sterilise by autoclaving.

6.1.2. CTAB/NaCl solution

hexadecyl-trimethyl-ammonium bromide	10.0 g
NaCl	4.1 g
milliQ water to	100 ml

Store at room temperature.

6.1.3. STT buffer

sucrose	8.0 g
1M Tris-HCl, pH 8.0	5.0 ml
Triton X-100	5.0 ml
milliQ water to	100 ml

Soter at room temperature.

6.1.4. Solution I

glucose	2.25 g
Tris base	0.98 g
0.5 M EDTA	5.0 ml
milliQ water to	250 ml

Sterilise by autoclaving. Store at 4°C.

6.1.5. Solution II

10% SDS	1.0 ml
milliQ water	8.6 ml
5 M NaOH	0.4 ml

Store at room temperature for up to one week.

6.1.6. Solution III

KAc	29.4 g
glacial acetic acid	11.5 ml
milliQ water to	100 ml

Sterilise by autoclaving and store at 4°C.

6.2. Agarose gel electrophoresis

6.2.1. 50 × TAE buffer

Tris base	242 g
$Na_2EDTA.(2H_20)$	37.2 g
glacial acetic acid	57.1 ml
milliQ water to	1000 ml

Store at room temperature.

6.2.2. Agarose gel loading buffer

bromphenol blue	10 mg
glycerol	2 ml
20 mg/ml RNase A	0.5 ml
milliQ water to	10 ml

Store at 4°C.

6.3. End-filling with Klenow fragment

6.3.1. 10 × Klenow buffer

1 M Tris-HCl, pH 8.0	20 µl
1 M $MgCl_2$	100 µl
milliQ water to	1000 µl

Store at –20°C. Dilute to 1 × and add 0.1U/µl Klenow DNA polymerase before use.

6.3.2. dNTP mix

10 mM dATP	1.25 µl
10 mM dCTP	1.25 µl
10 mM dGTP	1.25 µl
10 mM dTTP	1.25 µl
milliQ water to	100 µl

Store at –20°C.

6.4. Preparation of competent *E. coli*

6.4.1. Solution α

1 M KAc	3.0 ml
1 M KCl	10.0 ml
1 M $CaCl_2$	1.0 ml
1 M $MnCl_2$	5.0 ml
glycerol	15.0 ml
milliQ water to	100 ml

Sterilise by filtration. Store at room temperature.

6.4.2. Solution β

1 M MOPS	1.0 ml
1 M $CaCl_2$	7.5 ml
1 M KCl	1.0 ml
glycerol	15.0 ml
milliQ water to	100 ml

Sterilise by filtration. Store at room temperature.

6.5. Preparation of cosmid DNA libraries

6.5.1. Phage buffer

1 M Tris-HCl, pH 7.4	0.2 ml
5 M NaCl	0.2 ml
1 M $MgSO_4$	0.1 ml
milliQ water to	10 ml

Sterilise by filtration. Store at room temperature.

6.6. Polymerase chain reaction

6.6.1. 10 × PCR buffer

1 M KCl	5.0 ml
1 M Tris-HCl, pH 8.4	1.0 ml
1 M $MgCl_2$	150 µl
milliQ water to	10 ml

Sterilise by filtration. Store at –20°C.

6.7. DNA blotting and hybridisation

6.7.1. 20 × SSC

NaCl	175.3 g
sodium citrate	88.2 g
milliQ water to	1000 ml

Adjust pH to 7.0. Sterilise by autoclaving and store at room temperature.

6.7.2. Hybridisation solution – high stringency

20 × SSC	50.0 ml
10% sarcosyl	2.0 ml
10% SDS	0.4 ml
10% blocking reagent (Boehringer Mannheim)	20.0 ml
deionised formamide	80 ml
milliQ water to	200 ml

Store at 4°C.

6.7.3. Hybridisation solution – low stringency

20 × SSC	50.0 ml

10% sarcosyl	2.0 ml
10% SDS	0.4 ml
10% blocking reagent (Boehringer Mannheim)	20.0 ml
milliQ water to	200 ml

Store at 4°C.

6.7.4. Buffer 1

maleic acid	
NaCl	
milliQ water to	1000 ml

Adjust pH to 7.5 with solid NaOH. Store at room temperature.

6.7.5. Wash buffer

Buffer 1	1000 ml
Tween-20	3.0 ml

Store at room temperature.

6.7.6. Buffer 2

Buffer 1	500 ml
10% blocking reagent (Boehringer Mannheim)	50 ml

Store at 4°C.

6.7.7. Buffer 3

1 M Tris-HCl, pH 9.5	100 ml
5 M NaCl	20 ml
1M $MgCl_2$	50 ml
milliQ water to	1000 ml

Store at room temperature.

6.7.8. Lysis solution

10 M NaOH	25 ml
5 M NaCl	150 ml
milliQ water to	500 ml

Store at room temperature.

6.7.9. Neutralisation solution

Tris base	
NaCl	
milliQ water to	500 ml

Adjust pH to 7.5. Store at room temperature.

6.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

6.8.1. 2 × Lugtenberg's

1M Tris-HCl, pH 6.8	6.25 ml
10% SDS	20.0 ml
glycerol	10.0 ml
β-mercaptoethanol	5.0 ml
0.5% bromphenol blue	4.0 ml
milliQ water to	50 ml

Store at room temperature.

6.8.2. Stock 1 acrylamide

acrylamide	44.0 g
bis-acrylamide	0.8 g
milliQ water to	100 ml

Store protected from light at 4°C.

6.8.3. Stock 2 acrylamide

acrylamide	30.0 g
bis-acrylamide	0.8 g
milliQ water to	100 ml

Store protected from light at 4°C.

6.8.4. Resolving gel (15%)

stock 1 acrylamide	3.5 ml
0.75 M Tris-HCl, pH 8.8	5.5 ml
milliQ water	1.75 ml
10% SDS	235 µl
10% ammonium persulphate	50 µl
TEMED	8 µl

Mix gently and pipette into gel apparatus to within 1 cm from the bottom of the comb. Overlay gently with 0.1% SDS. Allow to polymerise for 30 min.

6.8.5. Stacking gel

stock 2 acrylamide	0.63 ml
0.25 M Tris-HCl, pH 6.8	1.88 ml
milliQ water	1.20 ml
10% SDS	38 µl
10% ammonium persulphate	25 µl
TEMED	8 µl

Mix gently. Remove 0.1% SDS overlay and pipette stacking gel onto resolving gel. Place comb and allow to polymerise for 30 to 60 min.

6.8.6. 5 × SDS-PAGE running buffer

Tris base	15.1 g
glycine	71.3 g
SDS	5.0 g
milliQ water to	1000 ml

Store at room temperature. Dilute to 1 × concentration before use.

6.8.7. Coomassie stain

Coomassie blue R250 (Bio-Rad)	2.5 g
methanol	400 ml
glacial acetic acid	100 ml
milliQ water to	1000 ml

Store at room temperature.

6.8.8. Coomassie destain

methanol	800 ml
glacial acetic acid	200 ml
milliQ water to	2000 ml

Store at room temperature.

6.8.9. Gel drying solution

methanol	300 ml
glycerol	100 ml
milliQ water to	600 ml

Store at room temperature.

6.9. Protein blotting and detection

6.9.1. Western transfer buffer

Tris base	3.03 g
glycine	14.40 g
milliQ water to	500 ml

Store at 4°C.

6.9.2. 20 × TBS

Tris base	48.5 g
NaCl	180.0 g
milliQ water to	1000 ml

Adjust pH to 7.5. Store at room temperature.

6.9.3. TTBS

20 × TBS	100 ml
Tween-20	1 ml
milliQ water to	2000 ml

Store at room temperature.

6.9.4. Blocking solution

bovine serum albumin	30 g
20 × TBS	50 ml
20% NaN_3	1 ml
milliQ water to	1000 ml

Store at 4°C.

6.9.5. Colour development solution

1 M Tris-HCl, pH 9.5	100 ml
5 M NaCl	20 ml
1M $MgCl_2$	50 ml
milliQ water to	1000 ml

Store at room temperature. Add 0.67 ml 5% NBT and 0.33 ml 5% BCIP per 100 ml immediately before use.

6.10. Expression and purification of recombinant TapA protein

6.10.1. Sonication buffer

1 M NaH_2PO_4, pH 7.8	25 ml
5 M NaCl	30 ml
milliQ water to	500 ml

Sterilise by autoclaving and store at room temperature.

6.10.2. Suspension buffer

1 M NaH_2PO_4, pH 7.8	50 ml
1 M Tris-HCl, pH 8.0	5 ml
milliQ water to	500 ml

Sterilise by autoclaving and store at room temperature.

6.11. Electron microscopy

6.11.1. Formvar

formvar	0.3 g
chloroform	100 ml

Store at room temperature.

6.11.2. TTB

1 M Tris-HCl, pH 8.2	2.0 ml
5 M NaCl	0.5 ml
bovine serum albumin	0.1 g
Tween-20	50 µl
milliQ water to	100 ml

Filter sterilise and store at 4°C.

6.11.3. Uranyl acetate

uranyl acetate	0.1 g
milliQ water to	10 ml

Store protected from light at 4°C.

6.12. Haemolysin assay

6.12.1. Alsever's solution

glucose	2.1 g
sodium citrate	0.8 g
NaCl	0.4 g
milliQ water to	100 ml

Filter sterilise, dispense into 10 ml aliquots and store at –20°C.

6.13. Cell culture

6.13.1. 10 × PBS

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
milliQ water to	1000 ml

Adjust pH to 7.4 and sterilise by autoclaving. Store at room temperature.

6.13.2. Trypsin solution

trypsin	0.1 g
PBS	100 ml

Sterilise by filtration. Dispense into 3 ml aliquots and store at –20°C.

6.13.3. pH 6.8 water

Buffer (pH 6.8) tablets	1
milliQ water to	1000 ml

Sterilise by autoclaving and store at room temperature.

6.13.6. May-Grunwald stain

May-Grunwald stain	30 ml
pH 6.8 water	30 ml

Prepare immediately before use and filter sterilise.

6.13.5. Giemsa stain

Geimsa stain	5 ml
pH 6.8 water	45 ml

Prepare immediately before use and filter sterilise.

Appendix 7. Oligonucleotides described in this thesis

Name	Description	T_m (°C)	Compan
M13f	5'-GTT TTC CCA GTC ACG AC-3' pUC/M13 forward (17mer) (-40) sequencing primer.	52	Beckmar
M13r	5'-CAG GAA ACA GCT ATG AC-3' pUC/M13 reverse (17mer) sequencing primer.	50	Beckmar
T7	5'-TAA TAC GAC TCA CTA TAG GG-3' T7 RNA polymerase promoter sequencing primer	56	Beckmar
P001	5'-ACC GCS GCS CCS AAG TTC TC-3' Degenerate oligonucleotide probe designed against Bfp pilin protein N-terminal amino acid sequence.	68	Amitof Biotech
P004	5'-AGG AGG ATC CAA ATG AAG AAA CAA TCG-3' Forward primer for amplifying *tapA* from *A. veronii* biovar sobria BC88 for cloning into pQE32. *BamH* I site is underlined.	56	Beckmar
P005	5'-TTA GGG CTA GAT ATC ATT ATT TGG-3' Reverse primer for amplifying *tapA* from *A. veronii* biovar sobria BC88 for cloning into pQE32. *EcoR* V site is underlined.	48	Beckmar
P006	5'-CCC CGA AAA GTG CCA CCT GCA GTC TAA GAA ACC-3' Forward primer for deletion of *bla* from pHC79. *Pst* I site is underlined.	82	Beckman
P007	5'-CTG ATT AAG CAT TGG TAA CTG CAG ACC AAG TTT ACT C-3' Reverse primer for deletion of *bla* from pHC79. *Pst* I site is underlined.	80	Beckman
P010	5'-CCG GAT CCT CCC AGG AAC CAA GG-3' Forward primer for amplifying *tapA* from *A. hydrophila* Ah65 for probe construction. *BamH* I site is underlined.	48	Beckman
P011	5'-CCA GGT ACC TAT TAG GGC TAG AGG-3' Reverse primer for amplifying *tapA* from *A. hydrophila* Ah65 for probe construction. *Kpn* I site is underlined.	46	Beckman
P013	5'-TGA AGA AAC AAC ATA TGT TTA CCC TTA TTG-3' Forward primer for amplifying *tapA*, without the leader sequence, from *A. veronii* biovar sobria BC88 for cloning into pET15b. *Nde* I site is underlined.	62	Beckman
P014	5'-CTA TTA GAT CTA GAG GTC ATT ATT TGG-3' Reverse primer for amplifying *tapA*, without the leader sequence, from *A. veronii* biovar sobria BC88 for cloning into pET15b. *Bgl* II site is underlined.	60	Beckman
P015	5'-GTT GCC ATG ACA CTC GCA CC-3' Forward primer for amplifying an internal 414 bp fragment of *tapD* from *A. hydrophila* Ah65 for probe construction.	64	Beckman
P016	5'-TCT GAT GGT GAT TGC GCA GC-3' Reverse primer for amplifying an internal 414 bp fragment of *tapD* from *A. hydrophila* Ah65 for probe construction.	62	Beckman

Name	Description	T_m (°C)	Compar
P019	5'-ATG ACI YTI ATH GAR YTI GT-3' Degenerate forward primer used to amplify N-terminal region of *bfp* from *A. veronii* biovar sobria BC88.	40	Gibco
P020	5'-ATH GTI ATH ATH ATH YTI GG-3' Degenerate forward primer used to amplify N-terminal region of *bfp* from *A. veronii* biovar sobria BC88.	40	Gibco
P021	5'-TGI ARR TTI ARR AAY TTI GG-3' Degenerate reverse primer used to amplify N-terminal region of *bfp* from *A. veronii* biovar sobria BC88.	40	Gibco
P022	5'-TTI ARR AAY TTI GGI GCI GC-3' Degenerate reverse primer used to amplify N-terminal region of *bfp* from *A. veronii* biovar sobria BC88.	40	Gibco
P023	5'-GCA AGC TTA TGA CIY TIA THG ARY TIG T-3' Degenerate forward primer used to clone N-terminal region of *bfp* from *A. veronii* biovar sobria BC88. *Hind* III site is underlined.	40	Gibco
P024	5'-GCT CTA GAT TIA RRA AYT TIG GIG CIG C-3' Degenerate reverse primer used to clone N-terminal region of *bfp* from *A. veronii* biovar sobria BC88. *Xba* I site is underlined.	40	Gibco
GM2	5'-CGA GCC ATA TCA GAT CGC-3' Primer used to sequence from pGO1.	56	Gibco
GM3	5'-GCC TGC TGA CCC TCT ATT TTC-3' Primer used to sequence from pGO1.	64	Gibco
GM4	5'-GGG TCT GGA CAA AGT TC-3' Primer used to sequence from pGO1.	52	Gibco

References

References

Abrami, L., Fivaz, M., Decroly, E., Seidah, N. G., Jean, F., Thomas, G., Leppla, S. H., Buckley, J. T., and van der Goot, F. G. 1998a. The pore-forming toxin proaerolysin is activated by furin. J. Biol. Chem. **273:**32656-32661.

Abrami, L., Fivaz, M., Glauser, P. E., Parton, R. G., and van der Goot, F. G. 1998b. A pore-forming toxin interacts with a GPI-anchored protein and causes vacuolation of the endoplasmic reticulum. J. Cell. Biol. **140:**525-540.

Agger, W. A. 1986. Diarrhea associated with *Aeromonas hydrophila*. Pediatr. Infect. Dis. **5:**S106-S108.

Aguilar, A., Merino, S., Rubires, X., and Tomás, J. M. 1997. Influence of osmolarity on lipopolysaccharides and virulence of *Aeromonas hydrophila* serotype O:34 strains grown at 37 degrees C. Infect. Immun. **65:**1245-1250.

Aho, E. L., Murphy, G. L., and Cannon, J. G. 1987. Distribution of specific DNA sequences among pathogenic and commensal *Neisseria* species. Infect. Immun. **55:**1009-1013.

Alexeyev, M. F., Shokolenko, I. N., and Croughan, T. P. 1995. Improved antibiotic-resistance gene cassettes and omega elements for *Escherichia coli* vector construction and *in vitro* deletion/insertion mutagenesis. Gene. **160:**63-67.

Allen, D. A., Austin, B., and Colwell, R. R. 1983. *Aeromonas media*, a new species isolated from river water. Int. J. Syst. Bacteriol. **33:**599-604.

Alm, R. A., and Mattick, J. S. 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa*. Gene. **192:**89-98.

Alm, R. A., Stroeher, U. H., and Manning, P. A. 1988. Extracellular proteins of *Vibrio cholerae*: nucleotide sequence of the structural gene (*hlyA*) for the haemolysin of the haemolytic El Tor strain 017 and characterization of the *hlyA* mutation in the non- haemolytic classical strain 569B. Mol. Microbiol. **2:**481-488.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.

Altwegg, M., Steigerwalt, A. G., Altwegg-Bissig, R., Luthy-Hottenstein, J., and Brenner, D. J. 1990. Biochemical identification of *Aeromonas* genospecies isolated from humans. J. Clin. Microbiol. **28:**258-264.

Asao, T., Kinoshita, Y., Kozaki, S., Uemura, T., and Sakaguchi, G. 1984. Purification and some properties of *Aeromonas hydrophila* hemolysin. Infect. Immun. **46:**122-127.

Atsumi, T., McCarter, L., and Imae, Y. 1992. Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. Nature. **355:**182-184.

Attridge, S. R., Manning, P. A., Holmgren, J., and Jonson, G. 1996. Relative significance of mannose-sensitive hemagglutinin and toxin- coregulated pili in colonization of infant mice by *Vibrio cholerae* El Tor. Infect. Immun. **64:**3369-3373.

Attridge, S. R., and Rowley, D. 1983. The role of the flagellum in the adherence of *Vibrio cholerae*. J. Infect. Dis. **147:**864-872.

Attridge, S. R., Voss, E., and Manning, P. A. 1993. The role of toxin-coregulated pili in the pathogenesis of *Vibrio cholerae* O1 El Tor. Microb. Pathog. **15:**421-431.

Ausubel, F. M., Brent, R., and Kingston, R. E. 1994. Current protocols in molecular biology, 2nd ed. John Wiley and Sons, Inc., New York.

Beard, M. K., Mattick, J. S., Moore, L. J., Mott, M. R., Marrs, C. F., and Egerton, J. R. 1990. Morphogenetic expression of *Moraxella bovis* fimbriae (pili) in Pseudomonas aeruginosa. J. Bacteriol. **172:**2601-2607.

Benitez, J. A., Spelbrink, R. G., Silva, A., Phillips, T. E., Stanley, C. M., Boesman-Finkelstein, M., and Finkelstein, R. A. 1997. Adherence of

Vibrio cholerae to cultured differentiated human intestinal cells: an *in vitro* colonization model. Infect. Immun. **65:**3474-3477.

Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513-1523.

Bogdanovic, R., Cobeljic, M., Markovic, M., Nikolic, V., Ognjanovic, M., Sarjanovic, L., and Makic, D. 1991. Haemolytic-uraemic syndrome associated with *Aeromonas hydrophila* enterocolitis. Pediatr. Nephrol. **5:**293-295.

Borrell, N., Acinas, S. G., Figueras, M. J., and Martinez-Murcia, A. J. 1997. Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. J Clin Microbiol. **35:**1671-4.

Bradley, D. E. 1980. A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. Can. J. Microbiol. **26:**146-154.

Brown, R. L., Sanderson, K., and Kirov, S. M. 1997. Plasmids and *Aeromonas* virulence. FEMS Immunol. Med. Microbiol. **17:**217-223.

Buckley, J. T. 1982. Substrate specificity of bacterial glycerophospholipid:cholesterol acyltransferase. Biochemistry. **21:**6699-6703.

Buckley, J. T., and Howard, S. P. 1999. The cytotoxic enterotoxin of *Aeromonas hydrophila* is aerolysin. Infect. Immun. **67:**466-467.

Burke, V., Gracey, M., Robinson, J., Peck, D., Beaman, J., and Bundell, C. 1983. The microbiology of childhood gastroenteritis: *Aeromonas* species and other infective agents. J. Infect. Dis. **148:**68-74.

Burke, V., Robinson, Berry, R. J., and Gracey, M. 1981. Detection of enterotoxins of *Aeromonas* hydrophila by a suckling-mouse test. J. Med. Microbiol. **14:**401-408.

Carnahan, A., Fanning, G. R., and Joseph, S. W. 1991a. *Aeromonas jandaei* (formerly genospecies DNA group 9 *A. sobria*), a new sucrose-negative species isolated from clinical specimens. J. Clin. Microbiol. **29:**560-564.

Carnahan, A. M., Behram, S., and Joseph, S. W. 1991b. Aerokey II: a flexible key for identifying clinical *Aeromonas* species. J. Clin. Microbiol. **29:**2843-2849.

Carnahan, A. M., Chakraborty, T., Fanning, G. R., Verma, D., Ali, A., Janda, J. M., and Joseph, S. W. 1991c. *Aeromonas trota* sp. nov., an ampicillin-susceptible species isolated from clinical specimens. J. Clin. Microbiol. **29:**1206-1210.

Carnahan, A. M., and Joseph, S. W. 1991. *Aeromonas* update: new species and global distribution. Experientia. **47:**402-403.

Carrello, A., Silburn, K. A., Budden, J. R., and Chang, B. J. 1988. Adhesion of clinical and environmental *Aeromonas* isolates to HEp-2 cells. J. Med. Microbiol. **26:**19-27.

Chaicumpa, W., and Rowley, D. 1972. Experimental cholera in infant mice: protective effects of antibody. J. Infect. Dis. **125:**480-485.

Chakraborty, T., Huhle, B., Bergbauer, H., and Goebel, W. 1986. Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in Escherichia coli K-12. J. Bacteriol. **167:**368-374.

Chakraborty, T., Huhle, B., Hof, H., Bergbauer, H., and Goebel, W. 1987. Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *A. hydrophila*- associated systemic infections. Infect. Immun. **55:**2274-2280.

Chakraborty, T., Montenegro, M. A., Sanyal, S. C., Helmuth, R., Bulling, E., and Timmis, K. N. 1984. Cloning of enterotoxin gene from *Aeromonas hydrophila* provides conclusive evidence of production of a cytotonic enterotoxin. Infect. Immun. **46:**435-441.

Champsaur, H., Adremont, A., Mathieu, D., Rottman, E., and Auzepy, P. 1982. Cholera-like illness due to *Aeromonas sobria*. J. Infect. Dis. **145:**248-254.

Cheasty, T., Gross, R. J., Thomas, L. V., and Rowe, B. 1988. Serogrouping of the *Aeromonas hydrophila* group. J. Diarrhoeal Dis. Res. **6:**95-98.

Chopra, A. K., Houston, C. W., Peterson, J. W., and Jin, G. F. 1993. Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. Can. J. Microbiol. **39:**513-523.

Chopra, A. K., Pham, R., and Houston, C. W. 1994. Cloning and expression of putative cytotonic enterotoxin-encoding genes from *Aeromonas hydrophila*. Gene. **139:**87-91.

Chuang, Y. C., Chiou, S. F., Su, J. H., Wu, M. L., and Chang, M. C. 1997. Molecular analysis and expression of the extracellular lipase of *Aeromonas hydrophila* MCC-2. Microbiology. **143:**803-812.

Colwell, R. R., MacDonell, M. R., and De Ley, J. 1986. Proposal to recognise the family Aeromonadaceae fam. nov. Int. J. Syst. Bacteriol. **36:**473-477.

Costello, G. M., Vipond, R., and MacIntyre, S. 1996. *Aeromonas salmonicida* possesses two genes encoding homologs of the major outer membrane protein, OmpA. J. Bacteriol. **178:**1623-1630.

Davin-Regli, A., Bollet, C., Chamorey, E., Colonna, D. i. V., and Cremieux, A. 1998. A cluster of cases of infections due to *Aeromonas hydrophila* revealed by combined RAPD and ERIC-PCR. J. Med. Microbiol. **47:**499-504.

de la Morena, M. L., Van, R., Singh, K., Brian, M., Murray, M. E., and Pickering, L. K. 1993. Diarrhea associated with *Aeromonas* species in children in day care centers. J. Infect. Dis. **168:**215-218.

de Lorenzo, V., and Timmis, K. N. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol. **235:**386-405.

Doig, P., Sastry, P. A., Hodges, R. S., Lee, K. K., Paranchych, W., and Irvin, R. T. 1990. Inhibition of pilus-mediated adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells by monoclonal antibodies directed against pili. Infect. Immun. **58:**124-130.

Doig, P., Todd, T., Sastry, P. A., Lee, K. K., Hodges, R. S., Paranchych, W., and Irvin, R. T. 1988. Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. Infect. Immun. **56:**1641-1646.

Donnenberg, M. S., Zhang, H. Z., and Stone, K. D. 1997. Biogenesis of the bundle-forming pilus of enteropathogenic *Escherichia coli*: reconstitution of fimbriae in recombinant E. coli and role of DsbA in pilin stability-a review. Gene. **192:**33-38.

Dorsch, M., Ashbolt, N. J., Cox, P. T., and Goodman, A. E. 1994. Rapid identification of *Aeromonas* species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental isolates. J. Appl. Bacteriol. **77:**722-726.

Duguid, J. P., and Old, D. C. 1994. Introduction: a historical perspective, p. 1-7. *In* P. Klemm (ed.), Fimbriae: adhesion, genetics, biogenesis, and vaccines. CRC Press, Inc., Boca Raton.

Dupuy, B., and Pugsley, A. P. 1994. Type IV prepilin peptidase gene of *Neisseria gonorrhoeae* MS11: presence of a related gene in other piliated and nonpiliated Neisseria strains. J. Bacteriol. **176:**1323-1331.

Elleman, T. C., Hoyne, P. A., Stewart, D. J., McKern, N. M., and Peterson, J. E. 1986. Expression of pili from *Bacteroides nodosus* in *Pseudomonas aeruginosa*. J. Bacteriol. **168:**574-580.

Elleman, T. C., and Peterson, J. E. 1987. Expression of multiple types of N-methyl Phe pili in *Pseudomonas aeruginosa*. Mol. Microbiol. **1:**377-380.

Ellis, A. E. 1991. An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. salmonicida. J. Fish Dis. **14:**265-277.

Esteve, C., Gutierrez, M. C., and Ventosa, A. 1995. *Aeromonas encheleia* sp. nov., isolated from European eels. Int. J. Syst. Bacteriol. **45:**462-466.

Fanning, G. R., Hickman-Brenner, F. W., Farmer, J. J. I., and Brenner, D. J. 1985. DNA relatedness and phenotypic analysis of the genus *Aeromonas*. Abstr. Annu. Meet. ASM. **C116:**319.

Figura, N., Marri, L., Verdiani, S., Ceccherini, C., and Barberi, A. 1986. Prevalence, species differentiation, and toxigenicity of *Aeromonas* strains in cases of childhood gastroenteritis and in controls. J. Clin. Microbiol. **23:**595-599.

Finlay, B. B., and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. **61:**136-169.

Francki, K. T., and Chang, B. J. 1994. Variable expression of O-antigen and the role of lipopolysaccharide as an adhesin in *Aeromonas sobria*. FEMS Microbiol. Lett. **122:**97-101.

Frey, J., and Krisch, H. M. 1985. Omega mutagenesis in gram-negative bacteria: a selectable interposon which is strongly polar in a wide range of bacterial species. Gene. **36:**143-150.

Fullner, K. J., and Mekalanos, J. J. 1999. Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae*. Infect. Immun. **67:**1393-1404.

Fyfe, J. A., Strugnell, R. A., and Davies, J. K. 1993. Control of gonococcal pilin-encoding gene expression in *Escherichia coli*. Gene. **123:**45-50.

Geiss, H. K., Fogel, W., and Sonntag, H. G. 1988. Frequency of *Aeromonas* species in the stools of healthy and diarrheic patients. Immun. Infekt. **16:**115-157.

Gilsdorf, J. R., McCrea, K. W., and Marrs, C. F. 1997. Role of pili in *Haemophilus influenzae* adherence and colonization. Infect. Immun. **65:**2997-3002.

Giron, J. A., Ho, A. S., and Schoolnik, G. K. 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science. **254:**710-713.

Gish, W., and States, D. J. 1993. Identification of protein coding regions by database similarity search. Nat. Genet. **3:**266-272.

Gordon, V. M., and Leppla, S. H. 1994. Proteolytic activation of bacterial toxins: role of bacterial and host cell proteases. Infect. Immun. **62:**333-340.

Gracey, M., Burke, V., and Robinson, J. 1982a. *Aeromonas*-associated gastroenteritis. Lancet. **2:**1304-1306.

Gracey, M., Burke, V., Rockhill, R. C., Suharyono, and Sunoto. 1982b. *Aeromonas* species as enteric pathogens. Lancet. **1:**223-224.

Grant, C. C., Konkel, M. E., Cieplak, W., Jr., and Tompkins, L. S. 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. **61:**1764-1771.

Haas, R., Schwarz, H., Meyer, T. F. 1987. Release of soluble pilin antigen coupled with gene conversion in *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. U. S. A. **84:**9079-9083.

Hall, R. H., Vial, P. A., Kaper, J. B., Mekalanos, J. J., and Levine, M. M. 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* 01. Microb. Pathog. **4:**257-265.

Haque, Q. M., Sugiyama, A., Iwade, Y., Midorikawa, Y., and Yamauchi, T. 1996. Diarrheal and environmental isolates of *Aeromonas* spp. produce a toxin similar to Shiga-like toxin 1. Curr. Microbiol. **32:**239-245.

Hazlett, L. D., Moon, M. M., Singh, A., Berk, R. S., and Rudner, X. L. 1991. Analysis of adhesion, piliation, protease production and ocular infectivity of several *P. aeruginosa* strains. Curr. Eye Res. **10:**351-362.

Heuzenroeder, M. W., Wong, C. Y., and Flower, R. L. 1999. Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: correlation with virulence in a suckling mouse model. FEMS Microbiol. Lett. **174:**131-136.

Hickman-Brenner, F. W., Fanning, G. R., Arduino, M. J., Brenner, D. J., and Farmer, J. J. d. 1988. *Aeromonas schubertii*, a new mannitol-negative species found in human clinical specimens. J. Clin. Microbiol. **26:**1561-1564.

Hickman-Brenner, F. W., MacDonald, K. L., Steigerwalt, A. G., Fanning, G. R., Brenner, D. J., and Farmer, J. J. d. 1987. *Aeromonas veronii*, a new ornithine decarboxylase-positive species that may cause diarrhea. J. Clin. Microbiol. **25:**900-906.

Hilton, S., McCubbin, W. D., Kay, C. M., and Buckley, J. T. 1990. Purification and spectral study of a microbial fatty acyltransferase: activation by limited proteolysis. Biochemistry. **29:**9072-9078.

Hirono, I., and Aoki, T. 1991. Nucleotide sequence and expression of an extracellular hemolysin gene of *Aeromonas hydrophila*. Microb. Pathog. **11:**189-197.

Hirono, I., and Aoki, T. 1993. Cloning and characterization of three hemolysin genes from *Aeromonas salmonicida*. Microb. Pathog. **15:**269-282.

Hirono, I., Aoki, T., Asao, T., and Kozaki, S. 1992. Nucleotide sequences and characterization of haemolysin genes from *Aeromonas hydrophila* and *Aeromonas sobria*. Microb. Pathog. **13:**433-446.

Ho, A. S., Mietzner, T. A., Smith, A. J., and Schoolnik, G. K. 1990. The pili of *Aeromonas hydrophila*: identification of an environmentally regulated "mini pilin". J. Exp. Med. **172:**795-806.

Ho, A. S., Sohel, I., and Schoolnik, G. K. 1992. Cloning and characterization of *fxp*, the flexible pilin gene of *Aeromonas hydrophila*. Mol. Microbiol. **6:**2725-2732.

Hobbs, M., Dalrymple, B. P., Cox, P. T., Livingstone, S. P., Delaney, S. F., and Mattick, J. S. 1991. Organization of the fimbrial gene region of *Bacteroides nodosus*: class I and class II strains. Mol. Microbiol. **5:**543-560.

Hobbs, M., and Mattick, J. S. 1993. Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. **10:**233-243.

Hokama, A., Honma, Y., and Nakasone, N. 1990. Pili of an *Aeromonas hydrophila* strain as a possible colonization factor. Microbiol. Immunol. **34:**901-915.

Hokama, A., and Iwanaga, M. 1992. Purification and characterization of *Aeromonas sobria* Ae24 pili: a possible new colonization factor. Microb. Pathog. **13:**325-334.

Honma, Y., and Nakasone, N. 1990. Pili of *Aeromonas hydrophila*: purification, characterization, and biological role. Microbiol. Immunol. **34:**83-98.

Horne, S. M., Kottom, T. J., Nolan, L. K., and Young, K. D. 1997. Decreased intracellular survival of an *fkpA* mutant of *Salmonella typhimurium* Copenhagen. Infect. Immun. **65:**806-810.

Howard, S. P., and Buckley, J. T. 1986. Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila*. Mol. Gen. Genet. **204:**289-295.

Howard, S. P., Critch, J., and Bedi, A. 1993. Isolation and analysis of eight *exe* genes and their involvement in extracellular protein secretion and outer membrane assembly in *Aeromonas hydrophila*. J. Bacteriol. **175:**6695-6703.

Howard, S. P., Garland, W. J., Green, M. J., and Buckley, J. T. 1987. Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. J. Bacteriol. **169:**2869-2871.

Howard, S. P., Macintyre, S., and Buckley, J. T. 1996. Toxins, p. 267-86. *In* B. Austin, M. Altwegg, P. J. Gosling and S. W. Joseph (ed.), The genus *Aeromonas*. John Wiley and Sons Ltd., Chichester, UK.

Hoyne, P. A., Haas, R., Meyer, T. F., Davies, J. K., and Elleman, T. C. 1992. Production of *Neisseria gonorrhoeae* pili (fimbriae) in *Pseudomonas aeruginosa*. J. Bacteriol. **174:**7321-7327.

Husslein, V., Bergbauer, H., and Chakraborty, T. 1991. Studies on aerolysin and a serine protease from *Aeromonas trota* sp. nov. Experientia. **47:**420-421.

Husslein, V., Chakraborty, T., Carnahan, A., and Joseph, S. W. 1992. Molecular studies on the aerolysin gene of *Aeromonas* species and discovery of a species-specific probe for *Aeromonas trota* species nova. Clin. Infect. Dis. **14:**1061-1068.

Husslein, V., Huhle, B., Jarchau, T., Lurz, R., Goebel, W., and Chakraborty, T. 1988. Nucleotide sequence and transcriptional analysis of the *aerCaerA* region of *Aeromonas sobria* encoding aerolysin and its regulatory region. Mol. Microbiol. **2:**507-517.

Huys, G., Kampfer, P., Altwegg, M., Kersters, I., Lamb, A., Coopman, R., Luthy-Hottenstein, J., Vancanneyt, M., Janssen, P., and Kersters, K.

1997. *Aeromonas popoffii* sp. nov., a mesophilic bacterium isolated from drinking water production plants and reservoirs. Int. J. Syst. Bacteriol. **47:**1165-1171.

Iredell, J. R., and Manning, P. A. 1997a. Outer membrane translocation arrest of the TcpA pilin subunit in *rfb* mutants of *Vibrio cholerae* O1 strain 569B. J. Bacteriol. **179:**2038-2046.

Iredell, J. R., and Manning, P. A. 1997b. Translocation failure in a type-4 pilin operon: *rfb* and *tcpT* mutants in *Vibrio cholerae*. Gene. **192:**71-77.

Iredell, J. R., Stroeher, U. H., Ward, H. M., and Manning, P. A. 1998. Lipopolysaccharide O-antigen expression and the effect of its absence on virulence in *rfb* mutants of *Vibrio cholerae* O1. FEMS Immunol. Med. Microbiol. **20:**45-54.

Iwanaga, M., and Hokama, A. 1992. Characterization of *Aeromonas sobria* TAP13 pili: a possible new colonization factor. J. Gen. Microbiol. **138:**1913-1919.

Janda, J. M. 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. Clin. Microbiol. Rev. **4:**397-410.

Janda, J. M., and Abbott, S. L. 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding Panorama of species, disease presentations, and unanswered questions. Clin. Infect. Dis. **27:**332-344.

Janda, J. M., and Kokka, R. P. 1991. The pathogenicity of *Aeromonas* strains relative to genospecies and phenospecies identification. FEMS Microbiol. Lett. **69:**29-33.

Jeanteur, D., Gletsu, N., Pattus, F., and Buckley, J. T. 1992. Purification of *Aeromonas hydrophila* major outer-membrane proteins: N- terminal sequence analysis and channel-forming properties. Mol. Microbiol. **6:**3355-3363.

Johnson, K., Parker, M. L., and Lory, S. 1986. Nucleotide sequence and transcriptional initiation site of two *Pseudomonas aeruginosa* pilin genes. J. Biol. Chem. **261:**15703-15708.

Jonson, G., Holmgren, J., and Svennerholm, A. M. 1991. Identification of a mannose-binding pilus on *Vibrio cholerae* El Tor. Microb. Pathog. **11:**433-441.

Jonson, G., Lebens, M., and Holmgren, J. 1994. Cloning and sequencing of *Vibrio cholerae* mannose-sensitive haemagglutinin pilin gene: localization of *mshA* within a cluster of type 4 pilin genes. Mol. Microbiol. **13:**109-118.

Jonsson, A. B., Nyberg, G., and Normark, S. 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. EMBO J. **10:**477-488.

Karaolis, D. K., Johnson, J. A., Bailey, C. C., Boedeker, E. C., Kaper, J. B., and Reeves, P. R. 1998. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc. Natl. Acad. Sci. U. S. A. **95:**3134-3139.

Karaolis, D. K., Somara, S., Maneval, D. R., Jr., Johnson, J. A., and Kaper, J. B. 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature. **399:**375-379.

Kaufman, M. R., Seyer, J. M., and Taylor, R. K. 1991. Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram- negative bacteria. Genes. Dev. **5:**1834-1846.

Kawagishi, I., Imagawa, M., Imae, Y., McCarter, L., and Homma, M. 1996. The sodium-driven polar flagellar motor of marine *Vibrio* as the mechanosensor that regulates lateral flagellar expression. Mol. Microbiol. **20:**693-699.

Kaznowski, A. 1998. Identification of *Aeromonas* strains of different origin to the genomic species level. J. Appl. Microbiol. **84:**423-430.

Kirov, S. M. 1997. *Aeromonas* and *Plesiomonas* species, p. 265-87. *In* M. P. Doyle, L. R. Beuchat and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. American Society for Microbiology, Washington, D. C.

Kirov, S. M., Hayward, L. J., and Nerrie, M. A. 1995a. Adhesion of *Aeromonas* sp. to cell lines used as models for intestinal adhesion. Epidemiol. Infect. **115:**465-473.

Kirov, S. M., Jacobs, I., Hayward, L. J., and Hapin, R. H. 1995b. Electron microscopic examination of factors influencing the expression of filamentous surface structures on clinical and environmental isolates of *Aeromonas veronii* biotype sobria. Microbiol. Immunol. **39:**329-338.

Kirov, S. M., O'Donovan, L. A., and Sanderson, K. 1999. Functional characterization of type IV pili expressed on diarrhea- associated isolates of *Aeromonas* species. Infect. Immun. **67:**5447-5454.

Kirov, S. M., Rees, B., Wellock, R. C., Goldsmid, J. M., and Van Galen, A. D. 1986. Virulence characteristics of *Aeromonas* spp. in relation to source and biotype. J. Clin. Microbiol. **24:**827-834.

Kirov, S. M., and Sanderson, K. 1996. Characterization of a type IV bundle-forming pilus (SFP) from a gastroenteritis-associated strain of *Aeromonas veronii* biovar sobria. Microb. Pathog. **21:**23-34.

Kirov, S. M., Sanderson, K., and Dickson, T. C. 1998. Characterisation of a type IV pilus produced by *Aeromonas caviae*. J. Med. Microbiol. **47:**527-531.

Klose, K. E., and Mekalanos, J. J. 1998. Differential regulation of multiple flagellins in *Vibrio cholerae*. J. Bacteriol. **180:**303-316.

Koga, T., Ishimoto, K., and Lory, S. 1993. Genetic and functional characterization of the gene cluster specifying expression of *Pseudomonas aeruginosa* pili. Infect. Immun. **61:**1371-1377.

Kokka, R. P., Vedros, N. A., and Janda, J. M. 1991. Characterization of classic and atypical serogroup O:11 *Aeromonas*: evidence that the surface array protein is not directly involved in mouse pathogenicity. Microb. Pathog. **10:**71-79.

Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., 2nd, and Peterson, K. M. 1994. pBBR1MCS: a broad-host-range cloning vector. Biotechniques. **16:**800-2.

Krovacek, K., Dumontet, S., Eriksson, E., and Baloda, S. B. 1995. Isolation, and virulence profiles, of *Aeromonas hydrophila* implicated in an outbreak of food poisoning in Sweden. Microbiol. Immunol. **39:**655-661.

Kuijper, E. J., Steigerwalt, A. G., Schoenmakers, B. S., Peeters, M. F., Zanen, H. C., and Brenner, D. J. 1989. Phenotypic characterization and DNA relatedness in human fecal isolates of *Aeromonas* spp. J. Clin. Microbiol. **27:**132-138.

Kuijper, E. J., Zanen, H. C., and Peeters, M. F. 1987. *Aeromonas*-associated diarrhea in the Netherlands. Ann. Intern. Med. **106:**640-641.

Laskos, L., Dillard, J. P., Seifert, H. S., Fyfe, J. A. M., and Davies, J. K. 1998. The pathogenic neisseriae contain an inactive *rpoN* gene and do not utilize the *pilE* sigma54 promoter. Gene. **208:**95-102.

Liles, M. R., Viswanathan, V. K., and Cianciotto, N. P. 1998. Identification and temperature regulation of *Legionella pneumophila* genes involved in type IV pilus biogenesis and type II protein secretion. Infect. Immun. **66:**1776-1782.

Lior, H., and Johnson, W. M. 1991. Application of the polymerase chain reaction (PCR) to detection of the aerolysin gene in whole cell cultures of beta-hemolytic *Aeromonas hydrophila*. Experientia. **47:**421-424.

Liu, Z., and Mishra, N. C. 1995. Single-tube method for plasmid miniprep from large numbers of clones for direct screening by size or restriction digestion. Biotechniques. **18:**214-216.

Ljungh, A., Wretlind, B., and Mollby, R. 1981. Separation and characterization of enterotoxin and two haemolysins from *Aeromonas hydrophila*. Acta. Pathol. Microbiol. Scand. [B]. **89:**387-397.

Lockman, H. A., and Curtiss, R. I. 1990. *Salmonella typhimurium* mutants lacking flagella or motility remain virulent in BALB/c mice. Infect. Immun. **58:**137-143.

Lockman, H. A., and Curtiss, R. I. 1992. Virulence of non-type 1-fimbriated and nonfimbriated nonflagellated *Salmonella typhimurium* mutants in murine typhoid fever. Infect. Immun. **60:**491-496.

Lu, H. M., Motley, S. T., and Lory, S. 1997. Interactions of the components of the general secretion pathway: role of *Pseudomonas aeruginosa* type IV pilin

subunits in complex formation and extracellular protein secretion. Mol. Microbiol. **25:**247-259.

Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P., and van Alphen, L. 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. FEBS Lett. **58:** 254-8.

Lutwyche, P., Exner, M. M., Hancock, R. E., and Trust, T. J. 1995. A conserved *Aeromonas salmonicida* porin provides protective immunity to rainbow trout. Infect. Immun. **63:**3137-3142.

Martinetti Lucchini, G., and Altwegg, M. 1992. rRNA gene restriction patterns as taxonomic tools for the genus *Aeromonas*. Int. J. Syst. Bacteriol. **42:**384-389.

Martinez-Murcia, A. J., Esteve, C., Garay, E., and Collins, M. D. 1992. *Aeromonas allosaccharophila* sp. nov., a new mesophilic member of the genus *Aeromonas*. FEMS Microbiol. Lett. **70:**199-205.

Mattick, J. A., and Alm, R. A. 1995. Common architecture of type-4 fimbriae and complexes involved in macromolecular traffic. Trends Microbiol. **3:**411-413.

Mattick, J. S., Anderson, B. J., Cox, P. T., Dalrymple, B. P., Bills, M. M., Hobbs, M., and Egerton, J. R. 1991. Gene sequences and comparison of the fimbrial subunits representative of *Bacteroides nodosus* serotypes A to I: class I and class II strains. Mol. Microbiol. **5:**561-573.

Mattick, J. S., Bills, M. M., Anderson, B. J., Dalrymple, B., Mott, M. R., and Egerton, J. R. 1987. Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*. J. Bacteriol. **169:**33-41.

McCardell, B. A., Madden, J. M., Kothary, M. H., and Sathyamoorthy, V. 1995. Purification and characterization of a CHO cell-elongating toxin produced by *Aeromonas hydrophila*. Microb. Pathog. **19:**1-9.

McIntosh, D., and Austin, B. 1991. Atypical characteristics of the salmonid pathogen *Aeromonas salmonicida*. J. Gen. Microbiol. **137:**1341-1343.

Menzl, K., Maier, E., Chakraborty, T., and Benz, R. 1996. HlyA hemolysin of *Vibrio cholerae* O1 biotype E1 Tor. Identification of the hemolytic complex

and evidence for the formation of anion-selective ion-permeable channels. Eur. J. Biochem. **240:**646-654.

Merino, S., Aguilar, A., Nogueras, M. M., Regue, M., Swift, S., and Tomás, J. M. 1999. Cloning, sequencing, and role in virulence of two phospholipases (A1 and C) from mesophilic *Aeromonas* sp. serogroup O:34. Infect. Immun. **67:**4008-4013.

Merino, S., Aguilar, A., Rubires, X., Abitiu, N., Regue, M., and Tomás, J. M. 1997a. The role of the capsular polysaccharide of *Aeromonas hydrophila* serogroup O:34 in the adherence to and invasion of fish cell lines. Res. Microbiol. **148:**625-631.

Merino, S., Aguilar, A., Rubires, X., and Tomás, J. M. 1998. Mesophilic *Aeromonas* strains from different serogroups: the influence of growth temperature and osmolarity on lipopolysaccharide and virulence. Res. Microbiol. **149:**407-416.

Merino, S., Rubires, X., Aguilar, A., and Tomás, J. M. 1996a. The O:34-antigen lipopolysaccharide as an adhesin in *Aeromonas hydrophila*. FEMS Microbiol. Lett. **139:**97-101.

Merino, S., Rubires, X., Aguilar, A., and Tomás, J. M. 1997b. The role of flagella and motility in the adherence and invasion to fish cell lines by *Aeromonas hydrophila* serogroup O:34 strains. FEMS Microbiol. Lett. **151:**213-217.

Merino, S., Rubires, X., Aguillar, A., Guillot, J. F., and Tomás, J. M. 1996b. The role of the O-antigen lipopolysaccharide on the colonization *in vivo* of the germfree chicken gut by *Aeromonas hydrophila* serogroup O:34. Microb. Pathog. **20:**325-333.

Nakasone, N., Iwanaga, M., Yamashiro, T., Nakashima, K., and Albert, M. J. 1996. *Aeromonas trota* strains, which agglutinate with *Vibrio cholerae* O139 Bengal antiserum, possess a serologically distinct fimbrial colonization factor. Microbiology. **142:**309-313.

Nandapalan, N., and Chang, B. J. 1989. Production and characterization of monoclonal antibodies to *Aeromonas sobria* surface antigens. FEMS Microbiol. Immunol. **1:**515-524.

Nataro, J. P., and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli.* Clin. Microbiol. Rev. **11:**142-201.

Nunn, D. N., and Lory, S. 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc. Natl. Acad. Sci. U. S. A. **88:**3281-3285.

Nunn, D. N., and Lory, S. 1992. Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. U. S. A. **89:**47-51.

Nunn, D. N., and Lory, S. 1993. Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -U, -V, and -W. J. Bacteriol. **175:**4375-4382.

Oakey, H. J., Ellis, J. T., and Gibson, L. F. 1996. A biochemical protocol for the differentiation of current genomospecies of *Aeromonas*. Zentralbl. Bakteriol. **284:**32-46.

Olsen, A., Jonsson, A., and Normark, S. 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli.* Nature. **338:**652-655.

Paranjpye, R. N., Lara, J. C., Pepe, J. C., Pepe, C. M., and Strom, M. S. 1998. The type IV leader peptidase/N-methyltransferase of *Vibrio vulnificus* controls factors required for adherence to HEp-2 cells and virulence in iron-overloaded mice. Infect. Immun. **66:**5659-5668.

Parker, M. W., van der Goot, F. G., and Buckley, J. T. 1996. Aerolysin - the ins and outs of a model channel-forming toxin. Mol. Microbiol. **19:**205-212.

Pazzaglia, G., Sack, R. B., Bourgeois, A. L., Froehlich, J., and Eckstein, J. 1990. Diarrhea and intestinal invasiveness of *Aeromonas* strains in the removable intestinal tie rabbit model. Infect. Immun. **58:**1924-1931.

Pearce, W. A., and Buchanan, T. M. 1978. Attachment role of gonococcal pili. Optimum conditions and quantitation of adherence of isolated pili to human cells *in vitro*. J. Clin. Invest. **61:**931-943.

Pepe, C. M., Eklund, M. W., and Strom, M. S. 1996. Cloning of an *Aeromonas hydrophila* type IV pilus biogenesis gene cluster: complementation of pilus assembly functions and characterization of a type IV leader peptidase/N-methyltransferase required for extracellular protein secretion. Mol. Microbiol. **19:**857-869.

Pollard, D. R., Johnson, W. M., Lior, H., Tyler, S. D., and Rozee, K. R. 1990. Detection of the aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. J. Clin. Microbiol. **28:**2477-2481.

Popoff, M. 1984. Genus III. *Aeromonas*. Kluyver and Van Niel 1936, 398^{AL}, p. 545-8. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore, MD.

Popoff, M., and Veron, M. 1976. A taxonomic study of the *Aeromonas hydrophila-Aeromonas punctata* group. J. Gen. Microbiol. **94:**11-22.

Popoff, M. Y., Coynault, C., Kiredjian, M., and Lemelin, M. 1981. Polynucleotide sequence relatedness among motile *Aeromonas* species. Curr. Microbiol. **5:**109-114.

Quinn, D. M., Atkinson, H. M., Bretag, A. H., Tester, M., Trust, T. J., Wong, C. Y., and Flower, R. L. 1994. Carbohydrate-reactive, pore-forming outer membrane proteins of *Aeromonas hydrophila*. Infect. Immun. **62:**4054-4058.

Quinn, D. M., Wong, C. Y., Atkinson, H. M., and Flower, R. L. 1993. Isolation of carbohydrate-reactive outer membrane proteins of *Aeromonas hydrophila*. Infect. Immun. **61:**371-377.

Reischl, U. 1998. Purification and immunological characterization of recombinant antigens expressed in the form of insoluble aggregates (inclusion bodies), p. 331-43. *In* U. Reischl (ed.), Molecular diagnosis of infectious diseases, vol. 13. Humana Press Inc., Totowa, NJ.

Robson, W. L., Leung, A. K., and Trevenen, C. L. 1992. Haemolytic-uraemic syndrome associated with *Aeromonas hydrophila* enterocolitis. Pediatr. Nephrol. **6:**221.

Rodriguez, L. A., Ellis, A. E., and Nieto, T. P. 1992. Purification and characterisation of an extracellular metalloprotease, serine protease and haemolysin of *Aeromonas hydrophila* strain B32: all are lethal for fish. Microb. Pathog. **13:**17-24.

Rood, J. I., Billington, S. J., and Johnston, J. L. 1997. Molecular pathogenesis of the ovine footrot pathogen *Dichelobacter nodosus*. Rev. Med. Microbiol. **8:**S31-S33.

Rothbard, J. B., Fernandez, R., Wang, L., Teng, N. N., and Schoolnik, G. K. 1985. Antibodies to peptides corresponding to a conserved sequence of gonococcal pilins block bacterial adhesion. Proc. Natl. Acad. Sci. U. S. A. **82:**915-919.

Rudel, T., Scheurerpflug, I., and Meyer, T. F. 1995. *Neisseria* PilC protein identified as type-4 pilus tip-located adhesin. Nature. **373:**357-359.

Ruehl, W. W., Marrs, C., Beard, M. K., Shokooki, V., Hinojoza, J. R., Banks, S., Bieber, D., and Mattick, J. S. 1993. Q pili enhance the attachment of *Moraxella bovis* to bovine corneas *in vitro*. Mol. Microbiol. **7:**285-288.

Sakazaki, R., and Shimada, T. 1984. O-serogrouping scheme for mesophilic *Aeromonas* strains. Jpn. J. Med. Sci. Biol. **37:**247-255.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Ney York.

Sanderson, K., Ghazali, F. M., and Kirov, S. M. 1996. Colonization of streptomycin-treated mice by *Aeromonas* species. J. Diarrhoeal Dis. Res. **14:**27-32.

Sar, N., McCarter, L., Simon, M., and Silverman, M. 1990. Chemotactic control of the two flagellar systems of *Vibrio parahaemolyticus*. J. Bacteriol. **172:**334-341.

Sato, M., Arita, M., Honda, T., and Miwatani, T. 1989. Characterization of a pilus produced by *Aeromonas hydrophila*. FEMS Microbiol. Lett. **50:**325-329.

Schubert, R. H., and Hegazi, M. 1988. *Aeromonas eucrenophila* species nova *Aeromonas caviae* a later and illegitimate synonym of *Aeromonas punctata*. Zentralbl. Bakteriol. Mikrobiol. Hyg. **268:**34-39.

Schultz, A. J., and McCardell, B. A. 1988. DNA homology and immunological cross-reactivity between *Aeromonas hydrophila* cytotonic toxin and cholera toxin. J. Clin. Microbiol. **26:**57-61.

Schweizer, H. P. 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene. **97:**109-121.

Schweizer, F. 1998. Interaction between the pili of *Pseudomonas aeruginosa* PAK and its carbohydrate receptor beta-D-GalNAc(1->4)beta-D-Gal analogs. Can. J. Microbiol. **44:**307-311.

Seifert, H. S. 1996. Questions about gonococcal pilus phase- and antigenic variation. Mol. Microbiol. **21:**433-440.

Sharma, D. P., Stroeher, U. H., Thomas, C. J., Manning, P. A., and Attridge, S. R. 1989. The toxin-coregulated pilus (TCP) of *Vibrio cholerae*: molecular cloning of genes involved in pilus biosynthesis and evaluation of TCP as a protective antigen in the infant mouse model. Microb. Pathog. **7:**437-448.

Shaw, J., Thornley, J., and Eley, A. 1997. Adherence and invasion of *Aeromonas caviae* to monolayer cells. Adherence and invasion of *Aeromonas caviae*. Adv. Exp. Med. Biol. **412:**217-219.

Shimada, T., Sakazaki, R., and Suzuki, K. 1985. Peritrichous flagella in mesophilic strains of *Aeromonas*. Jpn. J. Med. Sci. Biol. **38:**141-145.

Simon, R., Priefer, U., and Puhler, A. 1983. A broad hast range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology. **1:**784-791.

Singh, D. V., and Sanyal, S. C. 1992a. Enterotoxicity of clinical and environmental isolates of *Aeromonas* spp. J. Med. Microbiol. **36:**269-272.

Singh, D. V., and Sanyal, S. C. 1992b. Haemolysin and enterotoxin production by *Aeromonas caviae* isolated from diarrhoeal patients, fish and environment. J. Diarrhoeal Dis. Res. **10:**16-20.

Sohel, I., Puente, J. L., Ramer, S. W., Bieber, D., Wu, C. Y., and Schoolnik, G. K. 1996. Enteropathogenic *Escherichia coli*: identification of a gene cluster coding for bundle-forming pilus morphogenesis. J. Bacteriol. **178:**2613-2628.

Spangenberg, C., Fislage, R., Romling, U., and Tummler, B. 1997. Disrepectful type IV pilins. Mol. Microbiol. **25:**203-204.

Stelma, G. N., Jr., Johnson, C. H., and Spaulding, P. 1986. Evidence for the direct involvement of beta-hemolysin in *Aeromonas hydrophila* enteropathogenicity. Curr. Microbiol. **14:**71-77.

Stelma, G. N., Jr., Johnson, C. H., and Spaulding, P. L. 1988. Experimental evidence for enteropathogenicity in *Aeromonas veronii*. Can. J. Microbiol. **34:**877-880.

Strom, M. S., and Lory, S. 1991. Amino acid substitutions in pilin of *Pseudomonas aeruginosa*. Effect on leader peptide cleavage, amino-terminal methylation, and pilus assembly. J. Biol. Chem. **266:** 1656-1664.

Strom, M. S., and Lory, S. 1993. Structure-function and biogenesis of the type IV pili. Annu. Rev. Microbiol. **47:**565-596.

Studier, F. W., and Moffatt, B. A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113-130.

Tabor, S., and Richardson, C. C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. U. S. A. **82:**1074-1078.

Talon, D., Mulin, B., and Thouverez, M. 1998. Clonal identification of *Aeromonas hydrophila* strains using randomly amplified polymorphic DNA analysis. Eur. J. Epidemiol. **14:**305-310.

Tamamoto, T., Nakashima, K., Nakasone, N., Honma, Y., Higa, N., and Yamashiro, T. 1998. Adhesive property of toxin-coregulated pilus of *Vibrio cholerae* O1. Microbiol. Immunol. **42:**41-45.

Taylor, R. K., Miller, V. L., Furlong, D. B., and Mekalanos, J. J. 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. U. S. A. **84:**2833-2837.

Tennent, J. M., and Mattick, J. S. 1994. Type 4 fimbriae, p. 127-46. *In* P. Klemm (ed.), Fimbriae: adhesion, genetics, biogenesis, and vaccines. CRC Press, Inc., Boca Raton.

Thelestam, M., and Ljungh, A. 1981. Membrane-damaging and cytotoxic effects on human fibroblasts of alpha- and beta-hemolysins from *Aeromonas hydrophila*. Infect. Immun. **34:**949-956.

Thomas, L. V., Gross, R. J., Cheasty, T., and Rowe, B. 1990. Extended serogrouping scheme for motile, mesophilic *Aeromonas* species. J. Clin. Microbiol. **28:**980-984.

Thony, B., and Hennecke, H. 1989. The -24/-12 promoter comes of age. FEMS Microbiol. Rev. **5:**341-357.

Thornley, J. P., Shaw, J. G., Gryllos, I. A., and Eley, A. 1997. Virulence properties of clinically significant *Aeromonas* species: evidence for pathogenicity. Rev. Med. Microbiol. **8:**61-72.

Thornton, J., Howard, S. P., and Buckley, J. T. 1988. Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin-cholesterol acyltransferase and other lipases. Biochem. Biophys. Acta. **959:**153-159.

Towbin, H., Staehelin, T., and Gordon, J. 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology. **24:**145-149.

Umelo, E., and Trust, T. J. 1997. Identification and molecular characterization of two tandemly located flagellin genes from *Aeromonas salmonicida* A449. J. Bacteriol. **179:**5292-5299.

van der Goot, F. G., Lakey, J., Pattus, F., Kay, C. M., Sorokine, O., Van Dorsselaer, A., and Buckley, J. T. 1992. Spectroscopic study of the activation and oligomerization of the channel-forming toxin aerolysin:

identification of the site of proteolytic activation. Biochemistry. **31:**8566-8570.

Virji, M., Saunders, J. R., Sims, G., Makepeace, K., Maskell, D., and Ferguson, D. J. 1993. Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. Mol. Microbiol. **10:**1013-1028.

Waldor, M. K., and Mekalanos, J. J. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. **272:**1910-1914.

Wang, G., Tyler, K. D., Munro, C. K., and Johnson, W. M. 1996. Characterization of cytotoxic, hemolytic *Aeromonas caviae* clinical isolates and their identification by determining presence of a unique hemolysin gene. J. Clin. Microbiol. **34:**3203-3205.

Wang, J. T., Syed, A., and Gralla, J. D. 1997. Multiple pathways to bypass the enhancer requirement of sigma 54 RNA polymerase: roles for DNA and protein determinants. Proc. Natl. Acad. Sci. U. S. A. **94:**9538-9543.

Wang, L., and Gralla, J. D. 1998. Multiple *in vivo* roles for the -12-region elements of sigma 54 promoters. J. Bacteriol. **180:**5626-5631.

Watnick, P. I., Fullner, K. J., and Kolter, R. 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. J. Bacteriol. **181:**3606-3609.

Weiser, J. N., and Gotschlich, E. C. 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59:**2252-2258.

Whitchurch, C. B., and Mattick, J. S. 1994. *Escherichia coli* contains a set of genes homologous to those involved in protein secretion, DNA uptake and the assembly of type-4 fimbriae in other bacteria. Gene. **150:**9-15.

Wolff, K., and Stern, A. 1995. Identification and characterization of specific sequences encoding pathogenicity associated proteins in the genome of commensal *Neisseria* species. FEMS Microbiol. Lett. **125:**255-263.

Wong, C. Y., Heuzenroeder, M. W., and Flower, R. L. 1998. Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. Microbiology. **144:**291-298.

Wong, C. Y., Heuzenroeder, M. W., Quinn, D. M., and Flower, R. L. 1997. Cloning and characterization of two immunophilin-like genes, *ilpA* and *fkpA*, on a single 3.9-kilobase fragment of *Aeromonas hydrophila* genomic DNA. J. Bacteriol. **179:**3397-3403.

Wong, C. Y., Mayrhofer, G., Heuzenroeder, M. W., Atkinson, H. M., Quinn, D. M., and Flower, R. L. 1996. Measurement of virulence of aeromonads using a suckling mouse model of infection. FEMS Immunol. Med. Microbiol. **15:**233-241.

Woods, D. E., Straus, D. C., Johanson, W. G., Jr., Berry, V. K., and Bass, J. A. 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. **29:**1146-1151.

Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene. **33:**103-119.