Construction of a physical and preliminary genetic map of *Aeromonas hydrophila* JMP636

Helen N. Dodd and John M. Pemberton

Author for correspondence: John M. Pemberton. Tel: +61 7 33654640. Fax: +61 7 33654620.
e-mail: pemberto@biosci.uq.edu.au

Department of
Microbiology, University of
Queensland, Australia
4072

A physical and preliminary genetic map of the *Aeromonas hydrophila* JMP636 chromosome has been constructed. The topology of the genome was predicted to be circular as chromosomal DNA did not migrate from the origin during PFGE unless linearized by S1 nuclease. Cleavage of the chromosome with *PacI* and *PmeI* produced 23 and 14 fragments, respectively, and enabled calculation of the genome size at 4.5 Mb. Digestion of the chromosome with *I-CeuI* produced 10 fragments, indicating that 10 *rrf* (23S) genes were likely to be present. Hybridizations between DNA fragments generated with *PacI*, *PmeI* and *I-CeuI* were used to initially determine the relationship between these segments. To accurately map genes previously characterized from JMP636, the suicide vector pJP5603 was modified to introduce restriction sites for *PacI* and *PmeI*, producing pJP9540. Following cloning of genes into this vector and recombinational insertion into the JMP636 chromosome, *PacI* and *PmeI* cleavage determined the location of genes within macrorestriction fragments with the additional bands produced forming hybridization probes. From the data generated, it was possible to form a physical map comprising all the fragments produced by *PacI* and *PmeI*, and assign the contig of *I-CeuI* fragments on this map. The preliminary genetic map defines the location of six loci for degradative enzymes previously characterized from JMP636, while the locations of the 10 sets of ribosomal genes were assigned with less accuracy from hybridization data.

Keywords: *Aeromonas hydrophila*, extracellular enzymes, *rrn* operons, physical map, genetic map

INTRODUCTION

Traditionally, the genus *Aeromonas* has been grouped in the family *Vibrionaceae* on the basis of morphological and biochemical characteristics (Farmer, 1992). While this classification has been widely accepted, it has recently been determined that the evolution of the aeromonads is not adequately reflected by differentiation based on such criteria. Using molecular genetic data, Colwell *et al.* (1986) noted that *Aeromonas* had diverged from the *Vibrionaceae* and proposed that the genus should be removed and itself elevated to family status. More recent phylogenetic studies, based mainly on 16S rRNA sequencing have also supported the proposal to recognize the aeromonads as a separate family (Kita-Tsukamoto *et al.*, 1993; Ruimy *et al.*,

1994). Depending on the characteristic used, various studies have determined that *Aeromonas* has diverged equally from both the *Enterobacteriaceae* and *Vibrionaceae*, or that it is more closely related to one or the other of these families (Farmer, 1992). Therefore, while the genus *Aeromonas* forms a well defined taxon (Martinez-Murcia *et al.*, 1992), its relationship to its closest relatives has not been adequately resolved.

The type species of this genus is *Aeromonas hydrophila*, which has received recent attention as a medically important bacterium. Among the clinical manifestations associated with *A. hydrophila* are soft tissue infections and bacteraemia, usually in immunocompromised patients (Jones & Wilcox, 1995). There is also mounting evidence of the role of *A. hydrophila* as a gastrointestinal pathogen with some individuals able to mount a specific antibody response against the organism with which they have been infected (Janda, 1991; Jones & Wilcox, 1995). As the pathogenesis of other intestinal organisms is

Abbreviations: MCS, multiple cloning site; TAFE, Transverse Alternating-Field Electrophoresis.

related to extracellular factors, the abundant extracellular proteins produced by *A. hydrophila* have been well researched as potential virulence factors. These include aerolysin, pili, proteases and the S-layer (for review see Pemberton *et al.*, 1997).

Despite numerous genes for such determinants having been characterized, their organization within the genome has not been determined. The extent of genome mapping in *A. hydrophila* is the construction of a genetic linkage map for strain JMP636, which demonstrated that most of the detected amino acid biosynthesis markers were clustered in one region of the chromosome (Gobius & Pemberton, 1986). Since this work was completed, the increased use of PFGE has enabled the rapid construction of maps based on physical distances with this approach becoming favoured for bacterial genome analysis (Fonstein & Haselkorn, 1995). Genome structure and organization have been studied for members of both the *Enterobacteriaceae* and the *Vibrionaceae* (Perkins *et al.*, 1993; Majumder *et al.*, 1996), the nearest relatives of the aeromonads. Therefore, the study of the *A. hydrophila* genome would increase the knowledge of this medically important bacterium and provide further information on the molecular evolution of the genus *Aeromonas*.

In this paper, we have constructed a physical map for the circular 4.5 Mb genome of *A. hydrophila* JMP636, which to our knowledge constitutes the first such report for a member of this genus. The locations of genes for degradative enzymes previously characterized from this organism were mapped at defined locations within specific macrorestriction fragments by using a suicide vector and their arrangement around the chromosome was determined to be random. Cleavage of the chromosome with *I-CeuI* indicated that 10 *rri* (23S) genes were present in *A. hydrophila* JMP636, which is in contrast to the seven copies previously detected in other Gram-negative enteric organisms.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used or constructed during this study are described in Table 1.

All organisms were routinely cultured in Z medium (Gobius & Pemberton, 1986). *Escherichia coli* was grown at 37 °C and *A. hydrophila* at 32 °C unless otherwise stated. MacConkey agar was purchased from Becton-Dickinson, while X-Gal medium was prepared as described in Sambrook *et al.* (1989). Antibiotics were incorporated in media at the following final concentrations when required ($\mu\text{g ml}^{-1}$): ampicillin, 100; rifampicin, 100; kanamycin, 50 or 100 for *E. coli* and *A. hydrophila*, respectively. Conjugational transfer of plasmid DNA between strains was carried out by an overnight mating at 32 °C (Ingham & Pemberton, 1995).

Preparation of chromosomal DNA plugs. Chromosomal DNA plugs were prepared from 5 ml exponential-phase cultures (OD_{590} 0.5–0.7; LKB Ultraspec Plus) by using the method of Murray *et al.* (1990) with minor modifications. Briefly, harvested cells were washed once in cold PIV buffer (10 mM

Tris/HCl, 1.2 M NaCl, pH 7.6) prior to embedding in 1.6% low-melting-point agarose (Beckman pulsed-field-certified) with further manipulation according to the published procedure. DNA samples containing chromosomes which had been aligned at the origin of replication to ensure all parts of the genome were equally represented (Ohki & Smith, 1989) were prepared from exponential-phase cultures treated with 150 μg chloramphenicol ml^{-1} and grown for a further hour prior to harvesting.

PFGE. Chromosomal DNA plugs were washed twice for 30 min on ice in buffer (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.6) prior to digestion with *PmeI* or *PacI* (New England Biolabs) according to the manufacturer's instructions. Digestion with *I-CeuI* was performed according to the conditions described by Liu *et al.* (1993). The Beckman GeneLine II Transverse Alternating-Field Electrophoresis (TAFE) system was used for all electrophoretic runs with the varying pulse times used throughout this study selected from the manufacturer's recommendations. Optimum separation of a *PmeI* digest (bands *PmeA*–*PmeM*) was obtained using the following programme: 45 s pulse for 12 h at 370 mV; 60 s pulse for 6 h at 390 mV; 45 s pulse for 6 h at 390 mV. Fragments of a *PacI* digest (bands *PacA*–*PacS*) were routinely separated using a three-stage programme: 15 s pulse for 12 h at 350 mV; 30 s pulse for 12 h at 350 mV; 45 s pulse for 12 h at 370 mV. Optimum separation of an *I-CeuI* digest (bands *CeuA*–*CeuL*) was obtained using the following four-stage programme set at 350 mV: 8 s pulse for 12 h; 15 s pulse for 12 h; 30 s pulse for 8 h; 45 s pulse for 8 h. For all restriction digests, bands containing two DNA fragments of the same size were differentiated using a designation of '1' or '2', e.g. *PmeI*₁ and *PmeI*₂. Agarose gels (1% Bio-Rad pulsed-field-certified) were cast and run in TAFE buffer (45 mM Tris/borate, 1 mM EDTA, pH 8.3) at 13 °C. Gels were electrophoresed for 2 h prior to loading the DNA samples to reduce background (Maslow *et al.*, 1994). λ concatamers were used as the standard marker in most experiments (New England Biolabs). *HindIII*-cleaved λ DNA was used to estimate the sizes of smaller fragments. DNA was visualized following staining of gels in TAFE buffer containing 0.5 μg ethidium bromide ml^{-1} for 30 min followed by overnight destaining in distilled water at 4 °C. When DNA was to be isolated following TAFE runs, gels were cast using 1.2% low-melting-point agarose (Sigma type VII) and run without pre-electrophoresis, enabling DNA bands to be recovered by using agarase (Boehringer Mannheim).

Southern hybridization. DNA was immobilized on Hybond-N membrane (Amersham) by using conventional Southern transfer (Sambrook *et al.*, 1989) with membranes for hybridization usually containing one lane of a TAFE gel (1 × 15 cm strips). Probe DNA was labelled with biotin-14-dATP by using a nick-translation system according to the manufacturer's instructions (BRL). Hybridizations were performed at 65 °C and were followed by post-hybridization washes under stringent conditions. Visualization of positive signals used streptavidin-alkaline phosphate conjugate activity (BRL) on nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega).

DNA manipulations. Restriction enzymes, T4 DNA ligase and T4 DNA polymerase were purchased from Promega. All manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). Plasmids were extracted from *E. coli* by using alkaline lysis (Birnboim & Doly, 1979), while transformation was performed employing the one-step procedure of Chung *et al.* (1989). Isolation of DNA bands from

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
<i>A. hydrophila</i>		
JMP636	Wild-type	Gobius & Pemberton (1986)
JMP5520	Rf ^r mutant	This study
JMP5521	Rf ^r Km ^r ; JMP636::pJP9541	This study
JMP5522	Rf ^r Km ^r ; JMP636::pJP9542	This study
JMP5523	Rf ^r Km ^r ; JMP636::pJP9543	This study
JMP5524	Rf ^r Km ^r ; JMP636::pJP9544	This study
JMP5525	Rf ^r Km ^r ; JMP636::pJP9545	This study
JMP5526	Rf ^r Km ^r ; JMP636::pJP9546	This study
<i>E. coli</i>		
JM109 λ pir	JM109 (<i>recA1 endA1 hsdR17</i>) lysogenized with phage λ pir	Penfold & Pemberton (1992)
S17-1 λ pir	S17-1 (Pro ⁻ Res ⁻ Mod ⁺ ; integrated plasmid RP4-Tc::Mu-Kan::Tn7) lysogenized with λ pir	Herrero <i>et al.</i> (1990)
Plasmids		
pNEB193	Ap ^r , <i>lacZ'</i> ; pUC19-based vector with expanded MCS	New England Biolabs
pJP2529	Ap ^r ; 2.2 kb <i>chiA</i> insert in pUC19	Roffey & Pemberton (1990)
pJP3103	Ap ^r ; 2.1 kb <i>amyA</i> insert in pUC12	Gobius & Pemberton (1988)
pJP3201	Ap ^r ; 3.4 kb <i>amyB</i> insert in pUC18	Kidd (1998)
pJP5602	Km ^r ; <i>oriR6K</i> cloned to Tn5 <i>kan mobRP4</i>	Penfold (1994)
pJP9021	Ap ^r ; 3.4 kb <i>apl-1</i> insert in pUC18	Ingham & Pemberton (1995)
pJP9501	Ap ^r ; 4.2 kb <i>dnsH</i> insert in pUC19	H. N. Dodd & J. M. Pemberton, unpublished
pJP9521	Ap ^r ; 3.6 kb <i>nucH</i> insert in pUC19	Dodd & Pemberton (1996)
pJP9540	Km ^r ; <i>oriR6K mobRP4 lacZ'</i> ; MCS from pNEB193	This study
pJP9541	Km ^r ; 2.1 kb <i>SmaI</i> fragment from pJP3103 containing <i>amyA</i> in pJP9540	This study
pJP9542	Km ^r ; 2.2 kb <i>BamHI</i> fragment from pJP2529 containing partial <i>chiA</i> in pJP9540	This study
pJP9543	Km ^r ; 2.0 kb <i>BamHI</i> fragment from pJP9021 containing partial <i>apl-1</i> in pJP9540	This study
pJP9544	Km ^r ; 2.0 kb <i>SacI</i> fragment from pJP3201 containing partial <i>amyB</i> in pJP9540	This study
pJP9545	Km ^r ; 1.8 kb <i>SacI</i> fragment from pJP9521 containing partial <i>nucH</i> in pJP9540	This study
pJP9546	Km ^r ; 4.2 kb <i>KpnI</i> fragment from pJP9501 containing <i>dnsH</i> in pJP9540	This study

* Ap, ampicillin; Km, kanamycin; Rf, rifampicin.

agarose gels was performed using a Prep-a-Gene kit as per the manufacturer's instructions (Bio-Rad), while genomic DNA was prepared as previously described (Dodd & Pemberton, 1996).

Construction of a vector for genetic mapping. The vector pJP5603 has previously provided an efficient means of recombination into the *A. hydrophila* JMP636 chromosome (Ingham & Pemberton, 1995; Dodd & Pemberton, 1996) and was used to construct a genetic mapping vector for use in this organism. This was achieved by substitution of the multiple cloning site (MCS) region of pNEB193 (containing restriction sites for the rare-cutting enzymes *PacI*, *PmeI* and *AsclI*) for that of the pUC19 MCS present in pJP5603. pNEB193 was cleaved with *HaeII* to release a 460 bp fragment containing *lacZ'* and the MCS region. The protruding termini were blunt-ended using T4 DNA polymerase and the DNA sample was ligated to *SmaI*-cleaved pJP5602 (the precursor vector of pJP5603). Transformation into the host strain, JM109 λ pir, was per-

formed, with selection on X-Gal medium supplemented with kanamycin. Blue recombinants, which had acquired a functional β -galactosidase through α -complementation, and hence the *lacZ'* region, were screened by antibiotic susceptibilities and restriction enzyme profiles for the desired construct. One clone which could be linearized by *PmeI*, confirming the presence of the pNEB193 MCS, was retained and designated pJP9540.

Insertion of pJP9540 in the chromosome produced additional restriction fragments which were designated as 'I' for the smaller of the resultant bands and 'II' for the larger. In the case of *PmeC*, which was disrupted in two separate insertions, the four subfragments were designated *PmeC'* (100 kb), *PmeC^{II}* (150 kb), *PmeC^{III}* (430 kb) and *PmeC^{IV}* (480 kb).

DNA Amplification by PCR. The conditions of Giovannoni (1991) with an annealing temperature of 55 °C were used for the amplification of an *rrs* (16S) probe from *A. hydrophila* JMP636 by using the universal primers 27f [5' AGAGTT-

TGATC(A/C)TGGCTCAG 3'] and 1525r [5' AAGGAGGT-G(A/T)TCCA(A/G)CC 3'] (Lane, 1991).

RESULTS

The genome of *A. hydrophila* consists of a single chromosome

Electrophoresis of an intact *A. hydrophila* JMP636 genomic DNA plug produced a barely visible band at the limit of resolution of the gel which has previously been attributed to random double-stranded breakage of some circular DNA molecules during plug preparation (Jumas-Bilak *et al.*, 1995). However, the treatment of samples with S1 nuclease prior to electrophoresis (Barton *et al.*, 1995) produced a DNA band of increased intensity; a result consistent with a conversion from a circular to a linear form (not shown). Plasmids were not detected by either PFGE or alkaline lysis extraction, indicating that it is likely that the *A. hydrophila* JMP636 genome is a single circular chromosome.

A. hydrophila has a 4.5 Mb genome

As the genus *Aeromonas* has a high G+C content (Farmer, 1992), the AT-rich 8 bp recognition site endonucleases *PacI* and *PmeI* were expected to cut the chromosome relatively infrequently (McClelland *et al.*, 1987). Cleavage of JMP636 DNA with *PmeI* produced 13 distinct bands on electrophoresis (Fig. 1a and Fig. 3), ranging in size from 840 to 10 kb. *PmeI* was determined to be a doublet following recombination experiments (see below), giving a total of 14 *Pme* fragments. *PacI* cleavage of JMP636 produced 19 distinct bands on electrophoresis (Fig. 1b), ranging in size from 670 to 15 kb. Four bands, *PacF*, *M*, *Q* and *R*, were determined to be doublets from subsequent experiments, giving a total of 23 *Pac* fragments. The size of the JMP636 genome was calculated from the sum of the individual fragment sizes, with a minimum of three separate determinations performed for each DNA fragment. The mean size of each *Pac* and *Pme* band and calculated genome size are given in Table 2. These data indicate the genome size of *A. hydrophila* JMP636 is approximately 4.5 Mb.

Ten *rrl* (23S) genes as assessed by I-CeuI cleavage

I-CeuI is specific for cleavage in the *rrl* (23S) gene and has been used in the enumeration of *rrn* operons from a range of Gram-negative as well as Gram-positive organisms (Liu *et al.*, 1993; Toda & Itaya, 1995). Cleavage of the JMP636 chromosome with I-CeuI and electrophoresis under optimal conditions resolved nine distinct bands (Fig. 1c). The second smallest band was determined to be a doublet following the discrimination of two distinct fragments by using a TAFE programme targeted for separation in this region (not shown), confirming the presence of 10 I-CeuI cleavage sites.

The sizes of all but the largest of the nine well resolved bands were determined from three separate gels in

comparison to λ markers (Table 2). An attempt to size CeuA was made by comparing I-CeuI-cleaved JMP636 DNA to *Saccharomyces cerevisiae* chromosomes (separated according to conditions specified by Beckman) and this band found to be larger than the 2.2 Mb chromosome. The size of CeuA was therefore estimated as 2.9 Mb from the difference between the calculated genome size and the sum of the smaller Ceu fragments.

As there are only seven *rrn* operons in other related Gram-negative organisms (Liu *et al.*, 1993), a conventional ribotyping procedure was also used to confirm these data. A probe for the *rrs* (16S) gene was generated using PCR with extracted chromosomal DNA as template and universal primers under stringent conditions and a single expected fragment of 1.5 kb was produced. Extracted genomic DNA from JMP636 was cleaved with restriction enzymes which did not cut within the probe region and, following hybridization with the *rrs* (16S) gene, *SalI* and *SphI* were found to produce the best separation of positive bands among the enzymes tested. Only eight distinct bands were evident in each case; however, the two marked bands in each lane in Fig. 2 are of greater intensity and could conceivably contain two copies of the *rrs* (16S) gene. In addition, hybridization of this *rrs* (16S) probe against an I-CeuI digest demonstrated that all fragments were detected, with CeuH having an increased signal intensity (not shown). It is likely from these results that *A. hydrophila* JMP636 contains 10 *rrn* operons.

Preliminary physical map as determined by cross-hybridization

Cross-hybridizations between *Pme* and *Pac* fragments were used as the first step towards physical map construction for JMP636. *Pme* probes (bands A–M) and *Pac* probes (bands A–S) were isolated from low-melting-point gels and hybridized against heterologous restriction digests. These data are presented in Table 3. Examples of hybridizations against *PmeI*- and *PacI*-digested DNA are shown in Fig. 1(a) and (b), respectively. Ceu fragments B–I were also isolated from low-melting-point gels and were used in hybridizations against both *PacI*- and *PmeI*-digested DNA (Table 3). In this study, CeuA did not migrate well in TAFE gels under the electrophoresis conditions employed (possibly due to trapping related to its large molecular size; Gardiner, 1992) and could not be isolated in a sufficiently concentrated solution to be used as a probe. Cross-hybridizations against I-CeuI digests were also performed using only the *Pme* and *Pac* DNA bands which had previously returned a positive signal. Examples of hybridizations against I-CeuI-cleaved DNA are shown in Fig. 1(c). In addition, hybridization of the *rrs* (16S) probe against *PacI* and *PmeI* digests determined that *PmeA*, *B*, *C* and *F*, and *PacA*, *B*, *F* and *I* contained *rrn* genes.

Hybridizations with partial digestion fragments were used to determine the linkages of some *Pme* fragments. JMP636 was only partially cleaved with *PmeI* when

Fig. 1. Cleavage of *A. hydrophila* JMP636 DNA with restriction enzymes and examples of hybridization against cleaved DNA. (a) *PmeI* cleavage. Lanes: 1, *PmeI*-cleaved JMP636 DNA; 2, hybridization with PacN probe; 3, hybridization with PacO probe. (b) *PacI* cleavage. Lanes: 1, *PacI*-cleaved JMP636 DNA; 2, hybridization with 240 kb *PmeA'* probe from JMP5523; 3, hybridization with 260 kb *PmeB'* probe from JMP5526. (c) *I-CeuI* cleavage. Lanes: 1, *I-CeuI*-cleaved JMP636 DNA; 2, hybridization with *PmeI* probe; 3, hybridization with *PmeL* probe.

Table 2. Restriction fragments and calculation of *A. hydrophila* JMP636 genome size

Band	Size of band in restriction digest (kb)		
	<i>PacI</i>	<i>PmeI</i>	<i>I-CeuI</i>
A	670	840	> 2200
B	480	720	430
C	440	580	400
D	380	530	220
E	300	485	155
F	270 + 270	440	135
G	250	300	120
H	240	180	80 + 80
I	190	160 + 160	40
J	170	45	
K	160	30	
L	130	25	
M	115 + 115	10	
N	85		
O	80		
P	60		
Q	50 + 50		
R	30 + 30		
S	15		
Size of genome	4580	4505	ND

ND, Not determined.

Fig. 2. Southern hybridization of *rrs* (16S) probe against JMP636 DNA. Lanes 1–3 are restriction digests; 4–6 are hybridizations. Lanes: 1 and 4, *HindIII*-digested λ -DNA; 2 and 5, *SphI* digest; 3 and 6, *SalI* digest. Bands of increased intensity in lanes 5 and 6 are marked.

Mapping of homologous genes through use of insertion of new rare sites

Six genes encoding degradative enzymes have previously been cloned and characterized from *A. hydrophila* JMP636: *amyA* for amylase (Gobius & Pemberton, 1988), *chiA* for chitinase (Roffey & Pemberton, 1990), *apl-1* for phospholipase (Ingham & Pemberton, 1995), *nucH* for nuclease (Dodd & Pemberton, 1996), *amyB* for amylase (Kidd, 1998) and *dnsH* for nuclease (H. N. Dodd & J. M. Pemberton, unpublished). Sequencing upstream of the *dnsH* gene in pJP9501 has additionally detected homologues of the *E. coli* genes for transketolase (*tkt*) (GenBank L78439) and *S*-adenosyl-methionine synthetase (*metX*) (GenBank L78303). The

older stocks of enzyme were used, producing two additional visual bands of 600 and 460 kb which could be isolated from a TAFE gel (not shown). The larger of the partial products was found to hybridize to *PmeC* and *K*, while the smaller probe hybridized to *PmeF*, *G*, *I* and *L* and is likely to represent two separate linkages, *PmeF*–*L* (440 and 25 kb) and *PmeG*–*I* (300 and 160 kb).

Table 3. Cross-hybridization experiments

Pac probe	Positive bands		Pme probe	Positive bands		CeU probe	Positive bands	
	Pme	CeU		Pac	CeU		Pme	Pac
A	A, C, I, J	C, D	A	A, F, I	C, D, F, G, H, I	A	ND	ND
B	B, F, L, M	B, E	B	B, K, L, M, P, R	A, E	B	F	B, I, O
C	E, G	ND	C	A, D, M, R, S	C	C	A, C, I, J	A
D	C	ND	D	E, H, J	ND	D	A	A, F
E	D	ND	E	C, G, Q	ND	E	B, F, L, M	B
F	A	D, F, G, H, I	F	B, I, O	B, E	F	A	F
G	E, H	ND	G	C	ND	G	A	F
H	D, H	ND	H	G, H	ND	H	A	F
I	A, F	B, G	I	A, M, N, Q	A, C	I	A	F
J	D	ND	J	A	C			
K	B	ND	K	M	ND			
L	B	ND	L	B	E			
M	B, C, I, K	ND	M	B	E			
N	I	ND						
O	F	B						
P	B	ND						
Q	E, I	ND						
R	B, C	ND						
S	C	ND						

ND, Not determined.

Table 4. Band polymorphisms introduced by the insertion of pJP9540 vectors

Strain and gene	<i>PmeI</i> cleavage		<i>PacI</i> cleavage	
	Wild-type band	Resultant bands (kb)	Wild-type band	Resultant bands (kb)
JMP5521 (<i>amyA</i>)	C	480, 100	S	20
JMP5522 (<i>chiA</i>)	C	430, 150	D	280, 100
JMP5523 (<i>apl-1</i>)	A	600, 240	F	160, 110
JMP5524 (<i>amyB</i>)	I	120, 40	A	580, 100
JMP5525 (<i>nucH</i>)	D	No obvious polymorphism	E	280, 20
JMP5526 (<i>dnsH</i>)	B	460, 260	K	140, 20

construction of recombinant pJP9540 plasmids containing these genetic markers is outlined in Table 1. Plasmids pJP9541 to pJP9546 were separately transformed into the host S17-1 λ pir and the resultant strains were mated overnight with rifampicin-resistant JMP636 (JMP5520). Exconjugants were selected on medium containing kanamycin and rifampicin, with one exconjugant retained from each mating and designated as follows for each marker: JMP5521 (*amyA*), JMP5522 (*chiA*), JMP5523 (*apl-1*), JMP5524 (*amyB*), JMP5525 (*nucH*) and JMP5526 (*dnsH*). Each of these mutant strains was grown to exponential phase under antibiotic selective pressure and intact genomic DNA was prepared. DNA plugs were digested individually with *PacI*

and *PmeI* and electrophoresed using optimal conditions for each enzyme. The detected band polymorphisms are given in Table 4 and altered Pme banding patterns are shown in Fig. 3.

Construction of the Pme/Pac/Ceumap

A physical map was constructed using hybridization data and the insertion points of pJP9540-based vectors to anchor Pme and Pac fragments and determine the amount of overlap between them. To resolve the location of Pac fragments within PmeB, a *PacI* digest was hybridized with the two PmeB subfragments. The 260 kb PmeB^I probe linked PacK and M (Fig. 1b) and the

Fig. 3. *PmeI* digests of strains produced by the insertion of pJP9540-based vectors. Lanes: 1, λ concatamers; 2, JMP636; 3, JMP5521; 4, JMP5522; 5, JMP5523; 6, JMP5524; 7, JMP5525; 8, JMP5526. The variations in banding patterns from JMP636 to insertion mutant strains are marked.

Fig. 4. Physical and preliminary genetic map of *A. hydrophila* JMP636. The positions of PacP, R₁ and L, and PacN and Q₂ have been arbitrarily assigned within PmeB and PmeI₂, respectively. The locations of degradative enzyme loci have been determined with respect to Pme and Pac fragments, while the locations of *rrn* operons have been assigned on the basis of hybridization studies.

460 kb PmeB^{II} probe hybridized to PacB, L, P and R. The association of some bands within the large PmeA fragment was determined using the 240 kb PmeA^I band as probe, hybridizing to PacF and I (Fig. 1b) and CeuG, H and I. Distinction between Ceu fragments was attained using *apl-1* as probe with the gene located in a CeuH fragment (designated CeuH₁). Insertion of pJP9543 in this region of the chromosome also enabled separation of the PacF doublet, with the intact PacF band of JMP5523 (designated PacF₂) hybridizing to CeuD, F and H. CeuF was also used in hybridizations and found to link both PacF fragments by hybridizing to PacF₂ and the 110 kb PacF₁ band in JMP5523. The PmeI doublet was separated on insertion of pJP9544, with the intact PmeI band (designated PmeI₂) found to hybridize to PacM, N and Q. To further resolve the arrangement of fragments in this region of the chromosome, PmeJ was hybridized against *PacI*-cleaved JMP5524 and the 580 kb PacA^{II} fragment returned a positive signal. As both *amyA* and *chiA* were located on PmeC, hybridization of *chiA* against JMP5521 DNA and *amyA* against JMP5522 DNA (both cleaved with *PmeI*) was used to determine that these loci were approximately 330 kb apart. The 100 kb PmeC^I and 150 kb PmeC^{II} fragments were hybridized against *PacI*-cleaved DNA, detecting PacM and R, and PacA and D, respectively. The additional data enabled the order of Pme fragments to be determined as B-M-L-F-A-J-I₁-C-K-I₂-G-E-H-D and Pac fragments as M₁-K-(L,P,R₁)-B-O-I-F₁-F₂-A-D-S-R₂-M₂-(N,Q₁)-C-Q₂-G-H-J-E. The relative order of PacL, P and R₁, and of PacN and Q₁ could not be ascertained in this study. As insertion of pJP9545 occurred close to one end of PacE but produced no detectable polymorphism

in a *PmeI* digest, *nucH* was used as probe against *PmeI*-cleaved JMP5525 DNA. PmeD and a band of approximately 10 kb were detected. This indicates that a small region of overlap exists in this region of the chromosome which was not detected through hybridization and the physical map was drawn as circular to reflect the determined genome topology. A contig of Ceu fragments was determined as CeuE-B-G-I-H₁-F-H₂-C-D from hybridization data and is placed on the Pac/Pme map with slight overlap between PmeC and CeuC. The final Pme/Pac/Ceu physical and preliminary genetic map of the *A. hydrophila* JMP636 chromosome is shown in Fig. 4.

DISCUSSION

The study of the *A. hydrophila* JMP636 genome has provided an initial indication of genome structure and organization of this organism, a single, circular chromosome being predicted. This confirms the result obtained from previous genetic linkage studies (Gobius & Pemberton, 1986) and is in agreement with the genome topology determined for the nearest relatives of *A. hydrophila* and indeed most eubacteria (Perkins *et al.*, 1993; Majumder *et al.*, 1996). Prokaryotic genomes range from 600 kb in *Mycoplasma genitalium* to 9450 kb in *Myxococcus xanthus* (Cole & Saint Girons, 1994), the 4.5 Mb genome of JMP636 thus being in the middle of this observed range. Among the nearest relatives of *A. hydrophila*, most laboratory strains of *E. coli* have 4.5–4.7 Mb genomes, while environmental strains have genomes ranging from 4.6–5.3 Mb (Bergthorsson & Ochman, 1995). *Vibrio cholerae* strains have signifi-

cantly smaller genomes which range from 2.5 to 3.2 Mb (Roychoudhury *et al.*, 1994). The result obtained for JMP636 indicates that *A. hydrophila* has a genome size more similar to *E. coli* than *V. cholerae*.

Cleavage of *rrn* operons in eubacteria by I-CeuI occurs at a site within the *rrl* (23S) gene (Liu *et al.*, 1993). From examination of compiled sequence data, the 26 bp cleavage site occurs in a conserved region of this gene (Ludwig *et al.*, 1995) and has previously been detected in *A. hydrophila* (East & Collins, 1993). Although the *rrl* (23S) sequence has not been determined for JMP636, previous studies on 16S rDNA sequences in *Aeromonas* indicate that very few nucleotide differences are present even among different species (Martinez-Murcia *et al.*, 1992), supporting the notion that the I-CeuI cleavage site is conserved in JMP636. Related organisms *V. cholerae* and numerous members of the *Enterobacteriaceae* have been shown to possess seven *rrn* operons (Liu *et al.*, 1993; Majumder *et al.*, 1996). This has led to the hypothesis that in Gram-negative organisms, the number and location of *rrn* operons are conserved (Majumder *et al.*, 1996); however, the 10 sites detected in *A. hydrophila* JMP636 do not support this generalization.

Although some workers have reported variable restriction patterns with I-CeuI (Honeycutt *et al.*, 1993; Liu & Sanderson, 1995), reproducible results were obtained throughout this study. In addition, a ribotyping procedure was performed using an *rrs* (16S) rDNA probe in an attempt to independently confirm the result obtained by I-CeuI cleavage of JMP636. While only eight distinct bands were detected, it is believed that some contained more than one copy of the gene. A previous ribotyping study displayed results similar to those seen for JMP636, i.e. eight bands with two of obviously increased intensity and none smaller than the probe (Moyer *et al.*, 1992), supporting the notion that other aeromonads may have 10 *rrs* (16S) genes. Although differences in the number of *rrn* operons have been observed in the Gram-positive organisms *Streptomyces venezuelae* and *Bacillus cereus* (Johansen *et al.*, 1996; La Farina *et al.*, 1996), such variation has not been detected previously for Gram-negative enteric organisms.

While a correlation has been observed between the number of *rrn* operons in the genome of an organism and its generation time (Krawiec & Riley, 1990), the main advantage of additional *rrn* genes in *E. coli* has been shown to be a more rapid adaptation to changing environmental conditions (Condon *et al.*, 1995a). It is possible that given the ubiquity of *A. hydrophila* (Pemberton *et al.*, 1997), the increased number of *rrn* genes provides a selective advantage for this organism under various growth conditions. The clustering of *rrn* genes in one region of the genome is similar to that seen for *E. coli*, which also has its origin of replication in this region of the chromosome (Condon *et al.*, 1995b).

While PFGE has led to the rapid development of physical maps, the mapping of genetic loci has been achieved primarily through hybridization at fairly low resolution

(Fonstein & Haselkorn, 1995). The strategy of linking rare restriction sites to DNA insertion has been used previously for *E. coli* and *Lactococcus* strains to produce a much higher resolution for mapping of genes (Smith *et al.*, 1989; Le Bourgeois *et al.*, 1992). The vector pJP9540 constructed in this study has a number of advantages. In addition to the α -complementation cloning system available for this vector providing positive selection for DNA insertion, there are four restriction sites which can be used for megabase mapping (*AscI*, *PacI*, *PmeI* and *XbaI*) with cleavage sites of varying G+C content. Being based on *oriR6K*, pJP9540 can be expected to act as a suicide vector in most bacteria (Penfold & Pemberton, 1992). The Tn5 *kan* gene is a useful marker as it is widely expressed, but if required can also be replaced using the unique *BglII* and *NcoI* sites as done to produce the tetracycline-resistant vector pJP5608 (Penfold, 1994). By using both *PacI* and *PmeI* for physical map construction, insertion of pJP9540 was able to provide an anchor to determine the sizes of overlap between fragments generated with these two enzymes.

This study has produced, to our knowledge, the first reported physical map of an *Aeromonas* strain and has provided the necessary tools to enable further work on genome structure and organization in this organism to be performed. The suicide vector pJP9540 will enable mapping of genes which may be subsequently studied from JMP636. In addition, cosmid clones have previously been obtained from this organism (Gobius & Pemberton, 1988; Roffey & Pemberton, 1990) which may also be mapped to the chromosome, either by hybridization or by using pJP9540. The homology of regions of the *A. hydrophila* chromosome to previously characterized genes, as detected in the *dnsH* clone pJP9501, may mean that sequencing of random clones can also be used for further rapid genetic map expansion. The construction of a physical and preliminary genetic map of *A. hydrophila* JMP636 has provided an initial insight into the genome of this medically and ecologically important bacterium.

ACKNOWLEDGEMENTS

We gratefully acknowledge the initial screening of restriction enzymes for use with *A. hydrophila* by Dr Sarah Cherian, Dr Stephen Kidd for providing pJP3201 and Dr Mark Fegan for supplying PCR primers. H.N.D. was supported during this work as the recipient of an Australian Postgraduate Research Award.

REFERENCES

- Barton, B. M., Harding, G. P. & Zuccarelli, A. J. (1995). A general method for detecting and sizing large plasmids. *Anal Biochem* 226, 235–240.
- Bergthorsson, U. & Ochman, H. (1995). Heterogeneity of genome sizes among natural isolates of *Escherichia coli*. *J Bacteriol* 177, 5784–5789.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7, 1513–1523.

- Chung, C. T., Niemela, S. L. & Miller, R. H. (1989). One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci USA* 86, 2172–2175.
- Cole, S. T. & Saint Girons, I. (1994). Bacterial genomics. *FEMS Microbiol Rev* 14, 139–160.
- Colwell, R. R., MacDonell, M. T. & De Ley, J. (1986). Proposal to recognize the family *Aeromonadaceae* fam. nov. *Int J Syst Bacteriol* 36, 473–477.
- Condon, C., Liveris, D., Squires, C., Schwartz, I. & Squires, C. L. (1995a). rRNA operon multiplicity in *Escherichia coli* and the physiological implications of *rrn* inactivation. *J Bacteriol* 177, 4152–4156.
- Condon, C., Squires, C. & Squires, C. L. (1995b). Control of rRNA transcription in *Escherichia coli*. *Microbiol Rev* 59, 623–645.
- Dodd, H. N. & Pemberton, J. M. (1996). Cloning, sequencing and characterization of the *nucH* gene encoding an extracellular nuclease from *Aeromonas hydrophila* JMP636. *J Bacteriol* 178, 3926–3933.
- East, A. K. & Collins, M. D. (1993). Molecular characterization of DNA encoding 23S rRNA and 16S–23S rRNA intergenic spacer regions of *Aeromonas hydrophila*. *FEMS Microbiol Lett* 106, 129–134.
- Farmer, J. J., III (1992). The Family *Vibrionaceae*. In *The Prokaryotes*, 2nd edn, pp. 2939–2951. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Hardner & K.-H. Schleifer. New York: Springer.
- Fonstein, M. & Haselkorn, R. (1995). Physical mapping of bacterial genomes. *J Bacteriol* 177, 3361–3369.
- Gardiner, K. (1992). Transverse Alternating-Field Electrophoresis. In *Methods in Molecular Biology* Vol. 12: *Pulsed-field Gel Electrophoresis*, pp. 51–61. Edited by M. Burmeister & L. Ulanovsky. Totowa, NJ: Humana.
- Giovannoni, S. (1991). The polymerase chain reaction. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 177–203. Edited by E. Stackebrandt & M. Goodfellow. Chichester: John Wiley.
- Gobius, K. S. & Pemberton, J. M. (1986). Use of plasmid pULB113 (RP4::mini-Mu) to construct a genomic map of *Aeromonas hydrophila*. *Curr Microbiol* 13, 111–115.
- Gobius, K. S. & Pemberton, J. M. (1988). Molecular cloning, characterization, and nucleotide sequence of an extracellular amylase gene from *Aeromonas hydrophila*. *J Bacteriol* 170, 1325–1332.
- Herrero, M., deLorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* 172, 6557–6567.
- Honeycutt, R. J., McClelland, M. & Sobral, B. W. S. (1993). Physical map of the genome of *Rhizobium meliloti* 1021. *J Bacteriol* 175, 6945–6952.
- Ingham, A. B. & Pemberton, J. M. (1995). A lipase of *Aeromonas hydrophila* showing nonhaemolytic phospholipase C activity. *Curr Microbiol* 31, 28–33.
- 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.
- Johansen, T., Carlson, C. R. & Kolstø, A.-B. (1996). Variable numbers of rRNA gene operons in *Bacillus cereus* strains. *FEMS Microbiol Lett* 136, 325–328.
- Jones, B. L. & Wilcox, M. H. (1995). *Aeromonas* infections and their treatment. *J Antimicrob Chemother* 35, 453–461.
- Jumas-Bilak, E., Maugard, C., Michaux-Charachon, S., Allardet-Servent, A., Perrin, A., O'Callaghan, D. & Ramuz, M. (1995). Study of the organization of the genomes of *Escherichia coli*, *Brucella melitensis* and *Agrobacterium tumefaciens* by insertion of a unique restriction site. *Microbiology* 141, 2425–2432.
- Kidd, S. P. (1998). *Characterisation of amylases of Aeromonas hydrophila JMP636 and their regulation by catabolite repression*. PhD thesis, University of Queensland.
- Kita-Tsukamoto, K., Oyaizu, H., Nanba, K. & Simidu, U. (1993). Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16S rRNA sequences. *Int J Syst Bacteriol* 43, 8–19.
- Krawiec, S. & Riley, M. (1990). Organization of the bacterial chromosome. *Microbiol Rev* 54, 502–539.
- La Farina, M., Stira, S., Mancuso, R. & Grisanti, C. (1996). Characterization of *Streptomyces venezuelae* ATCC 10595 rRNA gene clusters and cloning of *rrnA*. *J Bacteriol* 178, 1480–1483.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: John Wiley.
- Le Bourgeois, P., Lautier, M., Mata, M. & Ritzenthaler, P. (1992). New tools for the physical and genetic mapping of *Lactococcus* strains. *Gene* 111, 109–114.
- Liu, S.-L. & Sanderson, K. E. (1995). I-CeuI reveals conservation of the genome of independent strains of *Salmonella typhimurium*. *J Bacteriol* 177, 3355–3357.
- Liu, S.-L., Hessel, A. & Sanderson, K. E. (1993). Genomic mapping with I-CeuI, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. *Proc Natl Acad Sci USA* 90, 6874–6878.
- Ludwig, W., Rosselló-Mora, R., Aznar, R. & 14 other authors (1995). Comparative sequence analysis of 23S rRNA from *Proteobacteria*. *Syst Appl Microbiol* 18, 164–188.
- McClelland, M., Jones, R., Patel, Y. & Nelson, M. (1987). Restriction endonucleases for pulsed field mapping of bacterial genomes. *Nucleic Acids Res* 15, 5985–6005.
- Majumder, R., Sengupta, S., Khetawat, G., Bhadra, R. K., Roychoudhury, S. & Das, J. (1996). Physical map of the genome of *Vibrio cholerae* 569B and localization of genetic markers. *J Bacteriol* 178, 1105–1112.
- Martinez-Murcia, A. J., Benlloch, S. & Collins, M. D. (1992). Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA–DNA hybridizations. *Int J Syst Bacteriol* 42, 412–421.
- Maslow, J. N., Slutsky, A. M. & Arbeit, R. D. (1994). Application of pulsed-field electrophoresis to molecular epidemiology. In *Diagnostic Microbiology – Principles and Applications*, pp. 563–572. Edited by D. H. Persing, T. F. Smith, F. C. Tenover & T. J. White. Washington, DC: American Society for Microbiology.
- Moyer, N. P., Martinetti, G., Lüthy-Hottenstein, J. & Altwegg, M. (1992). Value of rRNA gene restriction patterns of *Aeromonas* spp. for epidemiological investigations. *Curr Microbiol* 24, 15–21.
- Murray, B. E., Singh, K. V., Heath, J. D., Sharma, B. R. & Weinstock, G. M. (1990). Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. *J Clin Microbiol* 28, 2059–2063.
- Ohki, M. & Smith, C. L. (1989). Tracking bacterial DNA replication forks *in vivo* by pulsed field gel electrophoresis. *Nucleic Acids Res* 17, 3479–3490.
- Pemberton, J. M., Kidd, S. P. & Schmidt, R. (1997). Secreted enzymes of *Aeromonas*. *FEMS Microbiol Lett* 152, 1–10.

- Penfold, R. J. (1994).** *Development of genetic tools and their use in the analysis of photosynthesis in Rhodobacter sphaeroides*. PhD thesis, University of Queensland.
- Penfold, R. J. & Pemberton, J. M. (1992).** An improved suicide vector for construction of chromosomal insertion mutations in bacteria. *Gene* **116**, 145–146.
- Perkins, J. D., Heath, J. D., Sharma, B. R. & Weinstock, G. M. (1993).** *Xba*I and *Bln*I genomic cleavage maps of *Escherichia coli* K-12 strain MG1655 and comparative analysis of other strains. *J Mol Biol* **232**, 419–445.
- Roffey, P. E. & Pemberton, J. M. (1990).** Cloning and expression of an *Aeromonas hydrophila* chitinase gene in *Escherichia coli*. *Curr Microbiol* **21**, 329–337.
- Roychoudhury, S., Bhadra, R. K. & Das, J. (1994).** Genome size and restriction fragment length polymorphism analysis of *Vibrio cholerae* strains belonging to different serovars and biotypes. *FEMS Microbiol Lett* **115**, 329–334.
- Ruimy, R., Breittmayer, V., Elbaze, P., Lafay, B., Boussemart, O., Gauthier, M. & Christen, R. (1994).** Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas* and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int J Syst Bacteriol* **44**, 416–426.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Smith, C. L., Condemine, G., Chang, S.-Y., McGary, E. & Chang, S. (1989).** Insertion of rare cutting sites nearby genes allows their rapid physical mapping: localization of the *E. coli* *map* locus. *Nucleic Acids Res* **17**, 817.
- Toda, T. & Itaya, M. (1995).** I-CeuI recognition sites in the *rrn* operons of the *Bacillus subtilis* 168 chromosome: inherent landmarks for genome analysis. *Microbiology* **141**, 1937–1945.
-

Received 27 May 1998; revised 11 August 1998; accepted 13 August 1998.