Physical map of the chromosome of *Aeromonas salmonicida* and genomic comparisons between *Aeromonas* strains

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I-CeuI and PmeI physical maps of the *Aeromonas salmonicida* A449 chromosome were constructed using PFGE. The circular chromosome of *A. salmonicida* A449 was estimated to be 4658±30 kb. The approximate location of several genes, including those encoding proteins implicated in virulence, were identified. The map showed that the known virulence-factor-encoding genes were not clustered. The I-CeuI genomic digestion fingerprints of several typical and atypical strains of *A. salmonicida* were compared. The results confirmed the homogeneity of typical strains, which provided further support for the clonality of the population structure of this group. Extensive diversity was observed in the I-CeuI digestion fingerprint of atypical strains, although a clonality was observed in the strains isolated from diseased goldfish. The results suggest that comparison of I-CeuI digestion fingerprints could be used as a powerful taxonomic tool to subdivide the atypical strains and also help clarify some of the current confusion associated with the taxonomy of the genus *Aeromonas*.

Keywords: *Aeromonas salmonicida*, physical map, genetic map, circular chromosome, genome comparison

INTRODUCTION

Aeromonas salmonicida is a fish-pathogenic bacterium. A number of virulence-implicated factors have been identified in *A. salmonicida*, such as the paracrystalline tetragonal surface protein array (A-layer), several proteases and haemolysins, a glycerophospholipid cholesterol acyl transferase and LPS (reviewed by Noonan & Trust, 1995a). Although the genes encoding some of these factors have been cloned, sequenced and characterized, nothing is known about their location or organization on the chromosome.

Strains of *A. salmonicida* are broadly grouped either as typical or atypical, depending on several genotypic and phenotypic properties (Austin & Austin, 1993; Belland & Trust, 1988). The typical strains cause the septicæmic disease furunculosis in salmonids and are reported to be

a homogeneous group (Austin & Austin, 1993; Belland & Trust, 1988). The nutritionally fastidious atypical strains cause different types of diseases in a variety of fish and are very heterogeneous. The heterogeneity of atypical strains has made the subclassification of this group difficult. Indeed the atypical *A. salmonicida* strains grouped under the subspecies *nova* have been reported to have potential for further subdivision (Belland & Trust, 1988). The taxonomy of the genus *Aeromonas* as a whole is riddled with significant confusions because of poor correlation between results obtained using different classification methods (Austin *et al.*, 1989; Wilcox *et al.*, 1992). Austin and co-workers have suggested that the problems inherent in the current classification methods need to be addressed before a definitive classification for the genus *Aeromonas* can be made (Austin & Austin, 1993). Hence the need for better classification tools for the genus cannot be over-emphasized.

PFGE, which allows the resolution of megabase-sized DNA molecules (Schwartz *et al.*, 1983), has made it possible to construct genome maps of numerous micro-organisms. Using PFGE technology, the physical maps of organisms such as *Escherichia coli* (Smith *et al.*,

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1987), several *Salmonella* species (Liu *et al.*, 1993a), *Streptomyces* species (Lin *et al.*, 1993) and *Borrelia burgdorferi* (Casjens & Huang, 1993) have been determined. These were constructed by ordering high molecular mass genomic DNA fragments obtained following digestion with rare cutting restriction enzymes and separation by PFGE.

In this study, we have used PFGE and Southern blotting analysis to determine the physical and genetic map of the *A. salmonicida* A449 chromosome. Genes of *A. salmonicida*, including those encoding proteins implicated in virulence, were localized on the map. The I-CeuI digestion fingerprints of several typical and atypical strains of *A. salmonicida*, including representative strains from other *Aeromonas* species, were compared. The use of the I-CeuI digestion fingerprint as a taxonomic tool for *Aeromonas* was also investigated.

METHODS

Bacterial strains, vectors and growth conditions. The strains and vectors used in this study are listed in Table 1. All strains were cultured on trypticase soy agar or broth (TSA or TSB; BBL). Atypical strains of *A. salmonicida* were cultured on TSA supplemented with 5% horse blood or in TSB supplemented with 10 µg haemin ml⁻¹. The *Aeromonas* strains were grown at 20 °C for 24–36 h while *E. coli* strains were grown at 37 °C for 18 h. Antibiotics were used at the following concentrations (µg ml⁻¹): ampicillin, 100; chloramphenicol, 180.

Preparation of intact genomic DNA. *Aeromonas* strains were grown in 10 ml TSB to an OD₆₀₀ of 45 using the red 66 filter of the Klett–Summerson Photoelectric Colorimeter, model 800-3, corresponding to approximately 1.5 × 10⁸ c.f.u. ml⁻¹ and a yield of 10 µg DNA (Smith *et al.*, 1988). Chloramphenicol was added and the culture was grown for a further 1 h and then chilled on ice for 10 min. The cells were harvested and washed in 10 ml Pett IV (10 mM Tris/HCl, pH 7.6, 1 M NaCl). The cells were thoroughly resuspended in 1.6 ml Pett IV and warmed to 37 °C. An equal volume of 1% (w/v) low-melting-point agarose (Bio-Rad) warmed to 37 °C was added to the cells and the sample was mixed. The mixture was dispensed into 1 ml syringes and allowed to solidify at room temperature for at least 2 h. The agarose moulds containing the cells were pushed out of the syringes approximately 50 µl at a time and each small cylinder (insert) was sliced off with a scalpel. The inserts were placed into 6 ml EC lysis solution (6 mM Tris/HCl, pH 7.6, 1 M NaCl, 100 mM EDTA, pH 8.0, 0.5% Brij-58, 0.2% deoxycholate, 0.5% Sarkosyl) containing 1 mg lysozyme ml⁻¹ and 20 µg RNase ml⁻¹ and incubated at 37 °C overnight with shaking. The EC solution was replaced with 6 ml ESP solution (0.5 M EDTA, pH 9–9.5, 1%, w/v, lauryl sarcosine, 1 mg proteinase K ml⁻¹) and the sample was incubated at 50 °C for 48 h with shaking. The ESP solution was removed and the inserts were first incubated twice in 10 ml TE (10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA) containing 1 mM PMSF for 2 h and then three times in 10 ml TE for 2 h without PMSF. The inserts were stored in TE at 4 °C until use.

Restriction enzyme digestion and end-labelling of I-CeuI fragments. For complete DNA digestion with I-CeuI and PmeI, each insert was first incubated in 100 µl 2 × NEB buffer (New England Biolabs) for I-CeuI digestion or 2 × NEB buffer

4 for PmeI digestion for 15 min on ice. The solutions were replaced with 100 µl 1 × NEB buffer containing 2 U I-CeuI for I-CeuI digestion and 1 × NEB buffer 4 containing 5 U PmeI for PmeI digestion, followed by a 15 min incubation on ice. Complete digestion of A449 genomic DNA was carried out at 37 °C for 4 h. For PacI digestion, inserts were incubated twice in 100 µl 1 × NEB buffer 1 for 30 min on ice. A fresh 100 µl of 1 × NEB buffer 1 containing 10 U PacI was added to the insert and incubated at 4 °C overnight. Digestion was conducted at 37 °C for at least 4 h. For partial I-CeuI digestion of A449 genomic DNA, digestion was terminated after 40 min at 37 °C. After PFGE separation, the partially digested DNA fragments were excised from the gel under low wavelength UV light and then digested to completion with I-CeuI. After digestion, the DNA within the agarose pieces was end-labelled by blunt-ending the 5'-terminal I-CeuI overhangs using Klenow DNA polymerase and radioactive dNTPs. Each piece of agarose was first incubated in 100 µl 1 × Klenow buffer (New England Biolabs) on ice for 20 min. The buffer was replaced with a fresh 100 µl 1 × Klenow buffer containing 10 µCi (370 kBq) each of [α -³²P]dATP and [α -³²P]dTTP (Amersham), 2 mM dCTP, 2 mM dGTP and 5 U Klenow DNA polymerase and the sample was incubated on ice for 20 min. The end-labelling reaction was conducted at 18 °C for 2 h and the labelled DNA fragments were separated by PFGE. After separation, the gels were dried and exposed to X-OMAT AR film (Kodak).

PFGE conditions. DNA samples were analysed using the CHEF-DRRII PFGE system (Bio-Rad) connected to a model 1000 Mini chiller (Bio-Rad) to keep the buffer temperature at 14 °C. TBE buffer (0.5 × : 45 mM Tris/HCl, 45 mM borate, 1.0 mM EDTA, pH 8.3) and 0.7–1% agarose gels supplemented with 1.5 µg ethidium bromide ml⁻¹ were utilized in the separation of DNA. Using the long gel cast (21 × 14 cm; Bio-Rad), both PmeI and I-CeuI digests were separated using a pulse ramp of 10–80 s at 5.5 V cm⁻¹ for 53 h, unless otherwise specified. To separate PacI genomic DNA fragments, two windows of separation were utilized, first using a pulse ramp of 10–80 s at 5.5 V cm⁻¹ for 30 h and then using a pulse ramp of 5–10 s at 5.0 V cm⁻¹ for 30 h.

Southern blotting and DNA probes. Following PFGE, agarose gels were prepared as unblots (Tsao *et al.*, 1983). The gels were incubated twice for 30 min in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and twice for 30 min in neutralization solution (1.5 M NaCl, 1 M Tris/HCl, pH 8.0). After neutralization, the gels were dried under vacuum, first without heat for 30 min and then at 80 °C for 1 h. The dried gels (unblots) containing the DNA were subsequently probed in a Southern hybridization analysis as described by O'Toole *et al.* (1994). The DNA fragments utilized as probes in Southern blotting experiments were either cloned *A. salmonicida* genes, heterologous genes from *E. coli* or PCR fragments amplified using primers designed from published gene sequences of *A. salmonicida*. The PCR reactions were performed as previously described (Umelo & Trust, 1997). The amplified DNA fragments were confirmed by sequencing and then labelled for use as probes using standard methodology (Umelo & Trust, 1997).

Cloning of fragments from PmeI, PacI and I-CeuI genomic DNA digests. Specific A449 genomic DNA fragments obtained following either single or double digestion with I-CeuI, PacI and PmeI, were excised under low wavelength UV light. The pieces of agarose containing the DNA were individually incubated in 100 µl 1 × NEB buffer 3 twice on ice for 30 min. The solution was replaced with a fresh 100 µl of NEB buffer 3 containing 20 U each of PstI and BglII, incubated

Table 1. Bacterial strains and vector used in this study

Species	Strain	Source (other strain designations)
Typical <i>A. salmonicida</i>	A449	Brown trout, Eure, France
	A450	Brown trout, Tarn, France
	A488	Brook trout, USA
	A505	Salmonid fish, Japan
	A202	Salmonid fish, Japan
	A251	Atlantic salmon, UK (ex-NCMB 1102)
	A440	Brook trout, USA
	A438	British Columbia, Canada
	A447	Salmonid, Weymouth, UK
	A400	Goldfish, Boolara, Australia
Atypical <i>A. salmonicida</i>	A402	Goldfish, Boolara, Australia
	A419	Goldfish, Arkansas, USA
	A491	Goldfish, Maryland, USA
	A460	Atlantic salmon, Nova Scotia, Canada
	A461	Atlantic salmon, Nova Scotia, Canada
	A462	Atlantic salmon, Nova Scotia, Canada
	A477	European carp, Europe
	A480	European carp, The Netherlands
	A475	Norway
	A522	Herring, British Columbia, Canada
	A523	Herring, British Columbia, Canada
	A588	Atlantic cod, Nova Scotia, Canada
	A600	Shubunkin (Goldfish), Germany
	A601	Goldfish, Europe
	A602	Putative <i>Aeromonas</i> strain
<i>A. hydrophila</i>	TF7	Trout lesion, Quebec, Canada
	Ah300	Human diarrhoeal faeces
	Ah30	Salmonid fish, British Columbia, Canada
	Ah55	Salmonid fish, British Columbia, Canada
<i>A. sobria</i> biotype <i>veronii</i>	As701	Human septicaemia, USA
	As702	Human septicaemia, USA
<i>E. coli</i>	DH5 α	Hanahan (1983)
Vector	pBluescript	Stratagene

overnight at 4 °C and then at 37 °C for 16–24 h. After digestion, the agarose containing the DNA was broken into small pieces with a Pasteur pipette and *Pst*I/*Bam*HI-digested pBluescript was added to the sample. The DNA was extracted by phenol/chloroform extraction and precipitated by adding 0.1 vols 3 M sodium acetate and 2 vols 100 % ethanol followed by incubation at –70 °C for 1 h. The DNA was recovered by centrifugation, air-dried and resuspended in water. An aliquot of the DNA sample was ligated, electroporated into *E. coli* DH5 α and plated on selection medium. Cloned DNA fragments were used as probes in Southern blotting.

RESULTS

Estimation of chromosome size

The genomic DNA of *A. salmonicida* A449, with an estimated G + C content of 55 mol % (Belland & Trust, 1988), was digested with various enzymes to identify those which cleaved rarely. While the majority of the enzymes cleaved the A449 chromosome into too many fragments, two enzymes that produced a few fragments

suitable for size estimation were identified (Fig. 1). *I-Ceu*I, an intron-encoded enzyme which cleaves within the 23S rRNA-encoding gene (*rnl*) (Gauthier *et al.*, 1991; Liu *et al.*, 1993b), cleaved the A449 chromosome into nine fragments, the estimated sizes of which were 2800.00 $\pm$ 10.0, 515.25 $\pm$ 3.0, 436.50 $\pm$ 2.0, 238.06 $\pm$ 2.0, 186.50 $\pm$ 2.0, 178.50 $\pm$ 3.0, 130.94 $\pm$ 2.0, 76.50 $\pm$ 2.0 and 48.00 $\pm$ 3.0 kb. *Pme*I, which cleaves an AT-rich octanucleotide site, produced 11 fragments from A449, the estimated sizes of which were 876.00 $\pm$ 3.5, 766.00 $\pm$ 4.0, 710.81 $\pm$ 2.5, 670.00 $\pm$ 2.0, 525.50 $\pm$ 1.5, 460.75 $\pm$ 5.0, 315.60 $\pm$ 3.0, 160.75 $\pm$ 2.5, 144.00 $\pm$ 2.0, 68.00 $\pm$ 3.0 and 10.00 $\pm$ 2.0 kb. *Pac*I also cleaved an AT-rich octanucleotide site and produced at least 22 fragments from the A449 genomic DNA (Fig. 1). However, some fragments generated by *Pac*I digestion were used as tools to show the linkage between some *Pme*I- and *I-Ceu*I-derived fragments, as discussed below. By comparing the total fragment sizes obtained using *I-Ceu*I and *Pme*I, the mean size of the *A. salmonicida* A449 chromosome was estimated at 4658 $\pm$ 30 kb.

Construction of the I-CeuI physical map

The I-CeuI physical map of the *A. salmonicida* A449 chromosome was deduced using a combination of complete and partial digestion. Partial I-CeuI digestion of the A449 chromosome yielded the nine expected fragments, in addition to fragments corresponding to combinations of flanking fragments (Fig. 2). The partial fragments were isolated, digested to completion with I-CeuI and separated by PFGE adjacent to complete I-CeuI A449 genomic digests to aid identification of the constituent fragments. Since the resultant digests were often too faint to be visualized after ethidium bromide staining, the fragments obtained after complete digestion of partial fragments were end-labelled with radioactive nucleotides prior to being analysed by PFGE. Other I-CeuI-derived partial fragments obtained in this study include 3 + 5/6, 2 + 4 + 5/6 and 1 + 5/6 (data not shown). By comparison and alignment of the fragments obtained from complete digestion of the partials, the chromosomal order of the nine I-CeuI fragments was deduced as 1-5/6-3-7-9-8-5/6-4-2. Since Ceu5 and 6 migrated as a doublet under the separation conditions utilized in the partial digestion analysis, the identity of any fragment migrating at this size needed to be confirmed by a different approach.

Confirmation of the position of Ceu5 and 6 in the I-CeuI physical map

PacI-derived fragment 3 (Pac3) was used to confirm the position of Ceu5 and 6 in the deduced order of the *A. salmonicida* A449 I-CeuI-derived DNA fragments. Cleavage of Pac3 with I-CeuI yielded three subfragments (data not shown). One of the subfragments migrated at the same size as Ceu4, suggesting that the other two subfragments were part of the I-CeuI-derived fragments flanking Ceu4 on the A449 chromosome. DNA fragments unique to each of the three subfragments were cloned and utilized as probes on A449 I-CeuI genomic digests. The probes hybridized to Ceu2, 6 and 4. Thus, Pac3 revealed the order of the I-CeuI-derived fragments in that region of the chromosome to be 6-4-2. This suggested that Ceu5 was located at position 1-5-3 on the I-CeuI cleavage map of A449 chromosome. That Ceu5 was indeed located beside Ceu1, and Ceu6 beside Ceu4 was confirmed by the fact that digestion of Ceu6 with *PacI* yielded two subfragments, one of which corresponded to the same size as that obtained by the I-CeuI digestion of Pac3, while Ceu5 was not cleaved by *PacI* (data not shown). Therefore, the deduced order of the nine I-CeuI-derived fragments of *A. salmonicida* A449 chromosome was 1-5-3-7-9-8-6-4-2.

Circularization of the I-CeuI physical map

Because *B. burgdorferi* (Casjens & Huang, 1993) and several *Streptomyces* species (Lin *et al.*, 1993) have been shown to possess linear chromosomes, we sought to identify the conformation of the *A. salmonicida* A449 chromosome. Digestion of Pac2 with I-CeuI yielded two

Fig. 1. Genomic DNA of *A. salmonicida* A449 digested using *PacI* and I-CeuI (a), and *PmeI* and I-CeuI (b) separated on a 1% agarose gel. (a) Lanes: 1, λ concatamer; 2, *PacI* digest; 3, I-CeuI digest. (b) Lanes: 1, λ concatamer; 2, *PmeI* digest; 3, I-CeuI digest. The DNA in (a) was separated first using a pulse ramp of 5–10 s for 30 h at 5.0 V cm⁻¹ and then a 10–80 s pulse ramp for 30 h at 5.5 V cm⁻¹. The DNA in (b) was separated using a pulse ramp of 10–80 s at 5.5 V cm⁻¹ for 53 h.

Fig. 2. Partial I-CeuI digestion analysis of the genome of *A. salmonicida* A449. Lane 1, λ concatamer. In the lanes with the partial digests (2 and 3), digestion was terminated after 40 min and then analysed next to a complete digest (lane 4) conducted for 4 h. The partial fragments are indicated by arrows and the constituent I-CeuI fragments are indicated on the right. The fragments were separated using a pulse ramp of 5–50 s at 185 V cm⁻¹ for 20 h using a 0.7% agarose gel.

subfragments (data not shown). When DNA fragments cloned from each of the two Pac2 subfragments were used as probes on A449 I-CeuI digests, one hybridized to

Fig. 3. Physical and genetic map of the *A. salmonicida* A449 chromosome constructed using I-CeuI and PmeI. The approximate locations of some genes are shown. The PmeI map is represented by the inner circle and the I-CeuI map by the outer circle. The bars inside the inner circle represent the approximate positions of the PacI-derived DNA fragments used in linking the I-CeuI- and PmeI-derived fragments. The map was constructed using a combination of strategies such as partial and complete digestion analysis, alignment of fragments with common genes and use of fragments obtained with one enzyme as probes on the fragments obtained with the other enzymes.

Ceu1 while the other hybridized to Ceu2. This showed that *A. salmonicida* A449 possesses a circular chromosome and that Pac2 is located at the junction between Ceu1 and 2 (Fig. 3).

Determination of the PmeI physical map and its alignment to the I-CeuI map

The PmeI physical map was deduced and aligned to the I-CeuI map using several strategies. First, by comparing the I-CeuI- and PmeI-derived A449 genomic DNA fragments which had common genes (Table 2), 9 of the 11 PmeI fragments were aligned to their corresponding positions around the deduced I-CeuI physical map (Fig. 3). The alignment also gave a good indication of the PmeI fragments which were likely to be linked.

Isolation of Ceu1 and digestion with PmeI yielded six subfragments, four of which migrated at the size as Pme2, 5, 9 and 10, while the other two subfragments did not correspond to any PmeI-derived A449 genomic DNA fragments, suggesting that these two subfragments originated from the PmeI fragments located at the junctions of the I-CeuI, with one between Ceu1 and Ceu5 and the other between Ceu1 and Ceu2. Use of the two subfragments as probes on PmeI digests revealed they constituted part of Pme1 and 3.

To confirm the linkage between adjacent PmeI fragments, PacI-derived fragments which contained PmeI sites were utilized. Pac1, 2, 4, 5, 7 and 8/9 contained

PmeI sites and therefore could be used to link adjacent PmeI-derived fragments. When used as probe, Pac1 hybridized to Pme1 and 2, Pac2 linked Pme3 and 4, Pac4 and 5 linked Pme1, 11, 7 and 6, Pac7 linked Pme2 and 10, while Pac8/9 linked Pme10, 9 and 5. The PmeI fragment linkage order deduced using this approach was 6-7-11-1-2-10-9-5 and 3-4.

Finally, partial digestion analysis of *A. salmonicida* A449 with PmeI yielded several partial fragments, the size of two of which corresponded to Pme6 and 8, and Pme9 and 10. Thus, the PmeI cleavage map was shown to be 8-6-7-11-1-2-10-9-5. Since Pme8 hybridized to Ceu6 and Pme4 was localized to Ceu6, 4 and 2 by hybridization, this showed that the complete cleavage map of PmeI was 3-4-8-6-7-11-1-2-10-9-5. This accounted for the 11 PmeI fragments and the deduced linkage pattern was in agreement with the order predicted by comparison of PmeI and I-CeuI fragments with common genes. Since the *A. salmonicida* A449 chromosome has been deduced to be circular and the remaining linkage is localized within the single Ceu1 fragment, it follows that to complete the circle, Pme3 is linked to Pme5 (Fig. 3).

Mapping of virulence factor-encoding genes and others

Isolated genes of *A. salmonicida* were localized to the fragments generated by I-CeuI and PmeI by Southern blotting analysis. Radiolabelled λ DNA was included in the hybridization cocktail to aid in identifying the fragment to which the genes hybridized. Table 2 shows the genes analysed and the I-CeuI- and PmeI-derived A449 chromosomal fragments to which they localized. Since relatively few genes of *A. salmonicida* have been characterized, heterologous genes of *E. coli* were also used as probes to localize the homologous copies of these genes on the A449 chromosome. Using this approach, the PmeI- and I-CeuI-derived fragments of A449 which contained DNA homologous to the *E. coli* genes encoding UvrD helicase II, LytB and isoleucyl tRNA synthase were identified (Table 2).

Genomic comparison of *Aeromonas* strains

To examine the genomic relationship between typical and atypical strains of *A. salmonicida* isolated from diverse geographical origins and from different fish diseases, the genomic I-CeuI digestion fingerprints of nine typical and 16 atypical strains were compared (Fig. 4). In addition, the I-CeuI digestion fingerprints of four strains of *A. hydrophila* and two *A. sobria* biotype *veronii* strains were also compared to *A. salmonicida* strains. I-CeuI produced nine fragments with nearly identical digestion fingerprints from the genome of the typical strains examined (Fig. 4a). In contrast, extensive polymorphism was observed in the number and size of the fragments obtained following digestion of the genome of the atypical strains with I-CeuI (Fig. 4b and c, lanes 8–12), which is a good indication of significant genome diversity within this group. While nine of the atypical strains yielded nine fragments after I-CeuI

Table 2. Location of genes on I-CeuI- and PmeI-derived DNA fragments of the *A. salmonicida* A449 chromosome

I-CeuI	PmeI	Genotype	Phenotype	Reference
1	2	<i>flaA,B,G,H</i>	Flagellar proteins	Umelo & Trust (1997)
1	2	<i>aspA</i>	Serine protease	Whitby <i>et al.</i> (1992)
1	1	<i>asoA,B</i>	Secretion protein	Noonan & Trust (1996)
1	1	<i>lamB</i>	Maltose-inducible porin	Dodsworth <i>et al.</i> (1993)
1	10	<i>apsE</i>	A-protein specific secretion protein	Noonan & Trust (1995b)
1	10	<i>vapA</i>	A-protein subunit	Chu <i>et al.</i> (1991)
1	10	<i>abcA</i>	Transcriptional activator	Chu & Trust (1993)
1	5	<i>aroA</i>	5-Enolpyruvylshikimate-3-phosphate synthase	Vaughan <i>et al.</i> (1993)
2	4	<i>GCAT</i>	Glycerophospholipid-cholesterol acyltransferase	Nerland (1996)
2	4	<i>pilA,B,C,D</i>	Pilin subunit and prepilin peptidase	P. Lutwyche and others (unpublished data)
2	4	<i>recA</i>	RecA protein	Umelo <i>et al.</i> (1996)
2	4	<i>lytB</i>	Signals starvation stress	Gustafson <i>et al.</i> (1993)
2	4	<i>ileS</i>	Isoleucyl-tRNA synthase	Innis <i>et al.</i> (1984)
3	7	<i>exeD</i>	Secretion protein	Karlyshev & McIntyre (1995)
6	8	<i>ISAS1</i>	Insertion sequence	Gustafson <i>et al.</i> (1994)
7	6	<i>uvrD</i>	UvrD Helicase 11	This study
7	6	<i>pBSKpd</i>	Phosphogluconate dehydratase	This study
7	6	<i>pBSKrep</i>	Possible repressor	This study
Genes present on multiple fragments				
1	2, 5	<i>SsynA</i>	Siderophore synthase homologue	This study
1, 6, 8	2, 6, 8	<i>ISAS2</i>	Insertion sequence	Gustafson <i>et al.</i> (1994)
2, 3, 4, 5, 6, 7, 8, 9	1, 3, 4, 6	<i>rrs</i>	16S rRNA	Martinez-Murcia <i>et al.</i> (1992)
1, 2, 3, 4, 5, 6, 7, 8, 9	1, 3, 4, 6	<i>rrl</i>	23S rRNA	This study

digestion, A480 yielded 10 fragments, A600, A460, A461 and A462 yielded eight fragments, A475 yielded seven fragments and A602 yielded three fragments.

Like the typical strains of *A. salmonicida*, *A. sobria* biotype *veronii* genomic DNA was also cleaved into nine fragments and the two strains examined had an identical I-CeuI digestion fingerprint (Fig. 4c, lanes 2 and 3). There was also an observed similarity in size between the five smallest fragments of the *A. sobria* strains in comparison with the typical strains of *A. salmonicida*. In contrast, *A. hydrophila* strains showed polymorphism similar to that observed within the atypical strains of *A. salmonicida* (Fig. 4c, lanes 4–7). *A. hydrophila* strains Ah30 (lane 6) and Ah55 (lane 7) yielded 10 fragments upon digestion with I-CeuI, TF7 yielded eight fragments (lane 4) while Ah300 yielded seven fragments (lane 5). The I-CeuI digestion fingerprints of TF7 and Ah300 were practically identical, with the exception of the additional fragment produced from the TF7 genome.

DISCUSSION

This study presents the first chromosomal map of an *Aeromonas* species to be determined. The known genes which encode proteins implicated in the pathogenesis of

A. salmonicida A449, such as *aspA*, *aroA*, *vapA* and *GCAT*, are dispersed around the circular chromosome. The *exe* genes encoding proteins of the general secretory system and *apsE* encoding a protein involved in the specific transport of VapA, the subunit protein for the A-layer, are also far apart on the chromosome, showing a distinction in location in addition to function. While it was known that *abcA*, which encodes a protein involved in *vapA* regulation and LPS biosynthesis, was immediately downstream of *vapA* (Chu & Trust, 1993), the location of the *apsE* relative to the *vapA-abcA* loci was unknown. The map shows that *vapA*, *abcA* and *apsE* are clustered. The recently identified *fla* (Umelo & Trust, 1997) and pilin genes (P. Lutwyche and others, unpublished data) are widely separated on the *A. salmonicida* A449 chromosome. The two endogenous IS elements *ISAS1* and *ISAS2* of *A. salmonicida* (Gustafson *et al.*, 1994) are clustered with the exception that one of the three copies of *ISAS2* is located at a site distant from the others. While the copy numbers of *ISAS1* and *ISAS2* in the *A. salmonicida* chromosome were previously unknown, this study has revealed that there are at least three copies of *ISAS2* and possibly only one of *ISAS1*.

The attenuation of *A. salmonicida* by growth at high temperature was reported to be a result of insertion of *ISAS1* and *ISAS2* into *vapA* and its flanking DNA

Fig. 4. Comparison of the I-CeuI genomic digestion fingerprint of *Aeromonas* strains. (a) Typical *A. salmonicida* strains. Lanes: 1, A449; 2, A450; 3, A488; 4, A505; 5, A202; 6, A251; 7, A440; 8, A438; 9, A447. (b) Atypical *A. salmonicida* strains. Lanes: 1, A419; 2, A402; 3, A400; 4, A491; 5, A600; 6, A477; 7, A588; 8, A461; 9, A522; 10, A480; 11, A475. (c) Strains of other *Aeromonas* species. Lanes: 1, λ concatamer; 2 and 3, *A. sobria* biotype *veronii* strains As701 (2) and As702 (3); 4–7, *A. hydrophila* strains TF7 (4), Ah 300 (5), Ah30 (6) and Ah55 (7); 8–12, atypical strains of *A. salmonicida* A523 (8), A601 (9), A602 (10), A462 (11) and A460 (12); 13, λ concatamer. The DNA fragments were separated in a 1% agarose gel using a pulse ramp of 5–90 s at 185 V cm⁻¹ for 28 h.

the vicinity of *vapA*, two copies of *ISAS2* and the only copy of *ISAS1* are located approximately 2300 kb from *vapA*, yet they are both capable of translocating into *vapA* and its flanking DNA. This suggests interesting mechanisms for endogenous mutation.

The near constant I-CeuI digestion fingerprint of the typical strains of *A. salmonicida* analysed in this study confirms the homogeneity within this group as previously suggested (Belland & Trust, 1988; Boyd *et al.*, 1994; Nielson *et al.*, 1993). Because these typical strains were obtained from diverse geographical locations, these results provide further evidence that the genome of *A. salmonicida* is quite stable and also supports the suggestion that the population structure of typical strains is clonal (Boyd *et al.*, 1994). In contrast, the I-CeuI digestion fingerprints of the atypical *A. salmonicida* strains varied extensively, although there were clearly closer relationships between some strains than others. For example, *A. salmonicida* A400, A402, A419, A491, A600 and A601 which were all isolated from diseased goldfish from either Australia, USA or Europe had nine fragments in similar I-CeuI digestion fingerprints. These results therefore suggested a clonality for the goldfish isolates. These observations are in agreement with an earlier study by Belland & Trust (1988) which reported as much as $99 \pm 3.0\%$ homology between goldfish isolates from one location which had only an estimated $79 \pm 2.9\%$ identity with atypical strains isolated from other diseases and locations. From that study, Belland & Trust reported distinct groups corresponding to isolates from goldfish and carp. In this study, we examined two isolates from carp, A480 and A477. In contrast to the reported similarity between the carp isolates observed using the DNA–DNA hybridization technique, we did not observe a similarity between the I-CeuI digestion fingerprints of the two carp isolates examined. Not surprisingly, another group of strains which appeared to be clustered were A460, A461 and A462 which were all isolated from a single disease outbreak in Atlantic salmon in Nova Scotia, Canada.

The I-CeuI digestion fingerprints of the rest of the atypical strains examined in this study were highly variable and could not be grouped, suggesting they have diverged from other atypical strains. While firm conclusions cannot be made, considering the number of strains analysed in this study, the results suggest that comparison of the I-CeuI digestion fingerprints could help identify related strains and thus help to better classify and subdivide the atypical strains. By comparison of the I-CeuI digestion fingerprints, the *A. sobria* biotype *veronii* strains were more closely related to *A. salmonicida* strains than were the four *A. hydrophila* strains.

The variability in the number of fragments obtained from the I-CeuI digestion of atypical strains of *A. salmonicida* and the *A. hydrophila* strains was a surprising finding. Such a variation, to our knowledge, has not been reported for any other species. Liu *et al.* (1993c) reported that recombination events occurring

(Gustafson *et al.*, 1994). In this regard, it is of interest to note the chromosomal relationship between *vapA* and the IS elements. Although one copy of *ISAS2* is located in

within the *rrn* genes are common in bacteria and Sneath (1993) reported that genetic cross-overs have occurred between different *Aeromonas* species as a result of homologous recombination between *rrs* genes. It is possible that such recombination events, either within or between strains of different *Aeromonas* species, could lead to deletions or insertions, resulting in either an increase or decrease in the overall number of *rrn* operons. Differences in the number of *rrn* operons have been reported in two different *Rhodobacter* species. *Rhodobacter capsulatus* strain SB1003 possessed four *rrn* operons (Fonstein *et al.*, 1992) while *Rhodobacter sphaeroides* 2.4.1 possessed three *rrn* operons (Suwanto & Kaplan, 1989). However, there is generally an observed intraspecies conservation in genome backbone and *rrn* operons in species in which the genome maps of several strains have been constructed (reviewed by Cole & Saint Girons, 1994). It is therefore tempting to speculate that some strains currently classified as atypical *A. salmonicida* may not belong in this species. This could be a possible explanation for the difficulties which have been encountered in the classification of atypical *A. salmonicida* strains and other strains within the genus *Aeromonas*.

A. salmonicida contains several endogenous plasmids (Toranzo *et al.*, 1983). In *A. salmonicida* A449, three small plasmids of 5.0, 5.2 and 5.4 kb and a large plasmid of approximately 145 kb have been reported (Belland & Trust, 1989). The results obtained in this study show that these endogenous plasmids did not interfere in the linkage of both the *I-CeuI*- and *PmeI*-derived *A. salmonicida* A449 chromosomal DNA fragments. While the three small plasmids probably migrated off the gel under the conditions used in this study, the large plasmid might not. Indeed, a very faint band migrating at approximately 150 kb was sometimes seen in overloaded lanes containing *I-CeuI* digests and could be the linearized 145 kb plasmid of *A. salmonicida* A449. Neither of the genes used as probes in Southern analysis nor any of the fragments obtained during the *I-CeuI* partial digestion analysis hybridized to the faint apparent plasmid band.

The information provided in the map will be useful for the rational development of a live attenuated *A. salmonicida* vaccine strain. The map will also be useful for comparison of the chromosomal organization of *Aeromonas* species as more strain maps are determined. Finally, we have identified a tool that could have significant consequences in *Aeromonas* taxonomy, either in confirming the current classification of strains or in recognizing new isolates.

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