***Aeromonas salmonicida* Plasmids: Plasmid-directed Synthesis of Proteins *in vitro* and in *Escherichia coli* Minicells**

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Plasmid DNAs from 25 *Aeromonas salmonicida* strains (14 'typical' and 11 so-called 'atypical' strains) were analysed using two plasmid isolation techniques in conjunction with restriction enzyme digestion and agarose gel electrophoresis. 'Atypical' strains carried two to four plasmid species, which were different from the plasmids carried by 'typical' strains, and corresponded to isolate source and biotype, suggesting that plasmid content may be a useful epidemiological marker for 'atypical' *A. salmonicida*. The 'typical' group was extremely homogeneous in plasmid content. In addition to a single large (70–145 kb) structurally related plasmid species, all strains carried a highly conserved group of three low- M_r plasmids (pAsa1, 5.0 kb; pAsa2, 5.2 kb; pAsa3, 5.4 kb). The pAsa plasmids had different restriction maps, and directed the synthesis of four polypeptides when assayed *in vitro* using a transcription/translation system and [35 S]methionine radiolabelling. A partially conserved 6.0 kb plasmid directed synthesis of an additional polypeptide. Total cellular plasmid DNA from virulent 'typical' strain *A. salmonicida* A449 was also cloned in *Escherichia coli*, using the vector pBR322. When analysed using a minicell expression system, 17 plasmid-encoded proteins, ranging in size from 12 to 90 kDa, were identified, including two putative exported proteins, and a chloramphenicol acetyltransferase which was encoded by the 145 kb conserved, non-conjugative, plasmid.

INTRODUCTION

Aeromonas salmonicida is a worldwide pathogen of feral and cultured fish (Trust, 1986). Once thought to infect only salmonids, causing the systemic disease furunculosis, *A. salmonicida* is now commonly isolated from chronic, ulcerative diseases in both salmonid and non-salmonid fish (Bootsma *et al.*, 1977; Evelyn, 1971; Kimura, 1969; Paterson *et al.*, 1980; Shotts *et al.*, 1980; Smith, 1963). Isolates from these slowly progressing diseases produce a markedly different pathogenesis than 'typical' furunculosis strains. They are readily distinguishable by their slow growth, nutritional requirements, and biochemical properties, and are often referred to as the 'atypical' group of *A. salmonicida* (Ishiguro *et al.*, 1986; McCarthy, 1978; Trust *et al.*, 1980). DNA:DNA reassociation analysis of whole-cell DNA shows two groups of *A. salmonicida* which are related at the genus level, the genetically homogeneous 'typical' *A. salmonicida* subsp. *salmonicida*, and the genetically diverse 'atypical' strains, which differ from the 'typical' group at the subspecies level. The 'atypical' strains fall into two major taxa: *A. salmonicida* subsp. *achromogenes*, comprising isolates from salmonid fish, and *A. salmonicida* subsp. *nova*, comprising isolates from non-salmonid fish (McCarthy, 1978; Belland & Trust, 1988).

Plasmid carriage by 'typical' strains of *A. salmonicida* has been reported. For example, Toranzo *et al.* (1983) found that *A. salmonicida* strains carried as many as nine plasmids. They reported a similarity in low- M_r plasmid DNAs but did not characterize these plasmids further. Hackett *et al.* (1984) also observed a similarity in plasmid profiles, particularly of low- M_r DNAs.

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However, neither study included a broad geographical range of isolates and no functional characteristics were associated with the plasmids reported. Several R-plasmids have also been described in 'typical' *A. salmonicida* (Aoki *et al.*, 1979, 1986; Hedges *et al.*, 1985). As yet there have been no reports of plasmids in 'atypical' isolates from non-salmonid fish species.

To extend our genetic analysis of this important fish pathogen, we have examined plasmid carriage in a wide variety of *A. salmonicida* strains. Here we compare the plasmid DNA content of typical and atypical strains of *A. salmonicida*, and report the genetic characterization of a highly conserved group of three small plasmids found in all 'typical' isolates examined to date. We also report the expression of low- M_r *A. salmonicida* plasmid DNA in an *in vitro* transcription/translation system, and the cloning and expression of *A. salmonicida* plasmid DNA in *Escherichia coli*.

METHODS

Bacterial strains. *A. salmonicida* strains used in this study are listed in Tables 1 and 2. 'Typical' *A. salmonicida* strains were grown at 20 °C on tryptic soy agar (TSA, Difco) or in TS broth (TSB). 'Atypical' *A. salmonicida* strains were cultured at 20 °C on TSA supplemented with 5% human blood (TBA), as they have a requirement for added haemin (Ishiguro *et al.*, 1986). All bacterial strains were maintained at -70 °C in TSB containing 12% (v/v) glycerol.

Plasmid isolation. All isolates were screened using two isolation techniques, that of Portnoy *et al.* (1981) and that of Kado & Liu (1981), both essentially as described by Toranzo *et al.* (1983). For both procedures approximately 50 mg of cells (wet weight) were harvested from TSA or TBA plates and washed once with TS buffer (50 mM-Tris/HCl, 0.1 M-NaCl; pH 8.0).

When plasmids were isolated by the method of Portnoy *et al.* (1981), cells were resuspended in 100 µl TS buffer and lysed by the addition of 500 µl lysis buffer [50 mM-Tris/HCl, 0.1 M-NaCl, 3% (w/v) SDS; pH 12.4] at 37 °C for 30 min. To this, 30 µl neutralization buffer (2.0 M-Tris/HCl pH 7.0) was added, and the bulk of the chromosomal DNA was precipitated by the addition of 150 µl 5 M-NaCl. After incubation at 0 °C for at least 4 h, samples were centrifuged at 30000 *g* in a microfuge for 10 min. Supernatants were removed to new tubes and plasmid DNA precipitated by the addition of an equal volume of 2-propanol and incubation at 0 °C for 1 h. The resulting precipitate was collected by centrifugation in a microfuge at 15000 *g* for 5 min. Precipitated DNA was suspended in TE buffer (10 mM-Tris/HCl, 1 mM-EDTA; pH 7.6) in preparation for agarose gel electrophoresis.

To extract plasmid DNA by the technique of Kado & Liu (1981), washed cells were resuspended in 200 µl lysis buffer (50 mM-Tris/acetate, 3% SDS; pH 12.4) and incubated at 95 °C for 5 min. Following a brief extraction with an equal volume of phenol/chloroform (1:1, v/v) the emulsion was separated by centrifugation. The aqueous phase was removed to a new tube for agarose gel electrophoresis.

Plasmid DNA for restriction analysis, cloning, and *in vitro* expression experiments was purified by scaling up the Kado and Liu extraction technique (using sodium lauroyl sarcosinate in place of SDS) to 2 g cells and 25 ml lysis buffer. Phenol/chloroform-extracted plasmid-containing lysates were made to a density of 1.55 g ml⁻¹ by the addition of solid CsCl, and ethidium bromide was added to a final concentration of 600 µg ml⁻¹. Gradients were centrifuged to equilibrium at 134000 *g* for 36 h.

Plasmid DNA and restriction enzyme digestions of plasmid DNA were analysed by horizontal agarose gel electrophoresis. Voltage gradients of 12 V cm⁻¹ were used, except in the case of 'typical' isolates, where it was necessary to use much lower gradients (2 V cm⁻¹) to resolve plasmid DNA in the 5.0–5.4 kb range. DNA was visualized by soaking in a solution of 0.5 mg ethidium bromide ml⁻¹ for 30 min and viewing over a UV transilluminator. Photographs were made using an MP-4 camera head and Polaroid land film (type 665/667). Plasmid standards used in the study were pBR322 (4.36 kb), RSF1010 (13.2 kb), Sa (38 kb), RP4 (51.7 kb), R1 (92.7 kb) and R27 (170 kb).

Conjugal transfer of R plasmids. R-plasmids in *A. salmonicida* strains were detected by conjugal mating procedures (Curtiss, 1981) on solid media at 20 °C. Overnight broth cultures of *E. coli* HB101 were mixed with 48 h broth cultures of *A. salmonicida* and aseptically filtered onto sterile 25 mm membrane filters (Micron Sep Inc.) with a 45 µm pore size. Membrane filters containing the mating mixtures were incubated on TSA plates for 3 h at 20 °C, after which suitable dilutions were plated on TSA supplemented with tetracycline (15 mg ml⁻¹), chloramphenicol (30 mg ml⁻¹) or streptomycin (50 mg ml⁻¹) and incubated at 37 °C overnight to counterselect *A. salmonicida* (no growth at 37 °C). Donor and recipient controls were similarly treated.

Plasmid DNA cloning. Total plasmid DNA from 'typical' strain A449 was isolated by the scaled-up Kado and Liu extraction technique described above. After purification by CsCl/ethidium bromide equilibrium density gradient centrifugation, the plasmid DNA was digested with *Bam*HI (Pharmacia) and analysed by agarose gel electrophoresis. Digested plasmid DNA was then ligated into *Bam*HI-cleaved and dephosphorylated pBR322 and

transformed into the minicell-producing *E. coli* strain GK403 (*thr leu thi lacY gal minAB Tc^s rps*). Transformants were selected on agar supplemented with ampicillin and tetracycline, and putative recombinants identified by their Ap^r Tc^s phenotype were screened for the presence of inserts using the rapid plasmid screening technique of Portnoy *et al.* (1981) and restriction analysis with *Bam*HI. The presence of cloned *A. salmonicida* plasmid DNA sequences in recombinant plasmids was confirmed by dot-blot hybridization (Maniatis *et al.*, 1982) using ³²P-radiolabelled total *A. salmonicida* A449 plasmid DNA as a probe.

In vitro labelling of plasmid-encoded proteins. Purified plasmid DNA was dialysed against Tris/acetate buffer (10 mM, pH 8.0), and adjusted to 0.5 mg ml⁻¹. Plasmid-DNA-directed protein synthesis was done using a commercially available (Enzo-Biochem) lysate prepared according to a modification of the procedure of Yang *et al.* (1980). [³⁵S]Methionine was used for radiolabelling according to the instructions of the manufacturer, and plasmid-specified proteins were identified by SDS-PAGE (Laemmli, 1970) and autoradiography.

Minicells from *E. coli* GK403 derivatives, harbouring recombinant plasmids, were isolated following growth in rich medium, and purified by differential centrifugation in linear sucrose density gradients, as described by Dougan *et al.* (1983). Minicells, suspended to an OD₆₀₀ of 0.5 in M9 salts supplemented with 0.2% glucose and vitamin B1 (0.5 mg ml⁻¹), in the presence and absence of 10% (v/v) ethanol (Halegoua & Inouye, 1979; Palva *et al.*, 1981; Dougan *et al.*, 1983), were labelled in the presence of 0.25% methionine assay medium (Difco) with 5.6 mCi (207 MBq) [³⁵S]methionine (NEN) at 37 °C for 1 h, following a 30 min preincubation. Labelled minicells were harvested by centrifugation in a microfuge and suspended in a solubilization buffer (Tris/HCl, 60 mM; glycerol, 1%; 2-mercaptoethanol, 5%; SDS, 3%; bromophenol blue, 0.01%; pH 6.8) before boiling for 10 min. Samples were resolved on SDS-PAGE gels (12.5%, w/v, acrylamide) by the procedure of Laemmli (1970) and the gels, following a rapid stain, fix, and destain, were exposed directly to X-AR5 film (Kodak Eastman Co.) for autoradiography.

Acetyltransferase activity and cell fractionation. Acetyltransferase activity was assayed using the spectrophotometric procedure of Shaw (1975); cell inner and outer membrane, periplasmic and cytosol fractions were prepared essentially as described by Filip *et al.* (1973) and Willis *et al.* (1974).

RESULTS

Plasmid carriage

Plasmid DNA from each *A. salmonicida* strain was isolated by the techniques of both Kado & Liu (1981) and Portnoy *et al.* (1981) at least twice, and only strains giving identical patterns on each occasion are reported here. The yield of plasmid DNA from *A. salmonicida* varied according to the purification procedure employed. In general, the method of Kado & Liu (1981) was the more satisfactory isolation procedure as it consistently gave both high- (> 70 kb) and low-*M_r* plasmids. Lysing cells at 95 °C for 3–5 min gave the best yields of DNA; however, strains varied considerably in their susceptibility to lysis, probably in part due to the presence or absence of the surface protein array (A-layer) (Udey & Fryer, 1978; Kay *et al.*, 1981; Ishiguro *et al.*, 1981). High-*M_r* plasmids in *A. salmonicida* were especially difficult to isolate in large quantities, appearing to be particularly susceptible to shearing during the isolation procedures. The method of Portnoy *et al.* (1981) frequently gave a poor yield of high-*M_r* plasmid DNA. Indeed a slight alteration of the method (i.e. incubating cells in lysis buffer for 15 min at 0 °C) allowed the selective isolation of low-*M_r* plasmid DNA.

A total of 25 strains of *A. salmonicida* were examined: 14 'typical' and 11 'atypical' strains. All contained at least two plasmids, and plasmid sizes ranged from 3.5 to 150 kb as estimated by comparison with standard plasmid DNA isolated by the same techniques. Electrophoretic patterns of plasmid DNA from 'typical' strains were remarkably similar. 'Typical' strains of *A. salmonicida* contained from four to seven plasmid species, ranging from 3.5 to 145 kb in size, and all 14 strains possessed a group of three low-*M_r* plasmids of 5.0, 5.2 and 5.4 kb (Table 1). Ten of the 14 'typical' strains had a fourth low-*M_r* plasmid of 6.0 kb. Plasmids in Table 1 (columns A–F) had indistinguishable or very similar restriction digestion fingerprint patterns using *Bam*HI. R-plasmids which could be conjugally transferred to *E. coli* were found in 4/14 strains. Each of these four strains contained conjugative plasmids which coded for Tc^r, and strain A473 contained two R-plasmids, the larger of which was transferable to *E. coli* and coded for resistance to chloramphenicol and streptomycin.

Electrophoretic profiles of plasmid DNA isolated from 'atypical' strains, and restriction digestion fingerprint patterns using *Bam*HI, showed that the plasmids from 'atypical' strains

Table 1. *Plasmid carriage in 'typical' A. salmonicida isolates*

Strain	Source	A-layer	Plasmid size (kb)*						
			A	B	C	D	E	F	G
A251	NCMB 1102, Atlantic salmon, Scotland	+	5.0	5.2	5.4	6.0		120	
A362	Sea trout, England	+	5.0	5.2	5.4	6.0		75	
A437	Sockeye salmon, BC, Canada	+	5.0	5.2	5.4	6.0		130	
A438	Coho salmon, BC, Canada	—	5.0	5.2	5.4	6.0		130	
A440	ATCC 14174, Brook trout, USA	—	5.0	5.2	5.4		28(R)	120	
A449	Brown trout, Eure, France	+	5.0	5.2	5.4			145	
A450	Brown trout, Tarn, France	+	5.0	5.2	5.4			145	
A451	Rainbow trout, Manch, France	+	5.0	5.2	5.4	6.0		135	
A470	Rainbow trout, Wash. St., USA	+	5.0	5.2	5.4	6.0	28(R)	145	
A473	Coho salmon, Wash. St., USA	—	5.0	5.2	5.4	6.0	26(R)	135	95(R)
A482	Steelhead trout, BC, Canada	+	5.0	5.2	5.4			70	12
A488	Brook trout, New Hamp., USA	+	5.0	5.2	5.4	6.0		145	
A494	Coho salmon, Ore., USA	+	5.0	5.2	5.4	6.0		120	3.5
A500	Atlantic salmon, Mass., USA	+	5.0	5.2	5.4	6.0	30(R)	120	

* Plasmids in columns A–F show restriction fragment fingerprint similarities with the column. (R) indicates that these plasmids carry antibiotic resistance determinants that have been conjugally transferred to *E. coli* (resistances described in text).

Table 2. *Plasmid carriage in 'atypical' A. salmonicida isolates*

Strain	Source	A-layer	Plasmid size (kb)*			
			A	B	C	D
A400	Goldfish, Boolara, Australia	+	5.8 ^a	38 ^b	60 ^c	
A406	Goldfish, Boolara, Australia	—	5.8 ^a	38 ^b	60 ^c	150 ^d
A419	Goldfish, Ark., USA	+	5.8 ^a	38 ^b	60 ^c	
A491	Goldfish, Md., USA	+	5.8 ^a	38 ^b	60 ^c	140 ^d
A206	Masou, Japan	—	4.0	70		
A460	Atlantic salmon, NS, Canada	+	18	36	48	
A452	European carp, Netherlands	+	35	60	80	
A475	European carp, Norway	+	4.2 ^e	4.4 ^f	55	140
A436	Sablefish, Nanaimo, Canada	—	4.2 ^e	4.4 ^f		
A479	European carp, Denmark	+	8.4 ^g	55 ^h		
A480	European carp, Netherlands	+	8.4 ^g	60 ^h		

* Plasmids with the same superscripts (a–h) show restriction fragment fingerprint similarities.

differed significantly from the 'typical' strains, and also differed within the 'atypical' group itself. Plasmids isolated from 11 'atypical' strains are listed in Table 2. All 'atypical' strains examined possessed from two to four plasmid species, ranging in size from 4.0 to 150 kb. No detectable similarity in restriction fragment patterns was seen through the entire group of 'atypical' plasmids, although certain sub-groupings were apparent in electrophoretic plasmid profiles. Strains A400, A406, A419 and A491 had three plasmids of 5.8, 38 and 60 kb, two isolates (A475 and A436) had two small plasmids of 4.2 and 4.4 kb, while another two strains (A479 and A480) had a small (8.4) and an intermediate size plasmid (55–60 kb).

Small, conserved plasmid DNAs of 'typical' A. salmonicida

Small plasmid DNAs were selectively purified by incubating cells in Portnoy lysis buffer for 15 min at 0 °C followed by CsCl equilibrium density gradient centrifugation. The three small plasmids of 'typical' virulent strain A449 (pAsa1, 5.0 kb; pAsa2, 5.2 kb; pAsa3, 5.4 kb) were mapped simultaneously using a series of single, double and triple restriction enzyme digests. The mapping was confirmed following the cloning in pUC18 of: pAsa1 as a single 5.0 kb *Bam*HI fragment; a 4.2 kb *Hind*III/*Bgl*II fragment of pAsa2; and a 4.0 kb *Bgl*II fragment of pAsa3.

Fig. 1. Restriction maps of conserved low- M_r plasmids pAsa1, pAsa2 and pAsa3 from 'typical' *A. salmonicida* strain A449. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III.

Each pAsa plasmid exhibited a unique restriction map (Fig. 1). The synthesis of proteins directed by these three conserved low- M_r plasmids was examined using a commercially available transcription/translation lysate by labelling with [35 S]methionine. A pAsa1–3 plasmid DNA mixture from strain A449 directed the synthesis of four proteins, of M_r 25 500, 26 700, 28 000 and 32 300 (Fig. 2a, lane 1, open circles). Proteins of the same apparent M_r were seen when low- M_r plasmid DNA from strains A251, A451 and A488 was used in the transcription/translation system. Plasmid DNA from these three strains also contained the 6.0 kb plasmid and produced additional proteins of M_r 33 100 and 34 500 (Fig. 2, filled circles).

To determine coding differences between the low- M_r plasmids of the 'typical' and 'atypical' strains, the proteins specified by the 5.8 kb plasmids of 'atypical' strains A400 and A419, and the 8.4 kb plasmid of 'atypical' strain A479, were also examined. The 5.8 kb plasmid of strains A400 and A419 coded for proteins of M_r 14 000, 17 200, 30 000, 32 500 and 34 000, and the 8.4 kb plasmid of strain A479 coded for proteins of M_r 14 200, 30 100 and 33 300 (Fig. 2b).

Cloning and expression of A. salmonicida plasmid DNA in minicells

The expression of cloned *A. salmonicida* plasmid DNA from 'typical' virulent strain A449 was examined in minicells. In addition to the three small plasmids, pAsa1, 2 and 3, strain A449 carried large plasmid pAsa4 of about 145 kb. Strain A449 was resistant to chloramphenicol, tetracycline and streptomycin (MICs about 250, 250, and 300 mg ml $^{-1}$ respectively), but none of these resistances could be conjugally transferred or transformed into *E. coli* using plasmid DNA and standard techniques.

Following isolation, digestion, and ligation of total A449 cellular plasmid DNA to the vector pBR322, recombinant Amp r Tet s isolates were screened for the presence of inserts using a rapid plasmid isolation technique and dot-blot hybridization using 32 P-radiolabelled *A. salmonicida* A449 plasmid DNA as probe to confirm that the inserts were *A. salmonicida* plasmid DNA (Fig. 3a). In Fig. 3(a) the numbered fragments correspond to *A. salmonicida* plasmid DNA fragments cloned into pBR322 (plasmid constructs pEB1–15), and reacted with radiolabelled A449 plasmid DNA (Fig. 3b). Band 10 in Fig. 3(a) is a 5.2 kb plasmid (pAsa2) that has no *Bam*HI site and was therefore not represented in the plasmid library. Best estimates indicated that approximately 75 kb of an estimated 160.6 kb of A449 plasmid DNA, or approximately 47% of the plasmid genome, was cloned in this experiment. The disparity between the total amount of plasmid DNA available and that accounted for by agarose gel electrophoresis following digestion is probably due to the failure to separate fragments of similar size (e.g. it is likely that one or more of the bands 1–4 in Fig. 3(a) contain comigrating *Bam*HI fragments) and to the generation of low- M_r fragments that were too small to be retained in the gel.

The expression of cloned plasmid DNA from each recombinant strain was analysed in [35 S]methionine-radiolabelled *E. coli* minicells in the presence or absence of 10% ethanol, a

Fig. 2. Autoradiographs of [^{35}S]methionine-radiolabelled plasmid-DNA-encoded proteins resolved on 12.5% SDS-PAGE gels. Low- M_r plasmids from 'typical' *A. salmonicida* strains A449, A451, A488 and A251 were used in part (a), lanes 1-4 respectively. Low- M_r plasmids from 'atypical' strains A479, A400 and A419 were used in part (b), lanes 1-3 respectively. Lanes C in both (a) and (b) represent controls in which TE buffer was added to the lysate in place of plasmid DNA. Standard proteins were phosphorylase *b* (top), bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme; their sizes are given on the left.

Table 3. Recombinant plasmids containing *A. salmonicida* plasmid DNA directing synthesis of [^{35}S]methionine-labelled proteins

Plasmid	Insert size (kb)	$10^{-3} \times M_r$ of proteins expressed in minicells				
pEB1	15.0	23	90			
pEB2	12.0	12	14	15.8*	60	63*
pEB3	7.5	21	25			
pEB4	5.0	28	68			
pEB5	4.8					
pEB6	4.0	15	42			
pEB7	3.8	14.5				
pEB8	3.7	13.5				
pEB9	3.0					
pEB10	2.1	58				
pEB11	2.0					
pEB12	1.9					
pEB13	1.5					
pEB14	1.35	20				
pEB15	1.2					

* These proteins are presumed to be unprocessed export proteins.

Fig. 3. (a) *Bam*HI-digested plasmid DNA from *A. salmonicida* strain A449 (lane 2) resolved on a 0.8% agarose gel adjacent to DNA M_r standards [λ DNA cleaved with *Hind*III; fragment sizes are indicated (fragments of 0.56 and 0.13 kb are not visible)]. Numbered DNA fragments have been cloned into pBR322 with the exception of 10, which represents a small plasmid (pAsa2) with no *Bam*HI site. Numbers 1–9 indicate recombinant plasmids pEB1–9, and numbers 11–16 plasmids pEB10–15. (b) Recombinant plasmids [0.5 μ g, numbered as in (a)] dot-blotted onto Gene Screen Plus and probed with 32 P-radiolabelled A449 plasmid DNA. Control plasmid DNA (pBR322) is in the position labelled 'c'.

procedure known to prevent the removal of signal sequences from many exported proteins (Halegoua & Inouye, 1979; Palva *et al.*, 1981; Dougan *et al.*, 1983). A total of 17 proteins (a conservative estimate based on SDS-PAGE and autoradiography), ranging in size from M_r 12000 to M_r 90000 were expressed from *A. salmonicida* plasmid DNA (Fig. 4, Table 3). A single recombinant, pEB2, expressed two putative exported proteins (Fig. 4, lane 6 +/–), one with a mature M_r of 14000 (unprocessed M_r 15800) and the other with a mature M_r of 60000 (unprocessed M_r 63000). The pBR322 control reaction (Fig. 4, lane 1 +/–) showed that the processing of pre- β -lactamase (open triangles) to mature β -lactamase (closed triangle) was

Fig. 4. Autoradiograms of [^{35}S]methionine-radiolabelled proteins resolved on 12.5% SDS-PAGE gels. Labelled proteins were synthesized in *E. coli* minicells from recombinant plasmids containing *A. salmonicida* strain A449 plasmid DNA. Open circles indicate proteins encoded by *A. salmonicida* plasmid DNA. Experiments were done in the presence (+) or absence (-) of 10% ethanol. Open triangles indicate putative unprocessed or precursor forms of mature proteins (filled triangles) which predominate in samples labelled in the presence of 10% ethanol. The filled circle indicates a 25 kDa chloramphenicol acetyltransferase. Lanes: 1, pBR322; 2, pEB15; 3, pEB10; 4, pEB3; 5, pEB7; 6, pEB2; 7, pEB9; 8, pEB8; 9, pEB11; 10, pEB12; 11, pEB14; 12, pEB6; 13, pEB4; 14, pEB1; 15, pEB13; 16, pEB5.

almost completely prevented in the presence of ethanol. Western blotting experiments indicated that none of the expressed proteins were strong antigens when immunoblots were developed using antiserum raised to whole cells of strain A449 (data not shown). Another recombinant, pEB3, carrying a 7.5 kb insert (Fig. 4, lane 4 +/-) appeared to overexpress a number of

Table 4. Chloramphenicol acetyltransferase activity in cellular compartments of *E. coli* HB101(pBR322) and HB101(pEB3) assayed by the spectrophotometric technique of Shaw (1975)

Cellular compartment	10 ⁻² × Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	pBR322	pEB3
Cytosol	0.02	72.9
Periplasm	tr	1.2
Membranes (inner and outer)	tr	2.5
Supernatant	—	2.4

tr, trace; —, no activity detected.

proteins, ranging in size from M_r 14000 to M_r 25000. This recombinant plasmid was shown to confer high-level chloramphenicol resistance (MIC > 500 mg ml⁻¹) on the recipient strain. Further examination showed that the M_r 25000 protein was restricted to the cytosolic compartment, and was a very active acetyltransferase when assayed using the spectrophotometric procedure of Shaw (1975) (Table 4).

DISCUSSION

This study has provided further genetic evidence for a taxonomic separation of 'atypical' and 'typical' strains of *A. salmonicida*. The differences in plasmid content of the two groups coincide with recent genomic DNA:DNA reassociation evidence that *A. salmonicida* subsp. *salmonicida* is an extremely homogeneous taxon, while the 'atypical' strains are more diverse, but with distinct recognizable biotypes related to the fish species they were isolated from (Belland & Trust, 1988). The plasmid content of 'atypical' *A. salmonicida* strains showed much greater diversity in plasmid DNA content than the conserved population of plasmids in 'typical' strains. Within the 'atypical' group there were distinct sub-groups based on plasmid content that correlated with the fish species and diseases from which the strains were isolated. Most notable were strains isolated from goldfish. Despite diverse geographical sources, all four isolates had three plasmids with similar M_r values. Two isolates, one from the USA, the other from Australia, had a fourth high- M_r plasmid. This apparent conservation of plasmid DNA may be due to an involvement of these plasmids in the pathogenesis of goldfish ulcer disease, and indicates a common source for the goldfish ulcer disease strains, at least in the case of the Arkansas and Australian isolates which were from goldfish stocks originating in Japan (Shotts *et al.*, 1980; Whittington *et al.*, 1987). Two other pairs of 'atypical' strains showed similar plasmid profiles. In one case, both isolates were from erythrodermatitis in European carp (Bootsma *et al.*, 1977), and in the other case, both strains were isolated from marine fish species, i.e. sablefish and Pacific salmon (Evelyn, 1971), and Atlantic salmon. These results suggest that plasmid DNA content may be a useful epidemiological marker for 'atypical' *A. salmonicida*.

All 'typical' strains examined in this study contained at least four plasmid species. Using a genome size of 2.40×10^9 Da (Belland & Trust, 1988), plasmids represent approximately 20% of the *A. salmonicida* cellular DNA. The single large non-conjugative plasmid (> 70 kb) varied in size from strain to strain, but displayed considerable relatedness by restriction enzyme analysis. High- M_r conserved plasmids have been reported in a variety of other pathogens, including *Yersinia enterocolitica* (Portnoy *et al.*, 1981), *Salmonella typhimurium* LT2 (Jones *et al.*, 1982), *Bacillus thuringiensis* (Gonzales *et al.*, 1981), *Shigella* sp. (Hale *et al.*, 1983) and the fish-pathogenic *Yersinia ruckeri* (De Grandis & Stevenson, 1982) and *Vibrio anguillarum* (Crosa, 1980), and in all cases are important in pathogenesis. In this regard we have observed one strain of *A. salmonicida* (A451) from which two isolates with deletions in the large conserved plasmid have been found, and both display greatly reduced lethality in rainbow trout (unpublished data). To date, however, the phenotypic basis for this phenomenon has not been elucidated.

A partially conserved plasmid of 6.0 kb was found in 10 of 14 'typical' isolates examined. This plasmid does not appear to be required for *A. salmonicida* virulence, as all strains lacking it (with the exception of A440 [ATCC 14174]) were fully virulent (Belland & Trust, unpublished data). Indeed the 6.0 kb plasmid appears to have been lost from our isolate of A440 [ATCC 14174], and from the ATCC 14174 isolate studied by Hackett *et al.* (1984), as Toranzo *et al.* (1983) reported the presence of a 6.0 kb plasmid in their isolate of ATCC 14174. In contrast, our isolate of A440 [ATCC 14174] contained a 28 kb conjugative R-plasmid which was not seen in the other studies. Despite these conflicting data, it is not surprising that changes have occurred in this strain, which has been used as the archetypal *A. salmonicida* laboratory reference strain for over 20 years. Perhaps more surprising is the fact that despite decades of use as a laboratory strain, the plasmid profile is still very similar to that of recent isolates from nature.

The most remarkable conservation of plasmid DNA involved the three small plasmids pAsa1, pAsa2 and pAsa3. These three high-copy-number plasmids (about 55 copies per cell; Hackett *et al.*, 1984) were found in all 'typical' isolates examined despite their diverse geographical sources and isolation from a variety of fish species. Restriction enzyme analysis and protein expression assays showed that these plasmids were probably identical in all 'typical' strains. Small cryptic plasmids, generally very stable and found in high copy numbers, have also been found in a number of other bacterial species, including *Neisseria gonorrhoeae* (Korch *et al.*, 1985), *Shigella sonnei* (Croft *et al.*, 1983) and *E. coli* 15 (Cozzarelli *et al.*, 1968). In each case however the conserved elements are single plasmids, in contrast to the group of three plasmids in *A. salmonicida*.

In cell-free transcription/translation lysates the three small conserved *A. salmonicida* plasmids (purified as a group) directed the synthesis of four proteins ranging in M_r from 25 500 to 33 300. This seems a small number considering the coding capacity of 15.6 kb of plasmid DNA, but the overall efficiency of incorporation in our experiments was poor compared to controls of *E. coli* plasmid DNA. This may be due to a problem in either transcriptional or translational fidelity in an *E. coli* background. It is also important to note that estimates based on [35 S]methionine labelling can be low because some proteins contain few or no methionine residues (Glenn, 1976; Pollock & Richmond, 1962).

Three of the 'typical' strains examined in the transcription/translation assays also possessed the 6.0 kb plasmid, and in each case a single new protein was apparent. In two strains, the protein had an apparent M_r of 34 500 and in another an M_r of 33 100. The small plasmids isolated from 'atypical' strains were shown to code for different proteins than the pAsa plasmids when examined in the cell-free transcription/translation system. The 5.8 kb plasmid from 'atypical' strains A400 and A419 coded for four proteins ranging in M_r from 14 000 to 34 000, while the 8.4 kb plasmid from strain A479 directed the synthesis of three proteins ranging in M_r from 14 000 to 33 300. These results are in keeping with a similar expression of plasmid-encoded proteins from two small, conserved plasmids of *Chlamydia trachomatis* (pCTL2, 7.3 kb) and *Chlamydia psittaci* (pCPMn, 6.2 kb) which directed the expression of two (M_r 38 000 and 48 000) and three (M_r 22 000, 38 000 and 52 000) proteins respectively (Joseph *et al.*, 1986). Studies of very small, conserved plasmid DNA, such as pNZ500 (1.5 kb) in *S. sonnei*, have shown that these plasmids are totally reliant on the cellular machinery for replication, code for two or three proteins at most (involved in their mobilization by conjugative factors), and impart no phenotype on cells carrying them (Croft *et al.*, 1983). The pAsa plasmids appear to be large enough to provide a definite but as yet unidentified phenotype. The pAsa plasmids also have the ability to be mobilized by conjugative plasmids (unpublished observation).

When expression of plasmid DNAs of a representative virulent 'typical' strain was examined using a minicell labelling system, at least 17 plasmid-encoded proteins were identified. Many plasmids in other species have been shown to code for exported proteins, including toxigenic enzymes (Elwell & Shipley, 1980) and outer-membrane proteins (Elwell & Shipley, 1980; Hale *et al.*, 1983; Portnoy *et al.*, 1981). Two of the plasmid-coded *A. salmonicida* proteins also appeared to be exported proteins because they contained peptide regions which resembled the leader sequences of exported proteins in that they were cleaved by *E. coli* cellular machinery only in the absence of 10% ethanol. However, neither of the pEB2-encoded export proteins co-purified with

the outer membrane (unpublished observation), suggesting that they are either periplasmic constituents or are proteins exported across the outer membrane to the external medium. In this regard, Western blotting experiments indicated that none of the plasmid-coded *A. salmonicida* proteins were strong antigens.

One phenotypic property that was shown to be plasmid coded was the chloramphenicol resistance of strain A449. The Cm^r phenotype is imparted by a chloramphenicol acetyltransferase (CAT) enzyme similar to those described by Gaffney *et al.* (1978). The Cm^r gene must be carried by the high-*M_r* plasmid pAsa4 since the insert containing it is larger than any of the three low-*M_r* plasmids. Expression of the CAT gene in *E. coli* minicells was exceptionally high, and the CAT protein was the single predominant protein species in Coomassie-blue-stained whole-cell lysates. It is not known whether this reflects a peculiar alignment of the CAT gene with adjacent pBR322 sequences, or whether the *A. salmonicida* CAT gene in pAsa4 itself has a high level of expression due to an unusual promoter or genetic arrangement. High levels of expression were not found for other *A. salmonicida* plasmid genes examined.

The apparent universal carriage of plasmids by an obligate pathogen like *A. salmonicida*, the stability of the plasmids, and the inability of workers to cure plasmids in 'typical' strains of the organism, points to an important role for the plasmids in the survival of *A. salmonicida*. The ability to identify the products of *A. salmonicida* plasmid genes, as demonstrated in this study, will facilitate the directed generation of mutants in specific plasmid genes, and so increase our understanding of the role of plasmids in this important fish pathogen.

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