IncP-mediated transfer of loci involved with gas vesicle production in *Ancylobacter aquaticus*

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IncP plasmids were transferred by conjugation from *Escherichia coli* to gas-vacuolate strains of *Ancylobacter aquaticus*, between strains of *A. aquaticus*, and from *A. aquaticus* to *E. coli*. These plasmids would also mobilize the chromosome of *A. aquaticus*. In this way, *A. aquaticus* genes could complement mutations in either *A. aquaticus* or *E. coli*. A locus involved in gas vesicle production was co-transferred with rifampicin resistance in matings between strains of *A. aquaticus*.

Introduction

Ancylobacter aquaticus (Raj, 1983), formerly *Microcylus aquaticus*, is a Gram-negative heterotrophic bacterium that has been isolated from aquatic habitats. Some strains contain intracellular inclusions called gas vesicles (Konopka *et al.*, 1976; Van Ert & Staley, 1971), and these hollow proteinaceous structures can provide buoyancy if they occur in sufficient quantity (Walsby, 1971, 1978).

Gas vesicles occur in phylogenetically diverse species of bacteria, including many cyanobacteria (Walsby, 1981*a*), heterotrophic eubacteria (Walsby, 1981*b*), and archaeobacteria such as *Halobacterium* (Horne *et al.*, 1988) and methanogens (Zinder *et al.*, 1987). Genes encoding gas vesicle proteins have been cloned from *Calothrix* sp. PCC 7601 (Tandeau de Marsac *et al.*, 1985), *Anabaena flos-aquae* (Hayes *et al.*, 1988) and *Halobacterium halobium* (Horne *et al.*, 1988). These genes have been detected in a wide variety of cyanobacteria (Damerval *et al.*, 1989) and several isolates of halobacteria (Horne *et al.*, 1988).

Neither the cyanobacteria nor halobacteria from which gas vesicle genes have been cloned are amenable to genetic analysis. *Escherichia coli* clones containing gas vesicle genes have not been reported to produce gas vesicle protein. *Ancylobacter aquaticus* is a unicellular bacterium that grows on chemically defined media, and it may provide a useful system in which to conduct molecular and genetic analyses of gas vesicle assembly. In this report, we provide evidence that broad-host-range IncP plasmids (Thomas & Smith, 1987) can be used to transfer DNA (including chromosomal markers) between *A. aquaticus* strains and between *E. coli* and *A.*

aquaticus. Furthermore, we were able to complement non-gas-vacuolate *A. aquaticus* mutants with this system.

Methods

Bacterial strains and plasmids. Strains of *A. aquaticus* and *E. coli*, and IncP plasmids used in this study, are described in Table 1. Derivatives of *A. aquaticus* M1 typically have a non-motile phenotype, but phase variants which are motile (such as strain M7) can be selected (Lara & Konopka, 1987).

Media and growth conditions. *A. aquaticus* was grown in either CAGV or DM medium. CAGV contained (1⁻¹): 1 g glucose, 1 g Casamino acids, 20 ml Hutner's salts and 5 ml vitamin solution (Staley, 1968). DM contained (1⁻¹): 20 ml Hutner's salts, 5 ml vitamin solution, 1 g glucose, 0.025 g (NH₄)₂SO₄ and 0.07 g Na₂HPO₄. The pH was adjusted to 7.2 prior to autoclaving. *E. coli* strains were grown in L-broth (Miller, 1972) or Difco nutrient broth.

Antibiotics were added to media to the following concentrations: streptomycin (Str), 100 µg ml⁻¹, kanamycin (Km), 100 µg ml⁻¹, rifampicin (Rif), 30 µg ml⁻¹, ampicillin (Ap), 25 µg ml⁻¹, tetracycline (Tc) 20 µg ml⁻¹.

A. aquaticus strains were grown at 30 °C, and *E. coli* strains were grown at 37 °C. Broth cultures were incubated in a New Brunswick controlled-environment incubator-shaker.

Mating procedures. Matings were incubated at 30 °C for 24 h, unless otherwise indicated. For chromosome transfer between *A. aquaticus* strains, matings were left for 48 h. The data presented on mating experiments are the results from a typical experiment.

Non-quantitative patch matings were performed by picking individual colonies of donor and recipient with sterile toothpicks. These were mixed together on agar plates. L-agar or nutrient agar was used as the medium for matings which involved *E. coli*. CAGV agar was used when *A. aquaticus* strains were the donor and recipient.

For quantitative matings, cultures of the donor and recipient were grown in the appropriate liquid medium to densities of 10⁸ ml⁻¹. Cells were harvested by centrifugation and resuspended in medium to densities of 10⁹ ml⁻¹. A 0.1 ml sample of recipient was spotted onto a

Table 1. *Bacterial strains and plasmids*

Designation	Relevant markers and properties*	Comments and source
<i>A. aquaticus</i>		
M1	Wild-type Ves ⁺	Van Ert & Staley (1971)
M7	Ves ⁻ Mot ⁺	Lara & Konopka (1987)
M16	Ves ⁻	Spontaneous mutant of M1, this study
M100	Ves ^C	Konopka <i>et al.</i> (1976); formerly strain S1
M123	Ves ⁻ Str ^R	Derived from M16, this study
M125	Ves ⁺ Str ^R	Derived from M1, this study
M127	Ves ⁺ Rif ^R	Derived from M1, this study
M130	Ves ^C Rif ^R	Derived from M100, this study
M136	Ves ⁻ Str ^R	Derived from M22, a UV-induced Ves ⁻ mutant of M1, this study
M201	Ves ⁻ Rif ^R	Derived from M16, this study
<i>E. coli</i>		
CU981	<i>thr leu thi</i>	Obtained from H. E. Umbarger
HB5	<i>hag</i>	Obtained from P. Greenberg
HB101	<i>recA hsdM hsdR pro leu gal ara</i>	Obtained from A. Aronson
Plasmids		
RP4	Km ^R Ap ^R Tc ^R	Obtained from H. E. Umbarger
RP68.45	Km ^R Ap ^R Tc ^R Cma ⁺	Obtained from B. L. Marrs
pBLM2	Km ^R Ap ^R Cma ⁺	Marrs (1981)

* Abbreviations: Ves⁺, produces gas vesicles; Ves⁻, produces no gas vesicles; Ves^C, constitutive gas vesicle production; Mot⁺, motile; Str^R, streptomycin resistant; Rif^R, rifampicin resistant; Km^R, kanamycin resistant; Ap^R, ampicillin resistant; Tc^R, tetracycline resistant; Cma⁺, enhanced chromosome-mobilization ability.

plate and allowed to dry for 0.25–1 h. Then 0.1 ml of donor suspension was spotted over the recipient. The mating mix was incubated at 25 °C for 1–4 h, and then incubated at 30 °C. After the incubation period, the mating mix was scraped from the plate with an inoculating loop and resuspended in 3 ml of medium. Dilutions were made, and samples were spread onto selective solid media for enumeration. The frequency of transfer was calculated as the number of exconjugants per donor cell present at the beginning of the mating.

Matings on filters involved collection of 1×10^9 cells of both donor and recipient on a 0.45 µm pore size Gelman Metrical filter. The filter was placed on an L-agar plate and incubated for 24 h. Cells were removed from the filter by placing it in 5 ml sterile DM media and agitating on a vortex mixer for 3–5 min.

Antibody reactions. Antibody to *A. aquaticus* flagellar protein was provided by Dr J. C. Lara (Lara & Konopka, 1987). A 10 µl sample of each motile variant or recombinant to be tested was placed in each of two 1.5 ml Eppendorf tubes. One microlitre of antibody was added to one tube, and the samples were mixed and incubated for 30–60 min at 25 °C before examination by phase-contrast microscopy for loss of motility and clumping. The extent of immotility was compared to a positive control (motile *A. aquaticus* M7) and to the sample of motile culture that did not receive antibody.

Results

Matings between *E. coli* containing the IncP plasmids RP4, R68.45 or pBLM2 and *A. aquaticus* M1 all resulted in the isolation of Km^R clones of strain M1. The frequency of transfer for the three plasmids ranged from 10^{-2} to 10^{-3} (Table 2).

Several criteria were examined in matings between *E. coli* CU981 and *A. aquaticus* M1 to determine the optimal conditions for RP4 transfer between these species. The highest frequency of transfer occurred after 24 h; longer periods of mating decreased the number of Km^R exconjugants (Fig. 1). The frequency of plasmid transfer was 10^{-2} – 10^{-3} per donor cell when quantitative matings (see Methods) were performed on L-agar or nutrient agar, 10^{-6} on CAGV agar, and transfer was not observed when the strains were mated on DM + glucose. Matings placed directly on agar plates resulted in plasmid transfer frequencies of about 10^{-2} , whereas a transfer frequency of 10^{-4} – 10^{-5} was found when cells were concentrated on filters which were then placed on agar media. Donor:recipient ratios ranging from 3:1 to 1:10 yielded similar plasmid transfer frequencies, which ranged from 10^{-1} to 10^{-3} in individual experiments. However, ratios greater than 3:1 resulted in transfer frequencies of less than 5×10^{-5} .

Transfer of IncP plasmids between strains of *A. aquaticus* was attempted by mating at 30 °C for 24 h. Transfer of RP4, R68.45 and pBLM2 from a Str^S to a Str^R strain was confirmed by isolation of Str^R Km^R exconjugants at a frequency of approximately 10^{-2} for each of the three plasmids. The frequency of RP4 transfer was the same when matings were performed on L-agar or CAGV agar.

Table 2. Inter- and intra-generic transfer of *IncP* plasmids

Donor	Plasmid	Recipient	Transfer frequency* of Km ^R
<i>E. coli</i>		<i>A. aquaticus</i>	
CU981	RP4	M1	2×10^{-2}
HB101	R68.45	M1	2×10^{-3}
HB101	pBLM2	M1	2×10^{-3}
<i>A. aquaticus</i>		<i>A. aquaticus</i>	
M1	RP4	M123	1×10^{-2}
M1	R68.45	M123	1×10^{-2}
<i>A. aquaticus</i>		<i>E. coli</i>	
M130	RP4	HB101	10^{-2} – 10^{-4}
M130	R68.45	HB101	10^{-2} – 10^{-4}

* Transfer frequencies are expressed per donor cell. In matings between *E. coli* strains and *A. aquaticus* M1, exconjugants were selected on glucose minimal medium (DM) with Km. Matings between *A. aquaticus* strains in which M123 was the recipient used CAGV medium plus Str and Km to select exconjugants. *E. coli* exconjugants that arose from matings with *A. aquaticus* were detected on L-agar plus Km after 24 h. Slow-growing *A. aquaticus* required 3 d to form colonies on L-agar.

Patch matings were performed to determine whether these three plasmids could mobilize the chromosomal genes for Str or Rif resistance between strains of *A. aquaticus* (Table 3). Transfer of chromosomal markers for Str or Rif resistance did not occur unless matings were incubated for 36–48 h.

Selection for chromosomal transfer was made by plating onto CAGV agar containing either Str and Rif or Km + Str + Rif. All exconjugants selected on Str + Rif plates appeared to also contain the plasmid, because they were also Km^R. Quantitative matings indicated that the frequency of Rif^R transfer mediated by RP4 was 3×10^{-6} . The rate of spontaneous mutation to Rif^R in these strains was 300-fold lower (10^{-8}). Mobilization of Rif^R by R68.45 occurred at a frequency fourfold lower than with RP4. When pBLM2 was used as the mobilizing plasmid, the frequency of Rif^R recipients was similar to the rate of spontaneous mutation to Rif^R. When the complementary matings were performed (transfer of Str^R from a donor to a Rif^R recipient), only R68.45 and pBLM2 transferred Str^R at a frequency significantly greater than the rate of spontaneous Str resistance (10^{-9}).

In the experiments testing chromosome mobilization, the donor strains were Ves⁺, whereas the recipients were non-gas-vacuolate mutants. Co-transfer of the Ves⁺ phenotype with Rif^R occurred at frequencies up to 50% in exconjugants of *A. aquaticus* M123 or M136. The frequency of co-transfer varied in individual experiments from 5 to 50%. No co-transfer of the Ves⁺ phenotype was observed when transfer of Str^R to a Ves⁻ recipient was selected.

Fig. 1. Frequency of plasmid RP4 transfer between *E. coli* CU981 and *A. aquaticus* M1 as a function of the duration of mating. The transfer frequency is expressed as the number of Km^R exconjugants per 10^8 donor cells.

A. aquaticus M130 has a phenotype in which gas vesicle production is constitutive. In the wild-type, strain M1, the addition of L-lysine inhibits gas vesicle production. Linkage of the putative regulatory gene to the locus that converted strain M123 to the Ves⁺ phenotype was tested by mating M130(RP4) to M123. Transfer of Rif^R was selected on CAGV medium, which permits expression of the Ves⁺ phenotype. Ves⁺ exconjugants were tested for the ability to produce gas vesicles in media containing L-lysine. Ninety percent of them failed to produce gas vesicles under these conditions. Thus, there was not strong linkage between these two loci involved in gas vesicle production.

Six other Ves⁻ derivatives of *A. aquaticus* M1 were mated to strain M130(RP4), and Rif^R exconjugants were selected. We observed co-transfer of the Ves⁺ phenotype in only one of these strains.

Experiments were also performed to determine whether the *IncP* plasmids could be used to mobilize chromosomal markers of *A. aquaticus* that would complement mutations in *E. coli*. *E. coli* HB101 was chosen as the recipient, because it contains several mutations that might be complemented and it is defective in recombination and restriction. When *A. aquaticus* M130(RP4) was the donor, *E. coli* exconjugants were isolated in which the *pro*, *gal* or *ara* lesions had been complemented. With M130(R68.45), complementation of the *pro*, *gal* or *leu* loci was observed. The frequency of *gal* complementation in these experiments was measured to be 3×10^{-6} or 7×10^{-6} Gal⁺ exconjugants per donor cell for M130(RP4) or M130(R68.45), respectively. The frequency of reversion to Gal⁺ by HB101 was $<10^{-9}$.

We were also able to complement a defective flagellin gene in *E. coli* HB5 by mating with *A. aquaticus*. Gas-vacuolate strains of *A. aquaticus* are non-motile; however, flagellated non-gas-vacuolate variants can be

Table 3. Mobilization of chromosomal antibiotic resistance markers in *A. aquaticus* by IncP plasmids

Donor		Recipient		Rif ^R or Str ^R exconjugants per donor*	Percentage Ves ⁺
Strain	Phenotype	Strain	Phenotype		
M127	Rif ^R Ves ⁺ (RP4)	M136	Str ^R Ves ⁻	3×10^{-6}	50
M127	Rif ^R Ves ⁺ (RP68.45)	M123	Str ^R Ves ⁻	ND	50
M127	Rif ^R Ves ⁺ (RP68.45)	M136	Str ^R Ves ⁻	8×10^{-7}	10
M127	Rif ^R Ves ⁺ (pBLM2)	M123	Str ^R Ves ⁻	ND	20
M127	Rif ^R Ves ⁺ (pBLM2)	M136	Str ^R Ves ⁻	2×10^{-8}	5
M130	Rif ^R Ves ^C (RP4)	M123	Str ^R Ves ⁻	3×10^{-6}	25
M125	Str ^R Ves ⁺ (R68.45)	M201	Rif ^R Ves ⁻	1.4×10^{-6}	0
M125	Str ^R Ves ⁺ (pBLM2)	M201	Rif ^R Ves ⁻	2.0×10^{-6}	0
M125	Str ^R Ves ⁺ (RP4)	M201	Rif ^R Ves ⁻	1.2×10^{-9}	0

ND, Not determined.

* Exconjugants were selected on CAGV agar containing either Str and Rif or Km, Str and Rif.

selected (Lara & Konopka, 1987). Thus, phenotypically non-motile strains contain the genetic information for formation of flagella. *A. aquaticus* M100(RP4) was mated to a Str^R derivative of *E. coli* HB5, and exconjugants were selected on nutrient agar containing Kan and Str. Two of seventy colonies that were inoculated into 0.35% agar media exhibited diffuse growth in 10 d. Motility was confirmed when samples were examined microscopically.

Two pieces of evidence suggest that the motile derivatives of HB5 arose due to gene transfer from *A. aquaticus*. (1) No motile revertants of HB5 were isolated by repeated inoculation of 0.35% agar plates with HB5 alone. (2) Antibody to purified *A. aquaticus* flagellin inhibited motility of the exconjugants, but had no effect upon motility of wild-type *E. coli*.

Discussion

This study was undertaken to examine the feasibility of establishing genetic exchange in gas-vacuolate *A. aquaticus* using broad-host-range plasmids. We found that several IncP plasmids could be transferred between strains of *A. aquaticus* or between *A. aquaticus* and *E. coli*. This result was not surprising, because successful transfer of IncP plasmids has been reported between a wide variety of Gram-negative genera (Thomas & Smith, 1987).

The three IncP plasmids we employed were also capable of mobilizing chromosomal genes in *A. aquaticus*. Several *E. coli* mutations, responsible for defects in catabolic, biosynthetic or motility functions, were complemented with *A. aquaticus* DNA mobilized via IncP plasmids. Resistance to Str or Rif, which typically results from mutations in chromosomal genes (Gale *et al.*, 1981),

arose at a frequency 100–1000 times the spontaneous mutation rate in *A. aquaticus* when the appropriate strains of *A. aquaticus* were mated employing an IncP plasmid. Plasmids R68.45 and pBLM2 have been reported to have an enhanced ability to mobilize chromosomal genes in Gram-negative bacteria (Marrs, 1981; Holloway, 1969). However, in *A. aquaticus*, plasmid RP4 was just as effective in mobilizing the Rif^R locus as was R68.45. Note that R68.45 was the only one of the three plasmids with which we could transfer both the Str^R and Rif^R markers. The frequency of Rif^R transfer that was found using pBLM2 was not much greater than the frequency of spontaneous mutation in the recipient, but chromosome transfer may have occurred in this experiment, because a low percentage of the recipient cells also acquired the Ves⁺ phenotype (Table 3).

A locus involved in gas vesicle production was linked to the Rif^R locus. Two pieces of evidence suggest that the genes responsible for regulation and production of gas vesicles are not all clustered in one region of the genome. (1) We attempted to complement eight Ves⁻ mutants by selecting Rif^R exconjugants after mating to strain M130, but were only successful with three strains. (2) A putative regulatory locus, which confers constitutive expression of gas vesicles in strain M130 (Konopka, 1977), was not co-transferred to exconjugants in which the Ves⁻ mutation had been complemented.

These results demonstrate the feasibility of conducting genetic complementation in *A. aquaticus* and of shuttling DNA between *A. aquaticus* and *E. coli*. However, some difficulties remain in using *A. aquaticus* as a genetic system to study gas vesicle production in prokaryotes. This organism has been difficult to mutagenize. Ves⁻ mutants occur spontaneously at frequencies up to 10^{-2} in batch culture in late stationary phase (A. Konopka,

unpublished observation). Clones resistant to antibiotics such as Str and Rif are easily isolated. However, we have failed to isolate mutants defective in biosynthetic or catabolic capacity, or resistant to amino acid analogues after treatment with UV irradiation or chemical mutagens such as nitrosoguanidine, acridine orange or ethidium bromide (Bittle, 1986).

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