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ORIGINAL ARTICLE

Mesophilic strains of *Aeromonas* spp. can acquire the multidrug resistance plasmid pRAS1 in horizontal transfer experiments at low temperatures

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Abstract Both biotic and abiotic characteristics of an ecosystem play an important role in the horizontal transfer of DNA in nature. The abiotic factor temperature has a great impact on such transfers as it controls the metabolic activity of mesophilic microorganisms. Moreover, psychrophilic bacteria, which are not affected by low temperatures, are considered to be potential donors of DNA to mesophilic bacteria under temperature stress conditions. In our study, mesophilic *Aeromonas* spp. strains isolated from fresh fish were genotypically identified and used as recipients in in vitro conjugal transfer experiments using plasmid pRAS1 from psychrophilic strain *Aeromonas salmonicida* 718 at three different temperatures (8, 15 and 20 °C). The transfer of the plasmid was confirmed by identifying the elements of the integron in pRAS1. A low temperatures did not prevent the transfer of the pRAS1 plasmid to *Aeromonas veronii*, *A. media*, *A. hydrophila* and *A. caviae* strains, which showed detectable conjugation frequencies of 10^{-8} at 8 °C. In other strains of the same species, transconjugants were not detected, which indicated that the genetic background of each strain directly affected the ability to be a recipient of this plasmid at the temperatures tested. Our results demonstrate that mesophilic *Aeromonas* spp. strains are potential reservoirs of extrachromosomal genetic material. Implications of this plasmid transfer at low temperatures and its possible consequences for human health are discussed.

Keywords *Aeromonas* · Plasmid transfer · Low temperatures · Antimicrobials

Introduction

Plasmids play a crucial role in bacterial evolution and adaptation as they mediate the exchange of genetic material between microbial populations. The transfer of plasmids between bacterial strains is known as conjugation (Arutyunov and Frost 2013), and it is the main route for the transfer of genetic material in nature (Olsen et al. 2013). It has been shown that conjugation can link the gene pools of prokaryotic and eukaryotic organisms through the transfer of bacterial plasmids to higher organisms, such as filamentous fungi and yeasts (Mazodier and Davies 1991; Moriguchi et al. 2013). The study of this process at low temperatures is important as the storage of seafood only partially inhibits microbial activity (Coton et al. 2013), possibly still enabling the horizontal transfer of genetic material.

The pRAS1 plasmid is a 44-kb replicon which contains a class 1 integron containing the gene cassettes conferring resistance to trimethoprim and sulfamethoxazole and the *Tn1721* transposon conferring resistance to tetracycline (Rhodes et al. 2000). This plasmid was found in a psychrophilic strain of *Aeromonas salmonicida* 718, which is the causative agent of furunculosis in salmonids. *A. salmonicida* 718 (NVI 2402/89) was originally isolated from the head kidney of diseased Atlantic salmon in 1989 (Sandaa and Enger 1994) and subsequently deposited in the collection culture of the Norwegian School of Veterinary Science. pRAS1 has been successfully transferred to 46 microbial

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biotypes in marine sediments (Sandaa and Enger 1994) and to *Aeromonas hydrophila* strains under optimal temperature conditions (Bello–López et al. 2010). However, to date, it is unknown whether this plasmid can be transferred into mesophilic *Aeromonas* spp. strains at low temperatures.

Studying and quantifying the plasmid transfer process is important for ecological and health reasons. First, plasmids can be transferred between aquatic bacteria by conjugation (Bello–López et al. 2012). Second, a large number of the bacteria present in water systems possess conjugative R-plasmids (Del Castillo et al. 2013) and, therefore, can be considered potential donors. The aim of our study was to demonstrate the in vitro transfer of the pRAS1 plasmid in mesophilic strains of *Aeromonas* spp. isolated from fresh fish (*Cyprinus carpio* L.) at low temperatures, simulating the temperature conditions at which seafood for sale is stored.

Materials and methods

Isolation and genetic identification of mesophilic *Aeromonas* spp. strains

Mesophilic *Aeromonas* spp. strains were isolated from fresh fish (*Cyprinus carpio* L.) obtained in local markets in eastern Mexico City, as described by Sarria-Guzmán et al. (2014). The isolates were identified to the species level by analysis of the *rpoD* gene (Table 1) (Soler et al. 2004).

Strain characterization

All *Aeromonas* spp. strains of the same species were analyzed by enterobacterial repetitive intergenic consensus (ERIC)–PCR to differentiate unique isolates from clones. Total DNA was extracted using the QIAamp DNA Mini QIAcube Kit (QIAGEN, Venlo, The Netherlands). In the ERIC–PCR, the primers ERIC1R and ERIC2 were used for genotyping the strains (Table 1) (Versalovic et al. 1991). The total reaction volume was 25 μl that contained 18.5 μl distilled sterile water, 2.5 μl 10× PCR buffer with $MgCl_2$, 0.5 μl of 25 mM dNTPs, 1.0 μl of each primer, 0.5 U Taq DNA polymerase and 1 μl template DNA. The cycling program consisted of 30 cycles of pre-denaturation at 95 °C for 7 min, denaturation at 90 °C for 30 s, annealing at 58 °C for 1 min, and extension at 65 °C for 8 min, with a final extension at 68 °C for 16 min. Genetic profiles were analyzed visually by comparing the intra-gel patterns, and isolates representative of each ERIC–PCR pattern were selected for subsequent analyses.

Antimicrobial susceptibility

Resistance or sensitivity of all strains to tetracycline (30 μg) and ampicillin (30 μg) was determined using the disk diffusion method on Mueller–Hinton agar plates according to recommendations of The Clinical and Laboratory Standards Institute (CLSI 2007). *Pseudomonas aeruginosa* ATCC 25923 was used as the control. Results were interpreted as susceptible or resistant by measuring the diameter of the inhibition zone according to the criteria established by the CLSI.

Conjugal transfer experiments at low temperatures

Conjugal transfer experiments were performed in triplicate as described by Schmidt et al. (2001). In brief, the donor *A. salmonicida* psychrophilic strain 718 Amp^s carrying plasmid pRAS1 [IncU; class 1 integron (*IntI–dfrA16–qacEΔ1/sulI*), *Tn1721* (Tet^r)] and mesophilic *Aeromonas* spp. recipient strains were grown overnight in L-broth with shaking at the appropriate temperature (15 and 28 °C, respectively. The strains were adjusted to 1×10^9 CFU/ml in a spectrophotometer (model 3000 SmartSpec™ flow; Bio-Rad, Hercules, CA) at 600 nm. A 100-μl aliquot of donor and recipient cultures were mixed and placed on the surface of a sterile 0.22-μm filter (Millipore, Bedford, MA), positioned on the surface of an LB-agar plate and incubated immediately overnight at 20, 15 or 8 °C for 24 h. A bacterial mix from filters was suspended

Table 1 Primers used in this study

Primer	Sequence (5′→3′)	Reference
rpoD 70Fs	ACGACTGACCCGGTACGCATGTA	Soler et al. 2004
rpoD 70Rs	ATAGAAATAACCAGACGTAAGTT	
ERIC1R	ATGTAAGCTCCTGGGGATTCA	Versalovic et al. 1991
ERIC2	AAGTAAGTGACTGGGGTGAGC	
Rep-IncU-Fwd	CTGGCTGAAATGCTGTTGCC	Bello–López et al. 2012
Rep-IncU-Rev	GCTTCATAGGCTTCACGCTC	
IntI1-F	GTTCGGTCAAGGTTCTG	Zhang et al. 2004
IntI1-R	GCCAACTTTCAGCACATG	
in-F	GGCATCCAAGCAGCAAGC	Schmidt et al. 2001
in-B	AAGCAGACTTGACCTGAT	
qacEΔ1-F	ATCGCAATAGTTGGCGAAGT	Schmidt et al. 2001
sul1-B	GCAAGGCGGAAACCCGCGCC	

in 2-ml sterile phosphate buffered saline (PBS), pelleted and resuspended in 100 μl of the same buffer. Serial dilutions were spread onto selective L-agar plates containing tetracycline (25 μg/ml) and ampicillin (125 μg/ml) for transconjugants and ampicillin (125 μg/ml) for recipients. The plates were incubated at 28 °C for 24 h. Conjugal transfer frequencies were calculated by dividing the number of transconjugants by the number of *Aeromonas* spp. recipients. Clones of each *Aeromonas* spp. transconjugant were selected for further characterization.

Characterization of transconjugant pRAS1

Plasmid DNA extracted from transconjugants was purified using the QuickGene Plasmid kit S (Fujifilm, Tokyo, Japan) according to the manufacturer's protocol. The pRAS1 plasmid was used as the template for the screening of all elements of the class 1 integron, i.e. the integrase (*IntI*)–variable region–3′ end (*qacEΔ1–sulI*) and the *IncU* replicon using primers described previously (Table 1) (Schmidt et al. 2001; Zhang et al. 2004). Additionally, biochemical tests and genetic identification based on sequencing of the *rpoD* gene were performed to confirm the presence of transconjugants.

Results and discussion

Storage at low temperatures is one of the most common methods used to preserve seafood (Sánchez–Alonso et al. 2012; Mejlholm et al. 2012). In products of marine origin, however, psychrophilic bacteria can spread and cause alterations even under cold conditions, indicating that they remain metabolically active in the cold. The horizontal transfer of genetic material from psychrophilic bacteria at low temperatures has received little attention from researchers. However, this process is important in the area of food microbiology due to the emergence of multi-drug resistant strains.

We simulated the temperature at which seafood is stored awaiting sale, subjecting mesophilic strains of *Aeromonas* spp. to conjugation with the psychrophilic strain *A. salmonicida* 718 carrying plasmid pRAS1. This plasmid confers resistance to trimethoprim and sulphonamides and also to tetracycline due to the presence of a class 1 integron and to the truncated transposon *Tn1721*, respectively. We isolated a total of 13 mesophilic strains of *Aeromonas* spp. and identified these strains by conventional biochemical and morphological tests (i.e. Gram-negative, non-sporulating, oxidase positive, able to ferment glucose, no acid production from inositol and growth in 3 % NaCl but not in 6.0 % NaCl). Sequencing of the *rpoD* gene encoding the sigma subunit of the RNA polymerase allowed the isolates to be identified to the species level, namely, as *A. veronii* (n=3), *A. media* (n=2), *A. hydrophila* (n=4), *A. caviae* (n=2) and *A. jandaeii* (n=2). The clonal relationship between isolates was analyzed using ERIC-PCR. This probe showed that all 13 strains of *Aeromonas* spp. isolated were unique and not clones as the profiles obtained differed from each other. All strains showed sensitivity and resistance to tetracycline and ampicillin, respectively, and these markers were subsequently used for the selection of transconjugants derived from mating with *A. salmonicida* 718.

In our study, we demonstrated the ability of psychrophilic strain *A. salmonicida* 718 to in vitro transfer of the plasmid pRAS1 to mesophilic strains of *Aeromonas* spp. at low temperatures. The frequency of recovered transconjugants was 10^{-1}, 10^{-4} and 10^{-8} at 20, 15 and 8 °C, respectively. Strains BA3, BA4, BA10 and BA12 received the plasmid at all three temperatures, with a 1 to 3 log reduction at 15 °C and a 2 to3 log reduction at 8 °C, while strains BA1, BA2, BA5, BA6, BA7, BA11, BA15 and BC1 received the plasmid at 20 and 15 °C with log reductions of 2–4 at 15 °C (Table 2). Conjugal transfer experiments at 8 °C with strains BA1, BA2, SA5, BA6, BA7, BA11, BA15, BC1 and BC4 yielded no transconjugants even after plating of the entire conjugal mixture, indicating that conjugal transfer to these recipients was $<1\times10^{-9}$ at 8 °C (Table 2). No direct relationship between *Aeromonas* spp. strains and the ability to receive this plasmid was found, but it is clear that the genetic background of each strain is different and that this difference was decisive in determining the transfer frequencies.

Transfer frequencies with this plasmid with phylogenetically distant organisms have been reported in earlier laboratory studies. Sandaa and Enger (1994) reported transfer frequencies of pRAS1 to *Escherichia coli* HB101 of 1.4×10^{-2}, while Casas et al. (2005) obtained frequencies of 10^{-4} and 10^{-7} with the same strain. Although the frequencies reported in these studies were based on conjugation assays conducted at suboptimal temperatures (15 and 20 °C, respectively), Sandaa and Enger (1994) introduced tetracycline to induce selection pressure and therefore promote plasmid transfer. Additionally, they tested total DNA from each transconjugant for its ability to act as a template for PCR amplification of the *IntI* (923 bp), *dfrA16* (986 bp), *qacEΔ1–sulI* (800 bp) and *IncU* (589 bp) genes encoded in pRAS1 according to previously described amplification conditions (Schmidt et al. 2001; Zhang et al. 2004).

In our study, amplicons of the expected size were identified in all of the recovered clones, thereby confirming the identity of pRAS1 in the transconjugants compared to the positive control. These products were not amplified in the recipient strains (data not shown) (Fig. 1). Our conjugal transfer experiments clearly demonstrate the potential of pRAS1 to disseminate in strains belonging to the same microbial genus. This event acquires greater importance when these bacteria are in the same ecological niche.

Table 2 In vitro transfer frequencies[a] of the plasmid pRAS1 from *Aeromonas salmonicida 718* to different mesophilic *Aeromonas* spp. strains at three different temperatures

Isolate code	Genetic affiliation	Temperature		
		20 °C	15 °C	8 °C
BA1	*Aeromonas veronii*	$2.0\ (\pm10^{-3})\times10^{-4}$	$6.8\ (\pm10^{-2})\times10^{-7}$	ND [b]
BA2	*A. veronii*	$1.7\ (\pm10^{-4})\times10^{-4}$	$2.3\ (\pm10^{-3})\times10^{-6}$	ND
BA3	*A. veronii*	$5.6\ (\pm10^{-4})\times10^{-3}$	$2.4\ (\pm10^{-2})\times10^{-4}$	$5.6\ (\pm10^{-4})\times10^{-7}$
BA4	*A. media*	$1.0\ (\pm10^{-4})\times10^{-3}$	$6.7\ (\pm10^{-2})\times10^{-5}$	$1.0\ (\pm10^{-4})\times10^{-8}$
BA5	*A. media*	$1.3\ (\pm10^{-4})\times10^{-3}$	$2.1\ (\pm10^{-2})\times10^{-6}$	ND
BA6	*A. hydrophila*	$8.1\ (\pm10^{-4})\times10^{-4}$	$9.0\ (\pm10^{-3})\times10^{-8}$	ND
BA7	*A. hydrophila*	$8.0\ (\pm10^{-3})\times10^{-5}$	$4.3\ (\pm10^{-4})\times10^{-7}$	ND
BA10	*A. hydrophila*	$6.3\ (\pm10^{-5})\times10^{-4}$	$1.2\ (\pm10^{-3})\times10^{-6}$	$6.0\ (\pm10^{-5})\times10^{-8}$
BA11	*A. hydrophila*	$9.6\ (\pm10^{-6})\times10^{-5}$	$3.0\ (\pm10^{-2})\times10^{-8}$	ND
BA12	*A. caviae*	$8.0\ (\pm10^{-1})\times10^{-1}$	$2.7\ (\pm10^{-4})\times10^{-4}$	$8.0\ (\pm10^{-1})\times10^{-7}$
BA15	*A. caviae*	$7.3\ (\pm10^{-7})\times10^{-5}$	$4.5\ (\pm10^{-3})\times10^{-7}$	ND
BC1	*A. jandaeii*	$6.1\ (\pm10^{-1})\times10^{-4}$	$2.1\ (\pm10^{-2})\times10^{-7}$	ND
BC4	*A. jandaeii*	$2.6\ (\pm10^{-2})\times10^{-2}$	$5.6\ (\pm10^{-4})\times10^{-5}$	ND

ND, Not detected

Values are presented as the mean ± standard deviation

[a] Number of transconjugants relative to the number of recipients after the mating period

Previous studies have indicated that atypical strains, such as the psychrophilic *A. salmonicida* 718 strain, are important agents in the transfer of plasmids in nature (Sørum et al. 2003). Moreover, transcriptional analysis at low temperatures in *Yersinia enterocolitica* W22703 and NCTC10460 revealed the expression of the cold shock genes *cspA* and *cspb*, the glutamate–aspartate transporter and UhpABC, *ArcA* and MCPI, all of which are responsible for the synthesis of regulatory proteins and environmental sensors (Bresolin et al. 2006). Therefore, the transcription of genes involved in the transfer, replication and transcription of DNA in mesophilic strains of *Aeromonas* spp. was not inhibited at 8 °C. This was confirmed by transcriptional studies involving the construction of a mini-*Tn5–luxCDABE* mutant library in *Aeromonas* spp. with the aim of identifying genes expressed at low temperatures in the presence of the pRAS1 plasmid.

In recent years, the emergence of pathogenic and commensal bacteria resistant to multiple antibiotics has increased dramatically. Studies indicate that one of the reasons for this multidrug resistance is the horizontal transfer of plasmids due to the selection pressure exerted by antibiotics on bacteria, promoting the development of mechanisms of transfer. Irrational and indiscriminate use of antibiotics in food for livestock, fish farming and poultry are directly related to the

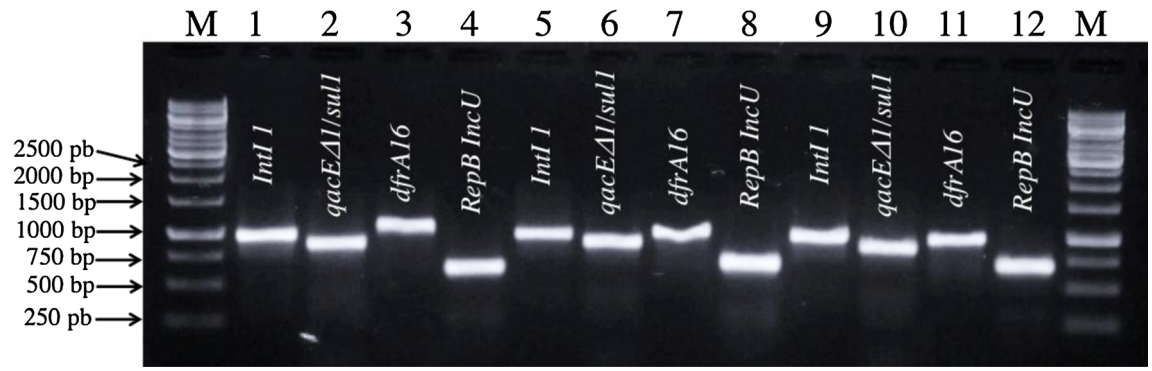

Fig. 1 Agarose gel electrophoresis of the PCR products of class 1 integron elements and the *RepB-IncU* replicon of a number of transconjugants of mesophilic *Aeromonas* spp. strains. *Lanes*: *M* GeneRuler 1-kb molecular size marker (Fermentas, Thermo Fisher Scientific, Waltham, MA), *1–4 A. veronnii* (BA1 strain), *5–8 A. media* (BA4 strain), *9–12* positive control, *A. salmonicida* 718. PCR products: *IntI* (923 bp), *dfrA16* (986 bp), *qacEΔ1–sulI* (800 bp), *RepB IncU* (589 bp)

emergence of antibiotic-resistant strains. For example, *Salmonella* spp. and *Campylobacter* spp. strains acquire resistance through the addition of antibiotics to fodder for livestock (Džidic et al. 2008), the use of tetracycline induces the emergence of tetracycline-resistant *Aeromonas* spp. strains in fish farms (Agersøa et al. 2007) and the use of ciprofloxacin is associated with the appearance of *E. coli* strains resistant to fluoroquinolones in poultry farms (Rice et al. 2003).

Our study on the horizontal transfer of DNA from mesophilic *Aeromonas* spp. strains isolated from fresh fish stored in ice is relevant because *A. salmonicida* shares the same ecological niche with species of *Aeromonas* spp. that are primary human pathogens. Our results suggest that non-pathogenic strains of humans have a greater potential to participate directly in the transfer of antibiotic resistance genes than pathogenic bacteria. This possibility should be considered when examining the potential of a psychrophilic microorganism to exchange extrachromosomal genetic material in nature.

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