BMC Microbiology

RESEARCH ARTICLE Open Access

Clustering subspecies of *Aeromonas salmonicida* using IS*630* typing

Nicole Studer[†], Joachim Frey[*] and Philippe Vanden Bergh[†]

Abstract

Background: The insertion element IS*630* found in *Aeromonas salmonicida* belongs to the IS*630*-Tc1-mariner superfamily of transposons. It is present in multiple copies and represents approximately half of the IS present in the genome of *A. salmonicida* subsp. *salmonicida* A449.

Results: By using High Copy Number IS*630* Restriction Fragment Length Polymorphism (HCN-IS*630*-RFLP), strains of various subspecies of *Aeromonas salmonicida* showed conserved or clustering patterns, thus allowing their differentiation from each other. Fingerprints of *A. salmonicida* subsp. *salmonicida* showed the highest homogeneity while 'atypical' *A. salmonicida* strains were more heterogeneous. IS*630* typing also differentiated *A. salmonicida* from other *Aeromonas* species. The copy number of IS*630* in *Aeromonas salmonicida* ranges from 8 to 35 and is much lower in other *Aeromonas* species.

Conclusions: HCN-IS*630*-RFLP is a powerful tool for subtyping of *A. salmonicida*. The high stability of IS*630* insertions in *A. salmonicida* subsp. *salmonicida* indicates that it might have played a role in pathoadaptation of *A. salmonicida* which has reached an optimal configuration in the highly virulent and specific fish pathogen *A. salmonicida* subsp. *salmonicida*.

Keywords: *Aeromonas salmonicida*, HCN-IS*630*-RFLP, IS element, Subtyping, Tc1 Mariner transposon, Salmonidae, Pathoadaptation

Background

Aeromonas salmonicida is one of the predominant bacterial species found in fish and water samples [1]. While some *Aeromonas* species are able to cause opportunistic disease in warm- and cold blooded vertebrates, *A. salmonicida* seems to be specific for fish. *Aeromonas salmonicida* subsp. *salmonicida* a specific primary pathogen of *Salmonidae* (salmon, trout and char) has been known for decades to cause furunculosis. This bacterial septicaemia has a significant economic impact on aquaculture operations as well as on the wild stock of salmonids and some other fish species [2]. Bergey's Manual of Systematic Bacteriology recognizes five subspecies of *A. salmonicida*: *salmonicida*, *achromogenes*, *smithia*, *pectinolytica* and *masoucida* [3]. *Aeromonas salmonicida* subsp. *salmonicida* is referred to as typical *Aeromonas salmonicida* by reason that these strains are very homogeneous and considered to be clonal [4,5]. Clinical strains that cannot be assigned to any of the known subspecies are referred to as *A. salmonicida* 'atypical'. In recent years, it has been recognized that 'atypical' strains cause diseases in *salmonidae* and other fish species that differ from furunculosis. Therefore their importance is being increasingly recognized. The most common clinical manifestation observed, following infections with such strains, is chronic skin ulceration [6]. Due to a convoluted history of nomenclature and taxonomy of *Aeromonas* sp., clear assignment of strains using currently available methods remains sometimes confusing and controversial which makes epidemiological studies difficult [7]. Intraspecies phenotypic variability also makes phenotypic identification challenging on the species level [8]. A variety of molecular genetic methods have been employed for genetic classification of Aeromonads including mol% G + C composition, DNA-DNA relatedness studies, restriction fragment length polymorphism, pulsed-field gel electrophoresis, plasmid analysis, ribotyping, multilocus sequence typing, PCR

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and more [3,5]. Combination of 16S rDNA-RFLP analysis and sequencing of the gene *rpoD* was proposed as a suitable approach for the correct assignment of *Aeromonas* strains [9]. Moreover, analyzing strains by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) with an extraction method revealed 100% genus-level accuracy and 91.4% accuracy at species level [10]. However, this method was not able to discriminate *A. salmonicida* at the subspecies level.

Currently, no molecular approach gives a clear genotypic distinction of strains among *A. salmonicida* species. For this reason we elaborated a molecular genetic technique to achieve an adequate subtyping of all *Aeromonas salmonicida* subspecies. This method, named High Copy Number IS-Element based Restriction Fragment Length Polymorphism (HCN-IS-RFLP), has been successfully applied in numerous epidemiological studies for other pathogenic bacteria [11-15].

Results

Optimization of HCN-IS*630*-RFLP conditions

IS*630* was selected because it is the IS element with the highest copy number in the genome of *A. salmonicida* [16]. Primers internal to the highly conserved IS*630* genes [GenBank: ABO88357.1] were designed to generate a probe on an intact IS fragment from the *A. salmonicida* subsp. *salmonicida* JF2267 genome. To obtain the most distinct banding pattern, the digestion by several restriction enzymes on a set of sequenced genomes (*A. salmonicida* subsp. *salmonicida* A449, *A. hydrophila* ATCC7966 and *A. veronii* B565) was predicted by computer analysis. XhoI that does not cut within our probe for IS*630* revealed a good resolution with a clear banding pattern and was therefore selected. A size window of 1375 bp to 21226 bp was defined on all southern blots as some hybridizing patterns with very large or small fragments were not sufficiently resolved (Figure 1). The genomic DNA sequence of *A. salmonicida* strain A449 [GenBank: CP000644.1] predicted that the probe would hybridize with 35 copies of IS*630* on XhoI fragments ranging from 1277 bp to 17948 bp (Additional file 1: Table S1).

We analyzed the IS*630* RFLP-fingerprints of 87 *Aeromonas* sp. strains of various geographical origins, which comprised 31 *A. salmonicida* subsp. *salmonicida*, 4 subsp. *achromogenes*, 4 subsp. *smithia*, 2 subsp. *masoucida*, one subsp. *pectinolytica*, 12 *A. salmonicida* atypical strains, 8 *A. popoffii*, 5 *A. sobria* and *A. bestiarum*, 2 *A. hydrophila*, one *A. trota*, *A. enteropelogenes*, *A. simiae*, *A. eucrenophila*, *A. ichthiosmia*, *A. jandaei*, *A. molluscorum*, *A. bivalvium*, *A. allosaccharophila*, *A. media*, *A. veronii*, *A. caviae* and *A.*

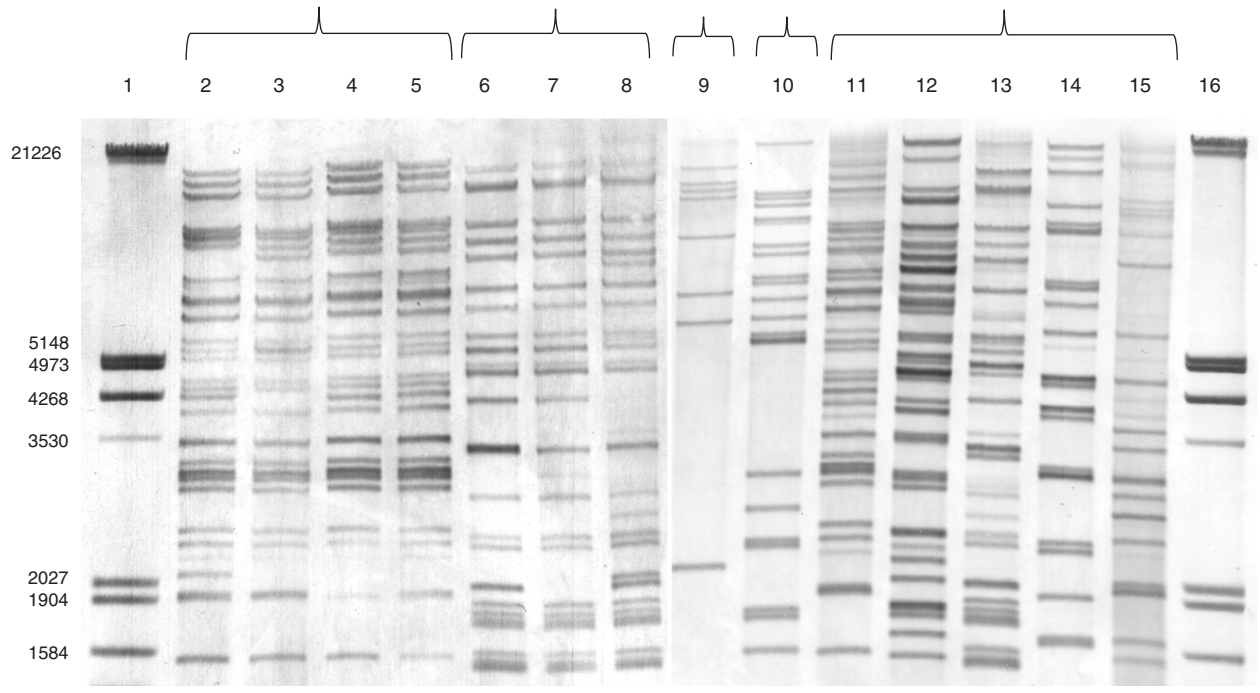

Figure 1 Southern blot of *Xho*-I digested DNAs from different *A. salmonicida* strains hybridized with an IS*630*-specific probe. Lanes 1 and 16, molecular size marker (sizes are indicated on the left in kilobase pairs); lanes 2 to 5 and 11, *A. salmonicida* subsp. *salmonicida* (JF2267, JF3224, JF3996, JF3507, JF3121 [formerly identified as atypical]); lanes 6 to 8 and 13, *A. salmonicida* subsp. *achromogenes* (JF3115, JF3116, JF2997, JF3123 [formerly identified as atypical]); lane 9, *A. salmonicida* subsp. *pectinolytica* (JF3120); lane 10, *A. salmonicida* subsp. *masoucida* (JF3118); lanes 12, 14 and 15, *A. salmonicida atypical* (JF3122, JF3124, JF3125).

Table 1 *Aeromonas* strains used in this study

JF N°	Synonyme	Species	Subspecies	Origin	Identified virulence characteristics	Pigment production (Day 6)	Ref
JF2996	Austin98	*salmonicida*	*salmonicida*	Sediment in Riccarton Loch, Scotland	*ascV-, ascU-, aexT+, aopP+, aopO-, aopH+*	+++	[17,18]
JF3507	ATCC 33658 T, NCIMB 1102 T	*salmonicida*	*salmonicida*	*Salmo salar*, Scotland	*ascV-, aexT+, aopP+, aopO+, aopH+, acrD-*	+++	[18-20]
JF3327	F330/04	*salmonicida*	*salmonicida*	Arctic char, Switzerland, 2004	*ascV+, aexT+, aopP+, aopO+, aopH+*	++++	[18]
JF3517	4757	*salmonicida*	*salmonicida*	Turbot, Norway	*ascV+, aexT+, aopP+, aopO+, aopH+*	++++	[18]
JF2267	Fi 94 G	*salmonicida*	*salmonicida*	Arctic char, Switzerland, 1999	*ascV+, ascU+, aexT+, aopP+, aopO+, aopH+, acrD+*	+++	[17,18,20]
JF2869	CCUG 47405 (A)	*salmonicida*	*salmonicida*	Arctic char (*Savelinus alpinus*)	*aexT+, SacrD 3′+*	++++	-
JF3223	Fi 210	*salmonicida*	*salmonicida*	White fish, Switzerland, 1997	*ascV+, aexT+, aopP+, aopO+, aopH+*	++++	[18]
JF3224	R04/170	*salmonicida*	*salmonicida*	Brown trout, Switzerland, 2004	*ascV+, ascU+, aexT+, aopP+, aopO+, aopH+*	++++	[17,18]
JF3518	4704	*salmonicida*	*salmonicida*	Turbot, Norway	*ascV+, aexT+, aopP+, aopO+, aopH+*	++++	[18]
JF2509	CC72 - D640	*salmonicida*	*salmonicida*	Atlantic salmon, Canada, before 1960	*ascV+, aexT+, aopP+, aopO+, aopH+, acrD+*	++++	[18,20]
JF3519	3294	*salmonicida*	*salmonicida*	Arctic char, Switzerland, 1986	*ascV-, aexT+, aopP+, aopO+, aopH-*	++++	[18]
JF2506	CC 27–80/9-1	*salmonicida*	*salmonicida*	Atlantic salmon Norway	*ascV+, aexT+, aopP+, aopO+, aopH+, acrD+*	++++	[18,20]
JF2507	CC 29 - 74/2	*salmonicida*	*salmonicida*	Atlantic salmon, Scotland	*ascV+, aexT+, aopP+, aopO+, aopH+, acrD+*	++++	[18,20]
JF2508	CC 63- D-615	*salmonicida*	*salmonicida*	Atlantic salmon, Canada	*ascV+, aexT+, aopP+, aopO+, aopH+, acrD+*	++++	[18,20]
JF2510	CC 23/8019-5	*salmonicida*	*salmonicida*	Atlantic salmon Norway	*ascV+, aexT+, aopP+, aopO+, aopH+, acrD+*	++++	[18,20]
JF3521	2265	*salmonicida*	*salmonicida*	Wild atlantic salmon, Norway 1991	*ascV-, aexT+, aopP+, aopO+, aopH-*	++++	[18]
JF3496	F05/160	*salmonicida*	*salmonicida*	Wild brown trout, Switzerland, 2005	*ascV+, aexT+, aopP+, aopO+, aopH+*	+++	[18]
JF3844	F06/417	*salmonicida*	*salmonicida*	Arctic char, Switzerland, 2006	*ascV+, aexT+, aopP+, aopO+, aopH+*	+++	[18]
JF2505	MT 44/SS 10	*salmonicida*	-	non virulent for trout, Canada	A+, LPS+, *acrD-*	+++	[20]
JF3791	F06/385	*salmonicida*	-	Arctic char *Salvelinus alpinus*, Switzerland, 2006	*ascV+, aexT+, aopP-aopO+, aopH+*	+++	[18]
JF4111	F07/357(NiA)	*salmonicida*	-	*Salvelinus*, Switzerland, 2007	ND	+++	-
JF4112	F07/357 (NiB)	*salmonicida*	-	*Salvelinus*, Switzerland, 2007	ND	+++	-
JF4113	F07/357 (NiC)	*salmonicida*	-	*Salvelinus*, Switzerland, 2007	ND	+++	-
JF3121	As209	*salmonicida*	*salmonicida* [formerly atypical]	Wolf fish, UK	*ascV-, ascU-*	+++	[17]

Table 1 *Aeromonas* strains used in this study *(Continued)*

JF4714	IMD1520	*salmonicida*	-	*Thymallus thymallus* (skin), Switzerland, 2009	ND	+++	-
JF4715	IMD 1521	*salmonicida*	-	*Thymallus thymallus* (kidney), Switzerland, 2009	ND	+++	-
JF4114	F07/357(LeA)	*salmonicida*	-	*Salvelinus* (liver), Switzerland, 2007	ND	+++	-
JF4115	F07/357 (LeB)	*salmonicida*	-	*Salvelinus* (liver), Switzerland, 2007	ND	+++	-
JF4116	F07/357 (LeC)	*salmonicida*	-	*Salvelinus* (liver), Switzerland, 2007	ND	+++	-
JF4118	F07/(MiB)	*salmonicida*	-	*Salvelinus* (kidney), Switzerland, 2007	ND	+++	-
JF4119	F07/357 (MiC)	*salmonicida*	-	*Salvelinus* (kidney), Switzerland, 2007	ND	+++	-
JF4117	F07/357 (MiA)	*salmonicida*	*salmonicida*	*Salvelinus* (spleen), Switzerland, 2007	ND	++++	-
JF3122	As204	*salmonicida*	atypical	Wrasse UK	*ascV+, aexT+, aopP + aopO-, aopH+*	++	[18]
JF3500	aAs 4143	*salmonicida*	atypical	Atlantic cod, Norway	*ascV+, aexT+, aopP + aopO+, aopH-*	++	[18]
JF3666	F06/211	*salmonicida*	atypical	Bleak (*Alburnus alburnus*), Switzerland, 2006	ascV+, aexT+, aopP- aopO-, aopH+	-	[18]
JF3124	As93	*salmonicida*	atypical	Plaice, Denmark	*ascV+, aexT+, aopP + aopO+, aopH+*	-	[18]
JF3520	4818	*salmonicida*	atypical	Atlantic Halibut, Norway, 2003	*ascV+, aexT-, aopP + aopO-, aopH+*	-	[18]
JF3115	ATCC 19261, NCIMB 1109	*salmonicida*	*achromogenes*	*Salmo trutta*	ND	+	-
JF3116	NCIMB 1110 T	*salmonicida*	*achromogenes*	Trout, Scotland	*ascV+, ascU+, aexT+, aopP+, aopO+, aopH+*	++	[17-19]
JF2997	F-265/87	*salmonicida*	*achromogenes*	Atlantic salmon, Iceland	*ascV+, ascU+, aexT+, aopP+, aopO+, aopH+*	++	[17,18]
JF3123	As183	*salmonicida*	*achromogenes* [formerly atypical]	Arctic char, Iceland	*ascV+, ascU+, aexT+, aopP+, aopO+, aopH+*	++	[17,18]
JF3499	aAs4101	*salmonicida*	*achromogenes*	Atlantic Cod, Iceland	*ascV+, aexT+, aopP + aopO+, aopH-*	-	[18]
JF3125	As 51	*salmonicida*	atypical	Rainbow trout, Norway	*ascV+, aexT+, aopP- aopO+, aopH+*	-	[18]
JF4097	-	*salmonicida*	*smithia*	*Salvelinus alpinus lepeschini*, Austria	*ascV+, aexT+, aopP+, aopO-, aopH+*	-	[21]
JF4460	-	*salmonicida*	*smithia*	*Salvelinus alpinus lepeschini*, Austria	*ascV-, aexT+, aopP+, aopO-, aopH+*	-	[21]
JF4439	-	*salmonicida*	*smithia*	*Salvelinus alpinus lepeschini*, Austria	*ascV+, aexT+, aopP+, aopO-, aopH+*	-	[21]
JF3117	NCMB13210, ATCC 49393	*salmonicida*	*smithia*	Roach, England	*ascV+, ascU+, aexT+, aopP-, aopO+, aopH+*	-	[17-19]
JF3126	As 54	*salmonicida*	atypical	Rainbow trout, Norway	*ascV-, aexT+, aopP-, aopO-, aopH-*	++	[18]
JF3502	aAs 4067	*salmonicida*	atypical	Spotted wolffish, Norway	*ascV+, aexT+, aopP+, aopO+, aopH+*	+	[18]

Table 1 *Aeromonas* strains used in this study *(Continued)*

JF3118	ATCC 27013 T	*salmonicida*	*masoucida*	Salmon, Japan	*ascV+, ascU+, aexT+, aopP-, aopO-, aopH+*	-	[17-19]
JF3119	NCMB 2020	*salmonicida*	*masoucida*	same as ATCC 27013 (salmon, Japan)	ND	-	-
JF2512	CC 30/8038	*salmonicida*	atypical	Atlantic salmon, Canada, before 1960	*ascV+, ascU+, aexT+, aopP+, aopO+, aopH+, acrD+*	-	[17,18,20]
JF2513	CC 34/8030	*salmonicida*	atypical	Atlantic salmon, Canada, before 1960	*ascV+, ascU+, aexT+, aopP+, aopO+, aopH+, acrD+*	-	[17,18,20]
JF3328	848 T	*molluscorum*	-	Type strain	ND	-	[22]
JF3071	ATCC 51106, bg sobria HG8	*veronii*	-	?	ND	-	[19]
JF2635	429/01 # 1c; official JF2635	*sobria*	-	*Perca fluviatilis*, Switzerland, 2001	*ascV+, ascU+, acrD+*	-	[17]
JF3326	-	*popoffii*	-	Urinary tract infection, France	ND	-	[23]
JF3120	DSM 12609 T	*salmonicida*	*pectinolytica*	River water	*ascV-, aexT-, aopP-, aopO-, aopH-*	++++	[17,19]
JF3240	LMG 17542, IK-B-r-15-1	*popoffii*	-	Drinking water production plant, Belgium	ND	-	[24]
JF2796	CECT 4199	*allosaccharophila*	-	Type strain	ND	-	[19]
JF3242	LMG 17547, AG-9	*popoffii*	-	Drinking water treatment plant, Scotland	ND	-	[24]
JF2797	LMG 17541^{T}, IK-0-a-10-3	*popoffii*	-	Drinking water production plant, Belgium	ND	-	[19,24]
JF3241	LMG 17544, IK-E-a- 14- 1	*popoffii*	-	Drinking water production plant, Belgium	ND	-	[24]
JF2905	Fi 125	*sobria*	-	Perch	*ascV+*	-	[25]
JF2791	ATCC 33907	*media*	-	Type strain NENT Nr. 2346-98	*ascV+, ascU+*	-	[17,19]
JF2899	F86/03-2	*sobria*	-	Perch	*ascV+*	-	[25]
JF2806	F533E	*popoffii*	-	Tap water, Switzerland, 2003	ND	-	[19]
JF2808	F600C	*popoffii*	-	Tap water, Switzerland, 2003	ND	-	[19]
JF2807	F548B	*popoffii*	-	Tap water, Switzerland, 2003	ND	-	[19]
JF 3954	868ET	*bivalvium*	-	Bivalve molluscs; Type strain	ND	-	[26]
JF2637	Fi 303	*hydrophila*	-	Ornamental fish	ND	-	-
JF2794	ATCC 49657, NENT Nr.2360-98	*trota* (*enteropelogenes*)	-	Human feces, India	ND	-	[19]
JF2785	CDC 9533-76	*bestiarum*	-	Type strain NENT Nr: N2341-98	ND	-	[19]
JF 4032	A28)A28B/1-1	*bestiarum*	-	Wild perch (*Perca fluviatilis*), Switzerland, 2007	ND	-	-
JF 4608	A28) 28B/1-1	*bestiarum*	-	Wild perch, Switzerland, 2009	*ascV+*	-	[22]
JF2804	F 530 D	*bestiarum*	-	Tap water	ND	-	-

Table 1 *Aeromonas* strains used in this study *(Continued)*

JF3018	68	*bestiarum*	-	River water	ND	-	-
JF3070	S 6874 T	*simiae*	-	Type strain	ND	-	[19]
JF2786	ATCC 15468	*caviae*	-	Type strain NENT Nr. N2344-98	ND	-	[19]
JF2789	ATCC 7966	*hydrophila*	-	Type strain NENT Nr. : N2339-98	ND	-	[19]
JF2793	CIP 7433; ATCC 43979	*sobria*	-	Type strain NENT Nr.2352	ND	-	[19]
JF2929	Fi 179a	*sobria*	-	Perch, Switzerland	*ascV* + *SacrD*+	-	[22]
JF2788	NCMB 74; ATCC 23309	*eucrenophila*	-	Type strain NENT Nr. N2348-98	ND	-	[19]
JF3069	ATCC 49904 T	*ichthiosmia*	-	Type strain Antonella Demarta	ND	-	-
JF2790	ATCC 49568	*jandaei*	-	Type strain NENT Nr. 2355-98	ND	-	[19]
JF3067	CIP 107763 T	*culicicola*	-	Type strain	ND	-	[19]
JF3068	ATCC 49803 T	*enteropelogenes*	-	Type strain	ND	-	-

ND: not determined.

culicicola (Table 1). The fingerprints (Figure 1) of the analyzed strains were subjected to similarity analysis and are shown in Figure 2.

HCN-IS*630*-RFLP profiles and stability of IS*630* insertions

A high degree of IS*630* polymorphism, both in a numerical and positional sense, was observed between the various *A. salmonicida* subspecies (Figure 1). However, the patterns revealed that IS*630* copy numbers and positions are well conserved within the given subspecies (Figure 1). The dendogram in Figure 2 is a RFLP tree that reveals the evolutionary relationship between strains analyzed. Strains of the subspecies *salmonicida*, *smithia*, *achromogenes* and *masoucida* each grouped together showing a similar banding pattern. The number of IS*630*-positive bands varied from 27 to 35 in *A. salmonicida* subsp. *salmonicida*, 23 to 33 in *achromogenes* and 19 to 21 in *smithia*. Within a subspecies, several bands were conserved: 21 in *salmonicida*, 20 in *achromogenes* and 13 in *smithia* subspecies. About 15 distinct patterns were observed in *A. salmonicida* subsp. *salmonicida* without showing geographical association. The IS*630* pattern of *A. salmonicida* subsp. *salmonicida* strain A449 as calculated from the genome sequence data closely clusters with these 15 patterns. In contrast, each pattern in the *achromogenes* cluster was different. In *A. salmonicida* subsp. *masoucida* 15 to 21 positive bands were detected and only 8 in the subspecies *pectinolytica*. Even though the copy numbers vary within the subspecies, the patterns form clusters for each subspecies. The most remarkable tight clustering was found for *A. salmonicida* subsp. *salmonicida*. This latter presents IS*630* patterns that only show minute differences among strains that were isolated from various continents and over a period of half a century. No pattern was specific of a given geographical region. The results showed also that strains JF3121 and JF3123, formerly classified as *A. salmonicida* atypical, clustered with *A. salmonicida* subsp. *salmonicida* (JF3121) and subsp. *achromogenes* (JF3123) (Figures 1 and 2) showing that they were misclassified previously.

The IS*630* pattern of *A. salmonicida* subsp. *salmonicida* strain JF 2267 that was subcultured for 4 days at 18°C and 25°C (in stressing conditions) to reach approximately 20 generations remained unchanged (results not shown) indicating a good stability of IS*630* under experimental growth conditions.

Copy number of the IS*630* element and RFLP among other *Aeromonas* species

Other *Aeromonas* species revealed lower copy numbers of IS*630*: 5 in *A. molluscorum*, 5 to 8 in clinical *A. sobria strains*, 9 in *A. veronii*, 5 in *A. allosaccharophila* and *A. media*. Only one copy was found in *A. bivalvium* and a clinical strain of *A. hydrophila*. No signal for IS*630* was obtained in *A. caviae*, *A. trota*, *A. simiae*, *A. eucrenophila*, *A. ichthiosmia*, *A. jandaei*, *A. culicicola*, *A. enteropelogenes*, *A. bestiarum* and the type strains of *A. hydrophila and A. sobria*. Among the 8 strains of *A. popoffii* we found 6 very distinct patterns.

Analysis of IS*630* abundance, localization and impact on the genome of *Aeromonas* species

In order to study the origin of IS*630* in *A. salmonicida*, we performed a profound analysis and comparison of published *Aeromonas* genomes (Additional file 2: Table S2). The genetic environment of IS*630* copies in the *A.*

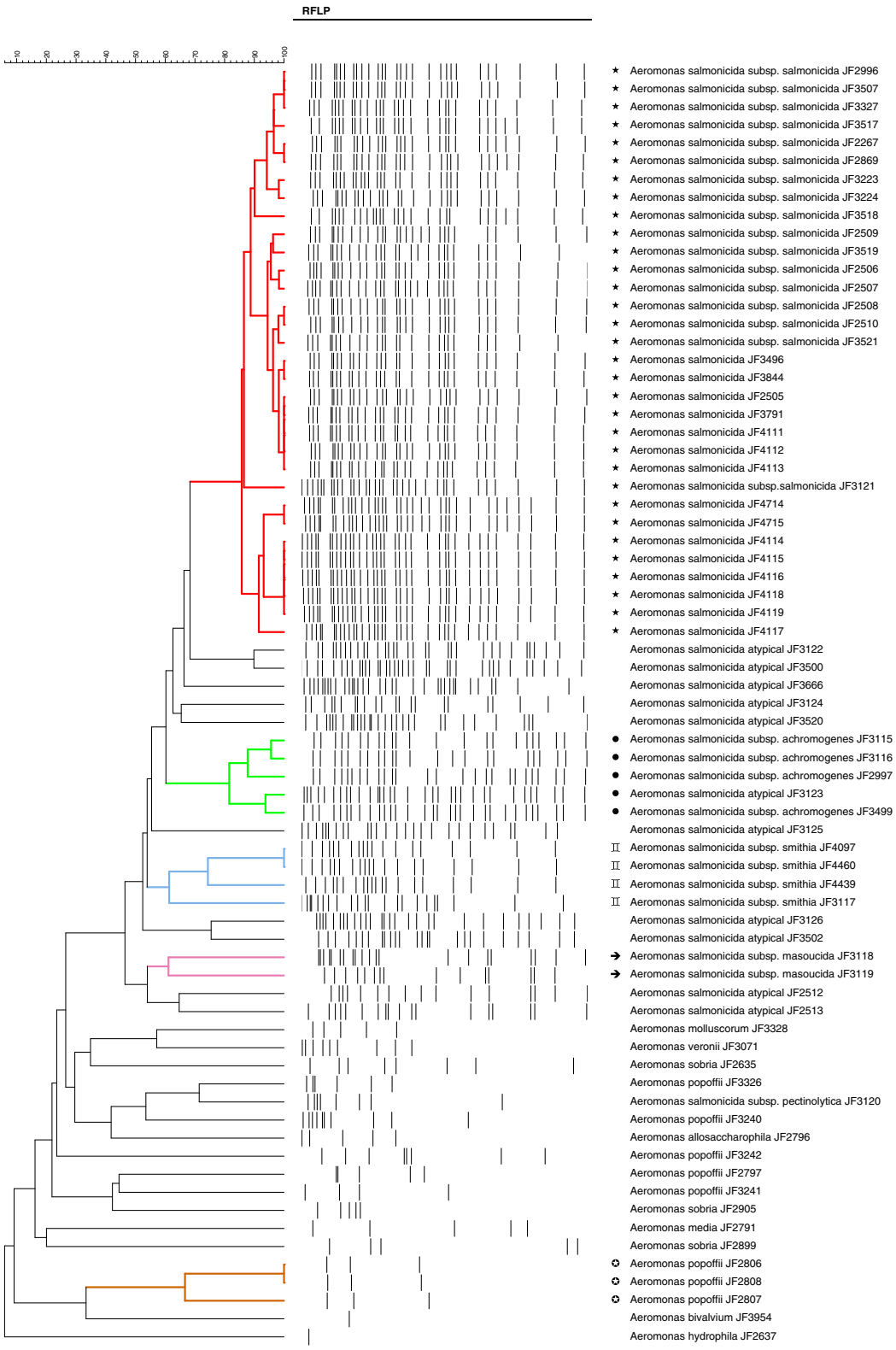

Figure 2 Dendogram generated from the IS*630*-RFLP patterns of the 87 *Aeromonas* strains used in this study. The tree was established by using the UPGMA clustering analysis with the BioNumerics software. In red (★), the *A. salmonicida* subsp. *salmonicida* cluster; in green (●), the *A. salmonicida* subsp. *achromogenes* cluster; in blue (Ⅱ), the *A. salmonicida* subsp. *smithia* cluster; in pink (➔), the *A. salmonicida* subsp. *masoucida* cluster; and in brown (✪), *A. popoffii* strains clustering together.

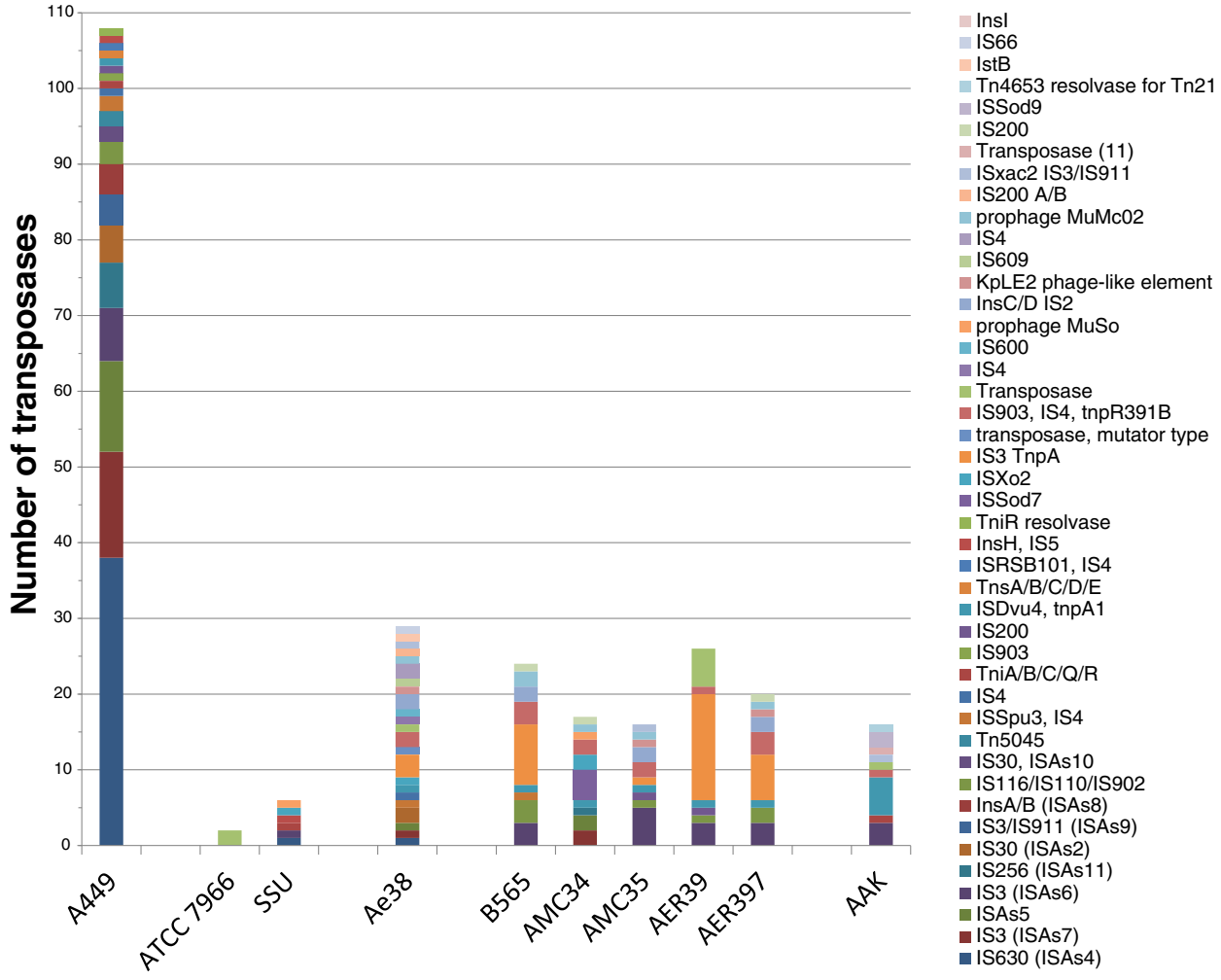

Figure 3 Number of transposases and IS family affiliation in *Aeromonas* sp. *A. salmonicida* A449 [GenBank: CP000644.1, CP000645.1 and CP000646.1], *A. hydrophila* ATCC 7966 and SSU [GenBank: CP000462.1 and AGWR00000000.1], *A. caviae* Ae398 [GenBank: CACP00000000.1], *A. veronii* B565, AMC34, AMC35, AER39 and AER397 [GenBank: CP002607.1, AGWU00000000.1, AGWW00000000.1, AGWT00000000.1 and AGWV00000000.1], and *A. aquarorium* AAK1 [GenBank: AP012343.1].

salmonicida subsp. *salmonicida* A449 genome is shown in detail in Additional file 1: Table S1. About 148 loci or DNA sequences forming 108 complete or partial IS units were found in the chromosome of *A. salmonicida* subsp. *salmonicida* A449 and on the plasmids pASA4/pASA5 [GenBank: CP000644.1, CP000645.1 and CP000646.1]. IS*630* (referred to as ISAs*4* in the Genbank genome annotation of *A. salmonicida* A449 and as ISAs7 in the corresponding manuscript [16]) was found to be present in 38 copies and was the most abundant family representing 35% of transposons in *A. salmonicida* A449 (Figure 3, Additional file 3: Table S3). The different copies are well-conserved and show 98% nucleotide sequences identity. The other 70 IS elements are ISAs7 (13%), ISAs5 (11%), ISAs6 (6%), ISAs11 (6%), ISAs2 (5%), ISAs9 (4%), ISAs8 (4%), and unclassified ISAs (16%) (Figure 3). 90% of the IS*630* copies reside in chromosomal regions that are specific to *A. salmonicida* subsp. *salmonicida* and were not found in other *Aeromonas*. Interestingly most of these loci correspond to known genes in bacterial genera other than *Aeromonas*. This is the case for instance for the hypothetical gene ASA_1385 (homology to VOA_002034 of *Vibrio* sp. RC586) that is directly linked to IS*630* in *A. salmonicida* subsp. *salmonicida* and is not found in other Aeromonads (Additional file 2: Table S2). In ISAs families other than IS*630*, 34 (31%) are directly adjacent to IS*630* showing that 66% of *A. salmonicida* A449 transposons are associated to genomic domains of variability. In comparison to other *Aeromonas* sp., *A. salmonicida* A449 contains 4 to 54 fold more transposases (Figure 3) which are not responsible for a genome-reductive evolution [27] because the total number of ORFs is stable in comparison to other Aeromonads (Figure 4). However they explain the high abundance of pseudogenes (170) in *A. salmonicida* subsp.

	Strain	Total ORFs	Common ORFs	%	Specific ORFs	%	Shared ORFs	%
A. salmonicida	A449	4472	2894	65	470	11	1108	25
	01-B526	4460	2894	65	502	11	1064	24
A. hydrophila	ATCC 7966	4175	2894	69	180	4	1101	26
	SSU	4390	2894	66	249	6	1247	28
A. caviae	Ae38	4148	2894	70	440	11	814	20
A. veronii	B565	4082	2894	71	355	9	833	20
	AMC34	4079	2894	71	433	11	752	18
	AMC35	4012	2894	72	437	11	681	17
	AER39	3909	2894	74	278	7	737	19
	AER397	3957	2894	73	337	9	726	18
A. aquarorium	AAK	4345	2894	67	294	7	1157	27

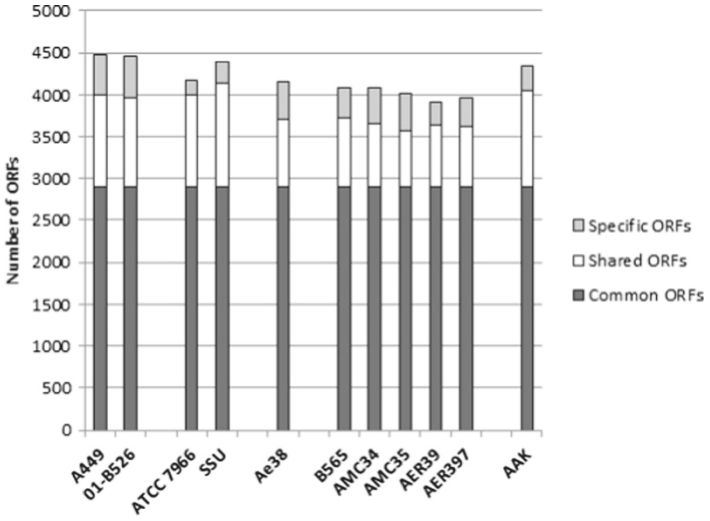

Figure 4 Numerical comparison of common, shared and specific ORFs between several *Aeromonas* species. The number of ORFs was calculated from Additional file 2: Table S2 without taking into account IS elements, tRNA and rRNA. In dark grey, the number of ORFs that are common among *Aeromonas* sp. In white, ORFs that are shared with at least one other *Aeromonas* species. In light grey, ORFs that are unique to the species. *A. salmonicida* subsp. *salmonicida* A449 and 01-B526, *A. hydrophila* ATCC 7966 and SSU, *A. caviae* Ae398, *A. veronii* B565, AMC34, AMC35, AER39 and AER397, and *A. aquarorium* AAK are illustrated in the graph.

salmonicida [16] in contrast to *A. hydrophila* ATCC 7966 which only contains 7 pseudogenes and 2 transposases.

Discussion

HCN-IS*6110*-RFLP has been applied as a standard method to subtype *Mycobacterium tuberculosis* strains for years [28]. Moreover, RFLP based on IS elements has been employed to type numerous other pathogenic bacteria [14,15,29-31]. The published genome of *A. salmonicida* subsp. *salmonicida* A449 shows numerous IS elements among which 38 belong to the IS*630* family [GenBank: CP000644.1]. We therefore used HCN-IS*630*-RFLP as a new typing methodology for *Aeromonas* species.

IS*630* was present in different copy numbers and integrated at various sites between the different *A. salmonicida* subspecies. On the other hand banding patterns were conserved within subspecies (Figure 1). HCN-IS*630*-RFLP revealed that IS*630* is abundant in all subspecies of *A. salmonicida* allowing a good accuracy for genomic fingerprinting. Our results showed that RFLP profiles can be used to distinguish subspecies of *A. salmonicida* and to differentiate *A. salmonicida* from other *Aeromonas* species. They also indicate a high variability among strains of 'atypical' *A. salmonicida*. All strains of yet unclassified 'atypical' *A. salmonicida* consisted of a high number of IS*630* copies and were effectively related to the *A. salmonicida* cluster. Our method demonstrates that such 'atypical' strains represent a heterogeneous group that does not fit into the classification of the five described *A. salmonicida* subspecies. These strains might represent various subtypes of *A. salmonicida* subsp. *salmonicida* or novel subspecies of *A. salmonicida* that have adapted to particular ecological niches or respective hosts. On the other hand, all *A. salmonicida subsp. salmonicida* isolated since the 1950s and originating from all over the world have very similar patterns, indicating that they form a single clone showing pathoadaptational stability. Altogether, our results confirm those of a previous study comparing genomic profiles of clinical isolates of *Aeromonas salmonicida* using DNA microarrays [32]. With the origin and intensification of fish farming, genetic

rearrangements occurring through IS transposition events could have been responsible for the selection and the emergence of this pathogenic fish specific clone. Such an adaptation process of a pathogenic bacterium towards its host was recently indicated in the *Mycoplasma mycoides* cluster for *Mycoplasma mycoides* subsp. *mycoides* [33]. Moreover, no unique pattern was associated to a specific geographical region of the world and we assume that this could be explained by the dissemination of *A. salmonicida* subsp. *salmonicida* strains between aquaculture countries via the intensification of the international trade in farmed salmon or by the natural migration of wild salmons.

Besides the epidemiologic and phylogenetic interests of IS*630* fingerprinting to subtype *A. salmonicida*, we studied the characteristics of this predominant IS element to reveal information concerning the pathoadaptation towards its specific host. Mobile genetic elements can exert different effects on bacterial genomes [11,34-36]. Through such genomic effects, IS*630* family has had an impact on the modulation of virulence genes in other bacteria [37-43]. In *A. salmonicida* 90% of the IS*630* copies reside in genomic regions that are variable between *Aeromonas* sp. (Additional file 1: Table S1) and 80% of these sites contain genes that are specific to *A. salmonicida* and are not encountered in other *Aeromonas* sp. suggesting that they constitute genomic islands. A part of these coding sequences are phages or hypothetical genes with homologues of characterized sequences in other environmental bacteria: i.e. the 'Vibrio Seventh Pandemic cluster I' (VSP-I), genes for the synthesis of polysaccharide capsule, lipopolysaccharide, S-layer, chitinase, cytolytic insecticidal delta-endotoxin, and some effectors (AopO and ApoH) of the type-three secretion system, the major virulence system of the bacterium. Based on these findings we assume that IS*630* elements could be used by environmental bacteria to exchange DNA fragments between each other by horizontal transfer. In the genomic islands where IS*630* is present, supplementary IS elements can be found, which might serve as hot spots for further insertions. This would allow the transposon and the genomic island to evolve with acquisition of new genes without disruption of existing loci. These observations could explain why the IS*630* elements remained stable within the *A. salmonicida* subsp. *salmonicida* genome.

Other interesting characteristics of IS elements homologous to IS*630* in *A. salmonicida* suggest that they could play a role in the co-adaptation of the bacterium with its host by trans-kingdom horizontal gene transfers through the bacterial T3SS: (i) such IS*630* elements are mostly present in Gram-negative bacteria that use a T3SS, (ii) their expression can be specifically induced or increased when bacteria are in direct contact with host cells [44] and (iii) several IS*630* are predicted to be T3SS effectors [45]. The Modlab® T3SS effector prediction software gives for *A. salmonicida* IS*630* a positive output at 0.69 which means, that the IS*630* itself is a potential T3SS effector. Hence, when the bacteria colonize the host, the IS*630* expression could be induced and they could begin to exert their transposase activity by excising the transposon (composite if associated to adjacent additional DNA fragments) from the bacterial genome. Subsequently, the transposase linked to its transposon could be translocated into the host cell by the T3SS, reach the host genome in the nucleus, and finally perform its transposition.

Bacterial IS*630* elements constitute with the Tc*1*/mariner eukaryotic DNA transposon family, a superfamily [46]. It was demonstrated *in vitro* that eukaryotic members of this family are able to transpose into prokaryotic genomes [46]. We suppose that the opposite could also be possible as IS*630* itself could be translocated via type three secretion system from the pathogen to its host. In this perspective, our assumption could explain how the adaptive horizontal transfer of a bacterial mannanase gene (HhMAN1) into the genome of an invasive insect pest of coffee (*Hypothenemus hampei*) occurred in the immediate genetic vicinity of a Tc1/mariner transposon [47].

Conclusions

In this study we describe HCN-IS*630*-RFLP as an adequate method for subtyping *A. salmonicida* strains and to differentiate *A. salmonicida* from other *Aeromonas* species. The high degree of conservation of HCN-IS*630*-RFLP profiles among strains of *A. salmonicida* subsp. *salmonicida* isolated from geographically most distant areas and over the period of half a century shows that practically all copies of IS*630* are stably integrated in this pathogen that has a well-defined host range. We therefore conclude that IS*630* might have contributed to the pathoadaptation of *A. salmonicida* to salmonidae and to the emergence of the subtype *A. salmonicida* subsp. *salmonicida*.

Methods

Bacterial strains and growth conditions

Aeromonas strains used in this study are listed in Table 1. Bacteria were grown on trypticase soy agar plates at 18°C for 3 to 6 days until sufficient bacteria were available for DNA extraction.

Southern blot analysis with *A. salmonicida* subsp. *salmonicida* IS*630* probe

Total DNA extraction from each strain was performed with the Peqgold Bacterial DNA extraction Kit (Peqlab Biotechnologie, Erlangen, Germany). One microgram of DNA from each sample was digested overnight with XhoI restriction enzyme (Roche Diagnostics, Mannheim, Germany), loaded on a 0.7% agarose gel and subjected

to electrophoresis for 4 to 5 hours. On each gel a DIG-labeled DNA Marker (Roche Diagnostics, Mannheim, Germany) and XhoI digested DNA from *A. salmonicida* subsp. *salmonicida* JF2267 were loaded for normalization. DNA bands were stained with ethidium bromide for control and transferred onto a nylon membrane (Roche Diagnostics, Mannheim, Germany) with a VacuGene apparatus (GE Healthcare Bio-Sciences). The IS*630* probe was prepared by PCR using primers Clust_asa1052_S6 (5′- AGGCAGAACTTGGGGTTCTT-3′) and Clust_asa-1052_R4 (5′- ACAAAAGCGGGTTGTCACTC-3′) and DNA of *A. salmonicida* subsp. *salmonicida* JF2267 as a template. PCR was performed in 30 μL which contained 0.5 μL of Taq DNA polymerase (5 units/μL) (Roche Diagnostics, Mannheim, Germany), 300 nM of each primer, 1.75 mM $MgCl_2$, 200 μM concentrations of each dNTP and 1 μl of the Digoxigenin-11-dUTP (1 nmol/μL) (Roche Diagnostics, Mannheim, Germany). Each reaction involved a denaturing step at 94°C for 5 min followed by 30 cycles of 10 sec at 94°C, 30 sec at 54°C and 60 sec at 72°C and a final extension step of 7 min at 72°C.

Bioinformatic analysis

The hybridization patterns were scanned and the data were analyzed using the BioNumerics software version 6.6 (Applied Maths, Kortrijk, Belgium). Bands automatically assigned by the computer were checked visually and corrected manually. Cluster analysis of the IS-RFLP patterns was done by the unweighted pair group method with average linkages (UPGMA) using the Dice coefficient and the following parameters: 0.5% Optimization, 0% Band filtering, 0.5% Tolerance and ignore uncertain bands.

Prediction of T3SS effectors was performed with the Modlab® online software (http://gecco.org.chemie.uni-frankfurt.de/T3SS_prediction/T3SS_prediction.html) [45].

Stability of IS*630* in cultured *A. salmonicida* subsp. *salmonicida*

The stability of IS*630* under growth conditions in TSB medium was assessed by daily 100x dilution of a culture of strain JF2267 at 18°C and at 25°C during 4 days to reach 20 generations. Every day DNA was extracted from 10^9 bacteria, digested with XhoI and submitted to southern blot hybridization.

Additional files

Additional file 1: Table S1. Table showing for each *A. salmonicida* A449 IS*630* copy, the size of the XhoI-digested DNA fragment containing the IS, the inter- or intragenic localization, the characteristics of the adjacent genes, and the association to a region of variability or to other IS elements.

Additional file 2: Table S2. Profound analysis and comparison of published *Aeromonas* genomes used for Figures 3 and 4. Grey: conserved ORFs; light green: ORFs specific of the species; yellow: IS*630*; pink: other IS elements; red: putative or characterized virulence factors; mauve: ORFs for resistance to antibiotic or heavy metal; dark green: ORFs associated to pili, fimbriae or flagella; blue: ORFs associated to phage; cyan: tRNA and rRNA; orange: ORFs with homology to eukaryotic genes.

Additional file 3: Table S3. Detail of loci corresponding to transposons in *Aeromonas* sp.

Abbreviations

HCN-IS-RFLP: High copy number insertion element restriction fragment length polymorphism; T3SS: Type-three secretion system; UPGMA: Unweighted pair group method with arithmetic mean.

Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

NS carried out the experiments, performed BioNumerics analysis and drafted the manuscript. JF participated in the coordination of the study and helped to draft the manuscript. PVB conceived of the study, participated in its design and coordination, carried out experiments, performed bioinformatic analysis and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was funded by the Swiss National Science Foundation grant no. 31003A-135808.

Received: 19 November 2012 Accepted: 5 February 2013
Published: 13 February 2013

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doi:10.1186/1471-2180-13-36
Cite this article as: Studer *et al.*: Clustering subspecies of *Aeromonas salmonicida* using IS*630* typing. *BMC Microbiology* 2013 **13**:36.