

Mechanisms of quinolone resistance and clonal relationship among *Aeromonas salmonicida* strains isolated from reared fish with furunculosis

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The mechanisms of resistance to quinolone and epidemiological relationships among *A. salmonicida* strains isolated from diseased fish in French marine farms from 1998 to 2000 were investigated. The quinolone resistance-determining regions of the *gyrA* and *parC* genes of 12 clinical *A. salmonicida* isolates with different levels of quinolone susceptibility were sequenced. MICs were determined in the presence of the efflux pump inhibitor (EPI) Phe-Arg β -naphthylamide and $E_{\max}$ values (MIC without EPI/MIC in the presence of EPI) were calculated. Isolates fell into two classes: (i) those that had a wild-type *gyrA* gene with oxolinic acid MIC ≤ 0.5 , flumequine MIC ≤ 1 and ciprofloxacin MIC $\leq 0.25 \mu\text{g ml}^{-1}$; and (ii) those that had a single mutation in *gyrA* encoding Asp-87 $\rightarrow$ Asn with oxolinic acid MIC ≥ 2 , flumequine MIC ≥ 4 and ciprofloxacin MIC $\geq 0.125 \mu\text{g ml}^{-1}$. No mutations were found in *parC*. High $E_{\max}$ values obtained for flumequine and oxolinic acid (up to 16 and 8, respectively, for the most resistant isolates of the two classes) indicated an important contribution of efflux to the resistance phenotype. Flumequine accumulation experiments confirmed that high $E_{\max}$ values were associated with a much lower level of accumulation. PCR/RFLP assays conducted on 34 additional isolates showed the presence of a mutation at codon 87 of *gyrA* in nearly all the quinolone-resistant isolates. This finding, together with PFGE typing results, strongly suggests a common clonal origin of these quinolone-resistant isolates.

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INTRODUCTION

Aeromonas salmonicida is the causal agent of furunculosis, a debilitating and lethal disease affecting numerous farmed fish species including salmonids, turbot and carp (Noga, 2000). With daily mortality rates typically between 0.1 and 1%, chronic furunculosis is responsible for massive economic losses for fish farms. Commercial vaccines and vaccination procedures have been developed for use in salmonid farming. However, they are still not widely used in France and offer poor protection in other fish species (Santos *et al.*, 2003). Furunculosis is frequently treated orally with quinolone antibiotics, principally oxolinic acid and flumequine. In France, the recommended treatment durations are 5 and 6–7 days for flumequine and oxolinic acid, respectively, as reported in the specifications for products

used to treat freshwater salmonids. Recommended dosages are $12 \text{ mg kg}^{-1} \text{ day}^{-1}$. Currently, extended treatment durations of up to 15 days and dosages up to $30 \text{ mg kg}^{-1} \text{ day}^{-1}$ have been observed in marine fish antibiotherapy for both quinolones. This use of quinolones has resulted in the emergence of *A. salmonicida* quinolone-resistant isolates, which have been isolated from diseased fish (Grant & Laidler, 1993; Hastings & McKay, 1987; Inglis & Richards, 1991; Oppegaard & Sorum, 1994; Tsoumas *et al.*, 1989; Wood *et al.*, 1986). In aquaculture, diagnoses are often presumptive and first-line treatments are administered in the absence of susceptibility data concerning the causative pathogen. The increasing prevalence of quinolone-resistant *A. salmonicida* strains has made the use of these antibiotics inappropriate in many cases. This misuse leads not only to therapeutic failures but also to the useless release of active ingredients into the aquatic environment, contaminating the surrounding water and sediments, either directly from uningested food or indirectly through excretion. Although the real selective pressure exerted by these antibiotics on the aquatic microflora is difficult to evaluate (Smith *et al.*, 1994), many studies

Abbreviations: EPI, efflux pump inhibitor; QRDR, quinolone resistance-determining region.

The GenBank accession number for the partial sequence of the *parC* gene of *A. salmonicida* ATCC 14174 is AF473701.

report an increase in the frequency of resistant bacteria isolated from water, sediments or wild animals in the vicinity of fish farms (Ervik *et al.*, 1994; Guardabassi *et al.*, 2000; Samuelsen *et al.*, 1992; Schmidt *et al.*, 2000; Spanggaard *et al.*, 1993). Guidelines for a rational use of antibiotics such as those available for the treatment of bacterial infections in humans and land animals are still poorly developed in the case of fish diseases. In particular, susceptibility criteria, which should be taken into account when selecting an antimicrobial agent, are not clearly defined for fish-pathogenic bacteria. The definition of such criteria requires the acquisition and integration of clinical, microbiological, pharmacokinetic/pharmacodynamic and molecular data on the interactions between the fish, their pathogens and aquacultural antibiotics. Whereas the use of fluoroquinolones was recently banned in the USA in many food-animal productions, including aquaculture, it is still authorized in the European Union. Policies are needed particularly to regulate the recent but increasing use of veterinary fluoroquinolones in aquaculture. Indeed, sarafloxacin has been approved for oral use in fish, and other veterinary fluoroquinolones like enrofloxacin and marbofloxacin may be used 'off-label' under the prescription of veterinarians (Martinsen & Horsberg, 1995; FAO, 2002). The main concern is the risk of selecting bacterial human pathogens, which are commonly found in and near aquaculture systems, with a cross-resistance to the fluoroquinolones used in human medicine (Angulo, 2000; Lehané & Rawlin, 2000; Twiddy, 1995).

The aim of this study was to investigate the mechanisms of resistance to quinolones in strains of *A. salmonicida* isolated from cultured fish with furunculosis. We assessed: (i) the presence of mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes, encoding the A subunits of DNA gyrase and topoisomerase IV, respectively, the target enzymes for quinolones (Hooper, 2001); and (ii) the presence and contribution of an efflux mechanism (Poole, 2000). The results led us to investigate the epidemiological relationship among the isolates using PFGE.

METHODS

Bacterial strains and growth media. A total of 46 *A. salmonicida* clinical strains and one reference strain, *A. salmonicida* ATCC 14174, were investigated. All clinical strains were isolated from diseased sea-water fish as part of an antibioresistance survey conducted between 1998 and 2000 in marine fish farms located on the west coast of France (Blanc *et al.*, 2002). A first set of 12 clinical strains spanning various levels and patterns of resistance to quinolones was chosen for sequencing of the topoisomerase genes and efflux experiments. For PCR/RFLP experiments, 34 other strains were randomly sampled from two groups corresponding to two different clinical contexts. Some of these strains had been isolated in the same farm, during apparently independent furunculosis outbreaks that occurred at different times. Twenty of the strains had been isolated during furunculosis outbreaks that had been successfully treated with oxolinic acid or flumequine (defined as a decrease in daily mortality rates to stable values less than 0.1 %, obtained within a treatment time of less than 15 days) and were thus considered to be clinically susceptible. The other 14 strains had been isolated at the beginning of outbreaks for which the quinolone treatment had resulted in a therapeutic failure. They were thus considered to be clinically

resistant. Considering the current treatment durations in France, the decrease in daily mortality rates within 15 days of treatment was retained as a preliminary indicator of a positive clinical outcome. All *A. salmonicida* strains were routinely grown at 22 °C on tryptic soy agar, on Mueller–Hinton agar or in Mueller–Hinton broth. *Escherichia coli* JM109 was used for cloning of the *parC* gene fragment of *A. salmonicida* ATCC 14174.

MIC determination. For all strains, MICs of oxolinic acid, flumequine and ciprofloxacin were determined by the 2-fold agar dilution method according to the guidelines defined by the working group on the development of standard reference methods for antimicrobial agent susceptibility testing for bacterial fish pathogens (CEFAS, 1999). MICs of the three quinolones were also determined for all of the strains used in sequencing experiments, in the presence of serial 2-fold dilutions (1–256 µg ml⁻¹) of the efflux pump inhibitor (EPI) Phe-Arg β-naphthylamide (Sigma). The plates were incubated for 48 h at 22 °C, after which $E_{\max}$ values were calculated. These were defined as the ratio between MIC without EPI and MIC in the presence of a maximal potentiating concentration of EPI (Lomovskaya *et al.*, 2001). Analytical-grade oxolinic acid and flumequine were purchased from Sigma and ciprofloxacin from Bayer.

DNA isolation. Chromosomal DNA was extracted from the strains by harvesting the cells from 1 ml of an overnight culture, resuspending the pellet in 100 µl sterile water and boiling for 3 min. After centrifugation for 3 min at 10 000 g, supernatants were collected and 1 : 100 dilutions in sterile water were used as a template for PCR.

Amplification and sequencing of the QRDR of the *gyrA* and *parC* genes. The sequences of primers used in the PCRs are given in Table 1. Primers ASGYRA1 and ASGYRA2 were designed from the nucleotide sequence of the *A. salmonicida gyrA* gene (GenBank accession no. L47978). PCRs were carried out in a total volume of 25 µl containing 25 pmol of each primer, 200 µM dNTPs, 1.5 mM MgCl₂, 0.5 U *Taq* polymerase (Qiagen) and 5 µl of the template DNA dilution. Standard PCR conditions were used: 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The 663 bp amplicons were purified using a QIAquick Spin PCR Purification kit (Qiagen) and sequenced with the primers used for PCR by the fluorescent dideoxy chain-terminating method using a Perkin-Elmer 3700 capillary device (Genome Express).

An alignment of ParC amino acid sequences of various bacterial species, obtained using CLUSTAL W (<http://www.infobiogen.fr/services/>), was used to design the degenerate primers ASPARC1 and ASPARC2, with the help of CODEHOP software (<http://blocks.fhcrc.org/codehop.html>). ParC sequences of the following species were included in the multi-alignment: *E. coli* (GenBank accession no. M58408), *Salmonella enterica* (NC_003197), *Vibrio parahaemolyticus* (AB023570) and *Pseudomonas aeruginosa* (AB003428). Amplification of a 447 bp amplicon overlapping the *parC* QRDR from *A. salmonicida* ATCC 14174 was possible using these degenerate primers. PCR conditions were the same as those described above, except that the annealing temperature was 68 °C. The PCR product was cloned into the pGEM-T Easy vector (Promega) and sequenced (Genome Express). Two specific primers, designated ASPARC3 and ASPARC4, were designed from this sequence to amplify the *parC* QRDR sequence of other *A. salmonicida* strains. PCR with primers ASPARC3 and ASPARC4 was carried out under the standard conditions described above, but with an annealing temperature of 60 °C. The 418 bp *parC* fragments were purified and sequenced directly, as for the *gyrA* sequence determination.

Sequence analysis. The *gyrA* and *parC* QRDR sequences and the putative amino acid counterparts of the 12 clinical strains studied and of *A. salmonicida* ATCC 14174 were aligned and compared using CLUSTAL W. Percentage identities were calculated using LEASTA.

Table 1. Oligonucleotide primers used for PCR amplifications and sequencing

Primer	Position	Sequence (5' → 3')
ASGYRA1	<i>gyrA</i> , nt 74–93	CCATGAGCGTGATCGTAGGA
ASGYRA2	<i>gyrA</i> , nt 717–736	CTTTGGCACGCACATAGACG
ASGYRA3	<i>gyrA</i> , nt 223–258	GGTAAATACCAACCCGACGGCGACAGTGCCGTGTCT
ASGYRA4	<i>gyrA</i> , nt 362–380	ATCCGCACCTCGGTGTAAC
ASPARC1	<i>ParC</i> , aa 35–42*	GGCGATGGCCTGAARCCNGTNCA
ASPARC2	<i>ParC</i> , aa 176–183*	GTGCGGCGGGATATCNGTNGCCAT
ASPARC3	<i>parC</i> , nt 126–143*	CAGCGGCGCATCATCTAC
ASPARC4	<i>parC</i> , nt 526–543*	GGATATCGGTGGCCATGC

*The positions of the degenerate primers ASPARC1 and ASPARC2 correspond to amino acid residues of *E. coli*. The positions of ASPARC3 and ASPARC4 correspond to nucleotide positions of the *E. coli parC* gene.

Detection of mutations at codon 87 by PCR/RFLP. Primers ASGYRA3 and ASGYRA4 were used to amplify a 158 bp PCR product (Fig. 1). The forward primer, ASGYRA3, whose sequence is different by one base from the *gyrA* gene sequence, was designed to introduce an artificial *Hpy188I* cleavage site overlapping the first two bases of codon 87, according to the primer-specified restriction site modification method (Haliassos *et al.*, 1989). PCR was performed as described for amplification of the sequenced fragments, with an annealing temperature of 58 °C. Three microlitres of the PCR product was digested with 1 U *Hpy188I* in a total volume of 8 µl. Digested PCR products were resolved in a 2.5 % agarose gel, alongside a lambda *EcoRI/HindIII* DNA marker. The presence of a mutation at codon 87 was detected by non-digestion of the 158 bp PCR product to two products of 122 and 32 bp (Fig. 2). This method makes it possible to detect all amino acid substitutions at position 87 with the exception of the Asp-87 → Glu substitution (which is due to a mutation of the third base of codon 87, outside the *Hpy188I* restriction site).

Flumequine accumulation experiments. Bacteria were grown to late exponential phase at 22 °C in Mueller–Hinton broth, harvested by

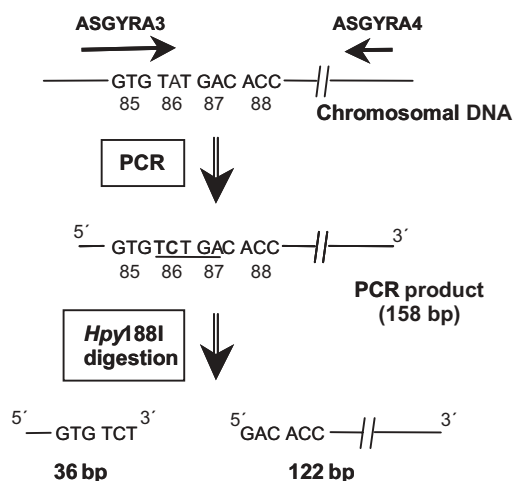


Fig. 1. Schematic diagram of the PCR/RFLP assay used to detect mutations at codon 87 of the *gyrA* gene of *A. salmonicida*. The case of a wild-type strain is shown. The A → C change directed by primer ASGYRA3 is shown in bold. The newly created *Hpy188I* restriction site in the PCR product is underlined.

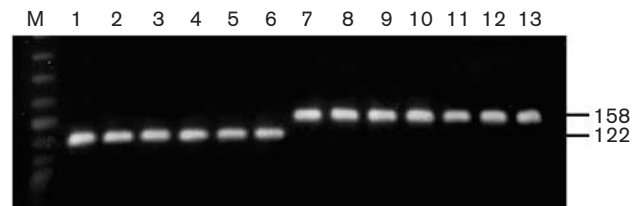


Fig. 2. Results of the PCR/RFLP assay conducted on a reference strain and 12 clinical isolates of *A. salmonicida*. The 36 bp restriction product is not shown. Lane M, molecular mass marker; lane 1, strain ATCC 14174; lanes 2–13, strains 12, 15, 2, 5, 3, 56, 13, 47, 77, 74, 44 and 41, respectively. Fragment sizes (nt) are indicated.

centrifugation, washed in 50 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer to an OD₆₀₀ of 4.0. The viable cells in this suspension were counted by plating the appropriate dilutions in triplicate on to tryptic soy agar plates after incubating overnight at 22 °C. The resuspended cells were equilibrated for 10 min at 22 °C in a water bath. Flumequine was then added to a final concentration of 10 µg ml⁻¹. Samples of 0.5 ml were taken 1, 2, 3 and 4 min after the addition of flumequine, diluted immediately in 1 ml ice-cold sodium phosphate buffer and centrifuged for 5 min at 5600 g. The pellet was washed once with 1 ml ice-cold buffer and resuspended in 1 ml 100 mM glycine hydrochloride (pH 3.0) for 15 h at room temperature. The samples were then centrifuged at 5600 g for 10 min at 4 °C. The concentration of flumequine in the supernatant was determined using an HPLC method adapted from Pouliquen & Pinault (1994). The results were expressed as ng flumequine incorporated (10⁹ c.f.u.)⁻¹.

PFGE. Strains were grown on tryptic soy agar (Bio-Rad) at room temperature for 48 h. After a purity check, several colonies were resuspended in cell suspension buffer (100 mM Tris/HCl, 100 mM EDTA, pH 8) at an OD₆₁₀ of 1.35–1.4. A volume of 120 µl of the cell suspension containing 10 µg lysozyme (Sigma-Aldrich) was mixed with an equal volume of 1.6 % Incert agarose (BMA) and allowed to solidify in 100 µl moulds. Plugs were incubated in 1 ml lysis buffer A (6 mM Tris/HCl, pH 7.6, 1 M NaCl, 0.1 M EDTA, 0.5 % Brij 58, 0.2 % deoxycholate, 0.5 % Sarkosyl) with 1 mg lysozyme and 20 µg DNase-free RNase. After 2 h at 37 °C, the lysis buffer was removed and the plugs were washed with 1 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and incubated with 1 ml lysis buffer B (0.25 M EDTA, 20 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, pH 9.0) supplemented with 500 µg proteinase K and 1 % Sarkosyl for

20 h at 50 °C. The plugs were washed three times with 1.5 ml TE buffer and three more times with distilled water and then digested with 30 U *Spe*I (Roche Diagnostics) overnight at 37 °C. Fragments of DNA were separated by PFGE in a 1% agarose gel (Seakem Gold; BMA) in 0.5 × TBE buffer (0.045 M Tris/HCl, pH 8, 0.045 M boric acid, 0.01 M EDTA) using a CHEF-DR III (Bio-Rad). The running conditions were 6 V cm⁻¹ at 12 °C for 20 h, with pulse times ramped from 2.2 to 63.8 s. Lambda Ladder PFG Marker (New England BioLabs) was used as the molecular size marker.

The gel was stained in 1 µg ethidium bromide ml⁻¹ and the gel image was captured electronically using a video camera interfaced to a microcomputer (ImageMaster VDS; Amersham-Pharmacia Biotech).

Patterns were compared using the BIO 1D++ Software (Vilber-Lourmat), based on the Dice similarity coefficients. A dendrogram was deduced from the matrix of similarities using the unweighted pair group method with the arithmetic mean (UPGMA) clustering algorithm.

RESULTS

Characterization of 12 selected *A. salmonicida* isolates

(i) **Quinolone MICs.** In order to study the mechanisms of resistance to quinolones of *A. salmonicida*, we selected 12 clinical isolates from our strain collection whose level of resistance to quinolones ranged from full susceptibility to clinically significant resistance, with all the intermediate levels represented. Oxolinic acid MICs ranged from 0.03 to 8 µg ml⁻¹, flumequine MICs from 0.06 to 32 µg ml⁻¹ and ciprofloxacin MICs from 0.015 to 1 µg ml⁻¹ (Table 2). The fluoroquinolone ciprofloxacin was the most active against all isolates, followed by oxolinic acid and flumequine.

(ii) **Nucleotide sequence of the *gyrA* and *parC* gene QRDRs.** The nucleotide sequences of the *gyrA* QRDR of 12 clinical isolates and *A. salmonicida* ATCC 14174 were determined by PCR amplification and direct sequencing. The isolates could be separated into two groups: (i) those that had a wild-type *gyrA* gene QRDR (identical to that of *A. salmonicida* ATCC 14174) and showed oxolinic acid MICs ≤ 0.5 and flumequine MICs ≤ 1 µg ml⁻¹ and (ii) those that had an identical single nucleotide mutation in *gyrA* leading to an Asp-87 → Asn (GAC → AAC) substitution and showed oxolinic acid MICs ≥ 2 and flumequine MICs ≥ 4 µg ml⁻¹ (Table 2). There was no clear distinction between the ciprofloxacin MIC of isolates harbouring the *gyrA* mutation and those of isolates with a wild-type *gyrA* QRDR. Indeed, isolate 56, which had the *gyrA* mutation, appeared equally or more susceptible to ciprofloxacin than some other isolates (2, 3 and 5) with no *gyrA* mutation.

Using degenerate primers (Table 1), we amplified, cloned and sequenced the *parC* QRDR of *A. salmonicida* ATCC 14174. This nucleotide sequence served to define primers specific to *A. salmonicida*, which made possible the amplification and direct sequencing of the *parC* QRDR of the 12 selected clinical isolates. The *parC* QRDR sequences of all the isolates were 100% identical to that of *A. salmonicida* ATCC 14174. The deduced amino acid sequences showed high identity with the corresponding ParC sequences of *E. coli* (88.1%) and *P. aeruginosa* (90.3%). Alignments over a 68 amino acid overlap with the ParC QRDR sequences of *Aeromonas sobria* and *Aeromonas hydrophila* (Goni-Urriza *et al.*, 2002) revealed 100 and 98.5% identity (one substitution), respectively.

(iii) **Active efflux analysis.** To evaluate the contribution of

Table 2. Amino acid substitutions in the GyrA and ParC QRDRs of 12 clinical *A. salmonicida* isolates compared with a wild-type ATCC strain, and MICs of three quinolones determined in the absence or presence of an EPI

MICs were determined in the absence (–EPI) or presence (+EPI) of a maximal potentiating concentration of Phe-Arg β-naphthylamide.

Strain	Amino acid changes in QRDR		Oxolinic acid MIC (µg ml ⁻¹)			Flumequine MIC (µg ml ⁻¹)			Ciprofloxacin MIC (µg ml ⁻¹)		
	GyrA	ParC	–EPI	+EPI	E _{max}	–EPI	+EPI	E _{max}	–EPI	+EPI	E _{max}
ATCC 14174	–	–	0.03	0.015	2	0.06	0.015	4	0.015	2 ⁻⁸	4
12	–	–	0.06	0.015	4	0.06	0.015	4	0.015	2 ⁻⁸	4
15	–	–	0.125	0.015	8	0.25	0.015	16	0.125	0.03	4
2	–	–	0.25	0.03	8	0.5	0.03	16	0.125	0.06	2
5	–	–	0.25	0.125	2	1	0.06	16	0.125	0.06	2
3	–	–	0.5	0.06	8	1	0.06	16	0.25	0.125	2
56	Asp-87 → Asn	–	2	0.5	4	4	0.5	8	0.125	0.06	2
13	Asp-87 → Asn	–	2	0.5	4	8	1	8	0.5	0.25	2
47	Asp-87 → Asn	–	2	0.5	4	8	1	8	0.5	0.25	2
77	Asp-87 → Asn	–	4	1	4	16	2	8	1	0.5	2
74	Asp-87 → Asn	–	4	0.5	8	16	1	16	1	0.5	2
44	Asp-87 → Asn	–	4	0.5	8	16	1	16	1	0.5	2
41	Asp-87 → Asn	–	8	1	8	32	2	16	1	0.5	2

active efflux to the quinolone-resistant phenotype of the 12 selected isolates, we compared MICs obtained in the presence and absence of the EPI Phe-Arg β -naphthylamide (Lomovskaya *et al.*, 2001) (Table 2). We assumed that active efflux and target gene mutations were contributing independently to phenotypic quinolone resistance. Consequently, we considered that $E_{\max}$ (i.e. the largest quinolone MIC reduction induced by the EPI) provided a measure of the contribution of active efflux to quinolone resistance, independently of the *gyrA* gene status.

Depending on the isolate, $E_{\max}$ was obtained with concentrations of EPI between 64 and 256 $\mu\text{g ml}^{-1}$ and we verified that EPI alone had no inhibitory activity within this range (data not shown). $E_{\max}$ calculated for flumequine and oxolinic acid reached values of 16 and 8, respectively. This was true for the most resistant isolates with the Asp-87 $\rightarrow$ Asn substitution (isolates 74, 44 and 41) as well as for the most resistant isolates harbouring a wild-type *gyrA* QRDR (isolates 2, 3 and 15). The EPI showed a weaker effect with fully susceptible strains (*A. salmonicida* ATCC 14174 and isolate 12) although it still induced hypersusceptibility to flumequine and oxolinic acid. In contrast, $E_{\max}$ for ciprofloxacin was as low as 2 for 10 out of the 12 isolates, including the most resistant. Surprisingly, this value was 4 for the two most susceptible strains, which were thus also made hypersusceptible to ciprofloxacin by the EPI.

Flumequine uptake experiments performed on four strains showed a correlation between $E_{\max}$ and levels of accumulation (Fig. 3). Indeed, strains 15 and 74, which had an $E_{\max}$ of 16, accumulated 10- to 20-fold less flumequine than *A. salmonicida* ATCC 14174, which had an $E_{\max}$ of 4. Intermediately, strain 56, with an $E_{\max}$ of 8, accumulated 3- to 4-fold less flumequine than did strain ATCC 14174.

Detection of mutations at codon 87 by PCR/RFLP

Considering the correlation between the presence of a mutation at codon 87 of *gyrA* and the level of resistance to quinolones that occurred among the 12 initially selected isolates, we developed a PCR/RFLP assay to detect mutations rapidly at this codon (Fig. 1). When performed on the clinical

and reference strains whose *gyrA* QRDR had been sequenced, the PCR/RFLP assay provided the expected restriction products (Fig. 2). The assay was further applied to a set of 34 clinically resistant or susceptible strains. The assay indicated the presence of a mutation at codon 87 for 13 of the 14 resistant strains. In contrast, the assay was negative for the 20 susceptible strains.

Strain typing by PFGE

PFGE patterns were obtained for all the clinical strains described in Table 2, except strain 41, which repetitively gave smeared patterns. All the strains displayed similar PFGE patterns, thus confirming the genetic homogeneity of typical *A. salmonicida* strains (Garcia *et al.*, 2000) (Fig. 4). However, as previously shown by Chomarat *et al.* (1998), restriction by *SpeI* was discriminative, providing unique patterns for all the strains, except strains 47 and 77, which were indistinguishable. Cluster analysis clearly distinguished the patterns of clinical strains from that of strain ATCC 14174 at a similarity level of about 50 % (Fig. 4). Among the clinical strains, those harbouring the mutation at codon 87 of *gyrA* and those with the wild-type *gyrA* gene clustered in two separate groups at a similarity level of 70 %.

DISCUSSION

Our study shows that the acquisition of resistance to quinolones by *A. salmonicida* is, as for other Gram-negative bacteria, mainly linked to the acquisition of mutations in the *gyrA* gene (Akasaka *et al.*, 2001; Bachoual *et al.*, 1998; Bagel *et al.*, 1999; Cloeckaert & Chaslus-Dancla, 2001; Deguchi *et al.*, 1997; Giraud *et al.*, 1999; Nakano *et al.*, 1997). However, it appears that the level of resistance can be significantly modulated by increases in efflux activity. Isolates sharing an identical status for the *gyrA* and *parC* gene QRDRs exhibited very different levels of resistance to quinolones. Although we cannot exclude the presence of quinolone resistance mutations outside the sequenced QRDR, our results show that these differences are probably due mainly to active efflux. Indeed, in strains with no mutation in the *gyrA* and *parC* QRDR, MICs measured in the presence of an EPI were restored to a full level of susceptibility, and even to a lesser level for some strains. This indicates that active efflux is not only involved in acquired quinolone resistance but also contributes significantly to the intrinsic level of activity of quinolones against susceptible strains. It has also been demonstrated that inhibition of efflux pumps significantly decreases the level of intrinsic resistance in *P. aeruginosa* (Lomovskaya *et al.*, 2001). In resistant strains possessing the *gyrA* mutation, EPI could not decrease MIC below values that may be interpreted as the MIC caused by the presence of this mutation alone. It is interesting to note that MICs observed in strains with no mutations in the *gyrA* and *parC* QRDR but with high $E_{\max}$ values are close to or even higher than MICs theoretically conferred by the presence of this *gyrA* mutation. This suggests that efflux upregulation, which is associated with a substitution in the GyrA QRDR in most resistant

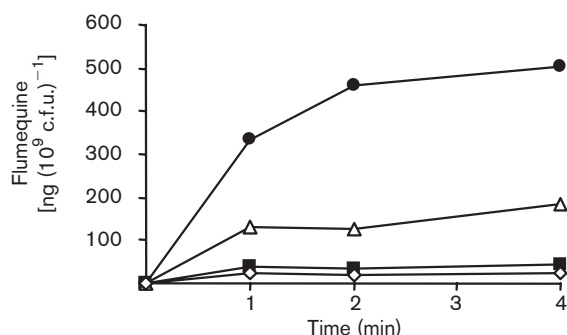


Fig. 3. Accumulation of flumequine by strains ATCC 14174 (●), 15 (■), 56 (△) and 74 (◇).

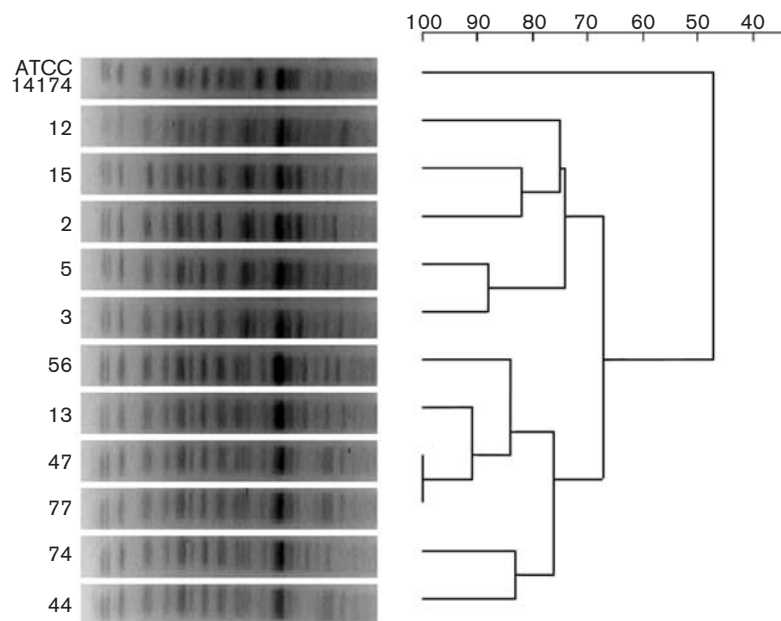


Fig. 4. *Spel* PFGE patterns of the *A. salmonicida* isolates described in Table 1 and dendrogram based on the UPGMA clustering of Dice similarity coefficient (expressed as percentages).

strains, may also be responsible by itself for significant levels of resistance to quinolones. More directly, flumequine accumulation experiments performed on selected strains confirmed the much lower level of accumulation of strains showing high $E_{\max}$ values (i.e. high efflux activity).

Mutations at codon 83 and 87 of *gyrA* (or homologous codons) are the most commonly observed in quinolone-resistant strains of Gram-negative bacteria. In the most resistant strains, they are often combined with *parC* mutations (Akasaka *et al.*, 2001; Bachoual *et al.*, 1998; Bagel *et al.*, 1999; Deguchi *et al.*, 1997; Gibreel *et al.*, 1998; Nakano *et al.*, 1997). Furthermore, different mutations affecting codons 83 and 87 and thus resulting in different amino acid substitutions may be responsible for the resistance to quinolones. However, the mutation that we found in the *gyrA* QRDR of most of the resistant isolates was of a single type, leading to an Asp-87 → Asn substitution in GyrA. This substitution has previously been identified in resistant strains of *P. aeruginosa* (Akasaka *et al.*, 2001). However, only mutations at codon 83 have been found in the *gyrA* QRDR of *Aeromonas* strains so far (Goni-Urriza *et al.*, 2002; Oppegaard & Sorum, 1994). In a recent study, mutations associated with quinolone resistance were investigated in riverine *Aeromonas* strains of mesophilic species other than *A. salmonicida* (*Aeromonas caviae*, *A. hydrophila* and *A. sobria*) (Goni-Urriza *et al.*, 2002). All the resistant strains carried a point mutation leading to Ser-83 → Arg or Ser-83 → Ile substitutions. In addition, some strains harboured a *parC* mutation at codon 80 or 84. Oppegaard & Sorum (1994) identified a Ser-83 → Ile substitution in quinolone-resistant *A. salmonicida* strains isolated from fish in Norway. The diversity of quinolone-resistance mutations (or combinations of mutations) in all the genera studied so far, and particularly in environmental *Aeromonas* strains, contrasts with the homo-

geneity of quinolone-resistant alleles that we observed in our study and that Oppegaard and Sorum observed in their study.

This homogeneity of quinolone-resistance mutations, together with the high similarity of the PFGE patterns, reveals a probable close epidemiological relationship between our isolates. Fry or fingerlings imported in fish farms from the same region are often purchased from a single producer. As a possible scenario, a clone possessing the Asp-87 → Asn substitution may have spread from a single source and then evolved differently in the different fish farms, through the occurrence of novel selection events. This could explain the various quinolone-resistance phenotypes observed among these isolates. Also, repeated isolation of resistant *A. salmonicida* strains showing the same *gyrA* mutation in the same fish farms and over periods spanning several years should raise questions about the persistence of resistant strains in the farm structures. Confirmation of epidemiological relatedness may have considerable implications in terms of improvement of sanitary practices on fish farms.

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