Antibiotic resistance due to an unusual ColE1-type replicon plasmid in Aeromonas salmonicida

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Aeromonas salmonicida subsp. salmonicida is a fish pathogen known to have a rich plasmidome. In the present study, we discovered an isolate of this bacterium bearing an additional unidentified small plasmid. After having sequenced the DNA of that isolate by next-generation sequencing, it appeared that the new small plasmid is a ColE1-type replicon plasmid, named here pAsa7. This plasmid bears a functional chloramphenicol-acetyltransferase-encoding gene (cat-pAsa7) previously unknown in A. salmonicida and responsible for resistance to chloramphenicol. A comparison of pAsa7 with pAsa2, the only known ColE1-type replicon plasmid usually found in A. salmonicida subsp. salmonicida, revealed that even if both plasmids share a high structural similarity, it is still unclear if pAsa7 is a derivative of pAsa2 since they showed several mutations at the nucleotide level. Transcriptomic analysis revealed that the cat-pAsa4 gene, another chloramphenicol-acetyltransferase-encoding gene, found on the large plasmid pAsa4, was significantly more transcribed than cat-pAsa7. This was correlated with a higher chloramphenicol resistance for isolates bearing pAsa4 compared with the one having pAsa7. Finally, a phylogenetic analysis showed that both CAT-pAsa4 and CAT-pAsa7 proteins were in different clusters. The clustering was supported by the identity of residues involved in the catalytic site. In addition, to give a better understanding of the large drug-resistance panel of A. salmonicida, this study reinforces the hypothesis that A. salmonicida subsp. salmonicida is a considerable reservoir for mobile genetic elements such as plasmids.
This bacterium has a notable plasmidome involved in antibiotic resistance in several isolates (Adams et al., 1998; L’Abée-Lund & Sørum, 2002; Piotrowska & Popowska, 2015; Reith et al., 2008; Sørum et al., 2003; Vincent et al., 2014), leading to a bottleneck in the effectiveness of antibiotic treatments. More precisely, pAsa4 (166.7 kb), pSN254b (152.2 kb), pAB559b (25.5 kb) and pAr-32 (~47 kb) are plasmids conveying resistance to chloramphenicol (CHL) in A. salmonicida subsp. salmonicida (Aoki et al., 1986; Reith et al., 2008; Vincent et al., 2014). For both pSN254b and pAB559b, the resistance is due to the floR gene, which encodes an efflux pump (Vincent et al., 2014). In the case of pAsa4 and pAr-32, the CHL resistance is produced by a cat gene, which encodes a CHL acetyltransferase (CAT) (Reith et al., 2008; Sørum et al., 2003).

In addition to the antibiotic-resistance-bearing plasmids, A. salmonicida subsp. salmonicida isolates usually have pAsa5, a plasmid bearing a type three secretion system (TTSS) (Reith et al., 2008; Vincent et al., 2015), pAsa1 bearing the aopP gene which encodes a TTSS effector (Fehr et al., 2006) and small cryptic plasmids pAsa1, pAsa2 and pAsa3 (Attéré et al., 2015; Boyd et al., 2003). These cryptic plasmids bear genes involved in replication, mobilization and stability. The plasmids pAsa1 and pAsa3 are ColE1-type replicons while pAsa2 is a CoE1-type (Boyd et al., 2003). In addition to these plasmids, a new, small plasmid was identified in the European isolate JF3791 during a recently published large-scale study (Attéré et al., 2015).

In the present study, we characterized this new CoE1-type replicon plasmid, named here pAsa7, as well as the CHL resistance due to its gene, cat-pAsa7, encoding a CAT. In addition, to give a better understanding of the considerable drug-resistance panel of A. salmonicida, this study adds further evidence of the large mobile-genetic-element repertoire of A. salmonicida subsp. salmonicida.

**METHODS**

**Bacterial strains and growth conditions.** JF3791 was isolated in 2006 from an Arctic char (Salvelinus alpinus) in Switzerland (Burr & Frey, 2007). JF3224 was isolated from a wild brown trout (Salmo trutta) captured in 2004 in a prealpine Swiss river (Burr et al., 2005). 01-B526 (Charette et al., 2012) is from the Province of Quebec (Canada), 2009-144K3 and 2004-05MF26 are from the Province of New Brunswick (Canada) (Vincent et al., 2014) and finally A449 and RS 534 are from France (Belland & Trust, 1987). All these A. salmonicida subsp. salmonicida isolates were grown on furunculosis agar (Hänninen & Hirvelä-Koski, 1997) at 18 °C for 24 to 72 h since it is at this temperature that this psychrophilic bacterium is usually most efficient in infecting fish (Beaz-Hidalgo & Figueras, 2012; Dacanay et al., 2006; Dautremepuis et al., 2006).

**Restriction fragment profiles.** A plasmid miniprep kit (Feldan) was used, as recommended by the manufacturer, to extract ~50 kb plasmids from isolates JF3791 and 01-B526, the latter being used as a control (Attéré et al., 2015). The extracts (25 µl) were digested with EcoRI (New England Biolabs) (Boyd et al., 2003) and then separated by gel electrophoresis (0.7 %) at 90 V for 80 min. The gel was stained with ethidium bromide to visualize the DNA bands under UV illumination.

**DNA extraction, sequencing and assembly.** The total genomic DNA of JF3791 was extracted using a DNeasy Blood and Tissue kit (Qiagen). The sequencing library was prepared using a KAPA Hyper Prep kit and was sequenced by next-generation sequencing (NGS) on a MiSeq instrument (Illumina) by the Plate-forme d’Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval, Canada). The resulting sequencing reads were assembled de novo to contiguous sequences using the A5-miseq pipeline version 20140401 (Coil et al., 2015). The complete draft genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and deposited in the public database GenBank under the accession number JXFG60000000. The sequence of pAsa7 was annotated manually with Artemis version 16.0.0 (Rutherford et al., 2000) and the NCBI Sequin version 13.70 and also deposited in GenBank under the accession number KU499859.

**Bioinformatics analyses.** In order to analyse the substitutions within the coding sequences of both pAsa2 and pAsa7, each nucleotide sequence was codon-aligned using MUSCLE version 3.8.31 (Edgar, 2004) through ParaMT.pl version 1.0 (Zhang et al., 2012). The synonymous and non-synonymous substitutions were evaluated by DAMBE version 6.1.6 (Xia, 2013).

The mean copy number per cell of pAsa2 and pAsa7 in JF3791 was estimated for both plasmids by using their relative coverage. Briefly, the sequencing reads were filtered using Trimmomatic version 0.32 (Bolger et al., 2014) and then mapped on a unique sequence for both pAsa2 and pAsa7 and also the sequence of the housekeeping gene gyrB using BWA (bwa-mem algorithm) version 0.7.9a-r786 (Li & Durbin, 2009). The result was then converted into a BAM file using SAMtools version 0.1.19-44428cd (Li et al., 2009). Finally, the mean copy number was computed for each plasmid by calculating the coverage, with Qualimap version 2.1.2 (García-Alcalde et al., 2012), for pAsa2 and pAsa7 as well as for the one of gyrB. The same procedure was performed for the large plasmids pAsa4, pSN254b and pAB59b of isolates RS 534, 2004-05MF26 and 2009-144K3, respectively. However, since the DNA of 2009-144K3 was sequenced by pyrosequencing (Vincent et al., 2015), the sequencing reads were converted from an sff to a fastq format by sff2fastq (https://github.com/indrael/sff2fastq) and the tag sequences removed using TagCleaner standalone version 0.16 (Schmieder et al., 2010).

The prediction of the secondary structures for the RNA I and II of both pAsa2 and pAsa7 was done with the mfold web server 2.3 (Zuker, 2003) and also deposited in GenBank under the accession number KU499859.

**Molecular phylogeny.** Molecular phylogeny analyses were done to find the evolutionary links of the translated cat gene sequences CAT-pAsa4 and CAT-pAsa7 with other CATs. A total of 96 cat translated sequences were downloaded from the ‘comprehensive antibiotic resistance database’ (CARD) (McArthur et al., 2013). The sequences were aligned using MUSCLE version 3.7 (Edgar, 2004). The resulting matrix was evaluated by ProtTest version 3.4 (Darriba et al., 2011) to find the best-fit model using the Bayesian information criterion (BIC). First, phylogenetic analysis was done by maximum-likelihood using RAxML version 8.1.17 (Stamatakis, 2014) under the LG+T model. Given this analysis, we found that both CAT-pAsa4 and CAT-pAsa7 were clearly not among the CATB, which formed a distinct cluster (Fig. S1, available in the online Supplementary Material). We consequently removed the sequences related to the CATB, as well as sequences annotated wrongly, that clustered among the CATB and also a sequence annotated as a CATS since it was likely truncated from both N- and C-terminals.
Finally, duplicated sequences were also removed, keeping only one representative sequence per unique protein sequence. The 34 remaining sequences were aligned using MUSCLE version 3.7 and evaluated by ProtTest as described above. The molecular phylogeny was performed by Bayesian inference by running five independent chains under the model LG+Γ for 10,000 cycles with PhyloBayes version 4.1 (Lartillot et al., 2009). A consensus topology was calculated from the saved trees using BPCOMP included in the package PhyloBayes after a burn-in of 2000 trees (20%). The largest discrepancy across all bipartitions (maxdiff) was 0.056, meaning that the convergence between the chains was achieved. Finally, a rapid bootstrap analysis (100 replicates) and a resolution of the polytomies were done by RAxML (Stamatakis, 2014) by using the fixed topology found by PhyloBayes. The tree was midpoint rooted using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/), exported in Newick format and finally visualized using the gttree package in the statistical framework R version 3.2.2 (R Core Team, 2015).

Molecular modelling. Amongst the 11 structures of CAT that are available in the Protein Data Bank (PDB), only two structures, a type 1 (Biswas et al., 2012) and a type 3 (Leslie, 1990) CAT from Escherichia coli are non-mutated WT enzyme bound to a CHL substrate (PDBID 3U9F and 3CLA, respectively). Primary sequences of CAT-pAsa4 and CAT-pAsa7 were aligned using MUSCLE version 3.7 (Edgar, 2004) with default parameters to primary sequences of structures 3U9F and 3CLA taken from UniProt (uniprotID: P62577 and P00484, respectively). This alignment showed an identity of 60.6 and 44.6 % and similarity of 74.4 and 64.3 % for CAT-pAsa7 with 3U9F and 3CLA, respectively, and an identity of 45.6 % and 40.7 and similarity of 64.0 and 63.1 % for pAsa4 with 3U9F and 3CLA, respectively. Important residues of the catalytic site were obtained from literature (Biswas et al., 2012). Percentages of identity and similarity based only on the catalytic residues were calculated. This showed an identity of 72.7 and 45.4 % and similarity of 81.8 and 63.6 % for pAsa7 with 3U9F and 3CLA, respectively, and an identity of 59.0 and 63.6 % and similarity of 77.2 and 77.2 % for CAT-pAsa4 with 3U9F and 3CLA, respectively. Based on catalytic site residues identity, PDB structure 3U9F was selected as the template for homology modelling for CAT-pAsa7 and PDB structure 3CLA for CAT-pAsa4. This primary structure alignment was used in MODELLER (Chemical Computing Group) for homology modelling with default parameters giving 10 side-chain positions for each model. The best model, based on the GB/VI score, was chosen. For each model, the CHL molecule was taken from its respective template and energy minimized in the catalytic site. Multiple sequence alignment representation, as well as calculation of identity and similarity based only on the catalytic residues were calculated. This showed an identity of 72.7 and 45.4 % and similarity of 81.8 and 63.6 % for pAsa7 with 3U9F and 3CLA, respectively, and an identity of 59.0 and 63.6 % and similarity of 77.2 and 77.2 % for CAT-pAsa4 with 3U9F and 3CLA, respectively. Based on catalytic site residues identity, PDB structure 3U9F was taken for homology modelling for CAT-pAsa7 and PDB structure 3CLA for CAT-pAsa4. This primary structure alignment was used in MODELLER (Chemical Computing Group) for homology modelling with default parameters giving 10 side-chain positions for each model. The best model, based on the GB/VI score, was chosen. For each model, the CHL molecule was taken from its respective template and energy minimized in the catalytic site. Multiple sequence alignment representation, as well as calculation of sequence identity and similarity, was done with TEXP Shade (Beitz, 2000). Structure images were generated with PyMOL 1.7.1.3 (Schrödinger, 2010).

Electroporation and CHL MIC. The plasmidic DNA of isolate JF3791 was extracted using a plasmid miniprep kit (Feldan) and introduced by electroporation into isolates 01-B526 and JF3224 using a previously published protocol (Dallaire-Dufresne et al., 2014b). CHL was used as selective agent. The presence of pAsa7 in 01-B526 and JF3224 was verified by restriction profiles and PCR assays (Boyd et al., 2003).

The protocol used to find the CHL MIC for the A. salmonicida subsp. salmonicida isolates has been published elsewhere (Vincent et al., 2014). Briefly, the isolates were recovered from frozen stocks and grown on furanoculosis agar at 18 °C for 48 to 72 h. Several colonies of each isolate were suspended in fresh LB medium (EMDI Millipore). The OD at 595 nm was measured for each bacterial suspension and diluted to 0.2, and 300 µl was deposited in the wells of 48-well microplates. CHL (Calbiochem) was serially diluted in LB medium, and an aliquot of each dilution was placed into a well of the 48-well microplates to obtain antibiotic concentrations ranging from 0 to 384 µg ml⁻¹ in a final volume of 600 µl. The plates were incubated at 18 °C for 48 h with shaking at 200 r.p.m. in a Tecan Infinite F200 PRO microplate reader. Growth was assessed at each 15 min for 48 h. Every assay was performed at least in duplicate.

RNA extraction, qRT-PCR and qPCR. The copy number of each transcript of cat-pAsa4 and cat-pAsa7 was determined using quantitative (q) reverse-transcription (RT)-PCR, whereas qPCR was used to assess the copy number of pAsa2 (orf3), pAsa4 (cat-pAsa4) and pAsa7 (cat-pAsa7). For every qRT-PCR and qPCR assay, a standard curve was generated using the appropriate qPCR primers (Table S1) by amplification of a serially diluted DNA template (10⁻⁷ to 10⁻¹ copies/µl). DNA templates were produced using specific primers (Table S1) by PCR from 01-B526 genomic DNA for gyrB, RS 534 genomic DNA for cat-pAsa4, JF3791 genomic DNA for cat-pAsa7 and orf3 for pAsa2. The confirmation that PCR generated a single amplicon was provided by electrophoresis on agarose gel, and PCR products were isolated using the Pure Link PCR Purification kit (Life Technologies). DNA concentrations were measured by a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). Each DNA template was confirmed by amplicon sequencing.

RNA extractions were performed from liquid cultures of isolates RS 534 and JF3791 grown for 15 h at 18 °C with agitation (200 r.p.m.) from identical inoculum of 0.05 at OD₆₅₀. The extractions were done in three biological replicates using a RiboPure RNA Purification kit, bacteria (Life Technologies) according to the recommendations from the manufacturer. Any residual genomic DNA contamination was removed using DNase I and the absence of DNA was confirmed by qPCR using the primers targeting the housekeeping gene gyrB (Table S1). The concentrations of extracted RNA were calculated by a NanoDrop 2000 UV-Vis Spectrophotometer. The integrity of the RNA was determined using an Agilent RNA 6000 Nano kit by the Plate-forme d’Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval, Canada). The RNA integrity number was between 9.5 and 9.8, whereas the RNA ratio [23S/16S] was between 1.8 and 2.2. All RNA samples were stored at −80 °C.

The copy number of each target (transcript or plasmid) was determined in three technical replicates using SYBR Select Master Mix (Life Technologies) and 50 ng of each biological replicate of cDNA (qRT-PCR) or DNA (qPCR) samples in a Rotor-Gene Q – Pure Detection (QIAGEN) with a standard curve. The housekeeping gene gyrB, which is present in a single copy on the chromosome of A. salmonicida subsp. salmonicida, was used to evaluate the copy number of chromosomes by qPCR. The copy number of each plasmid (pAsa2, pAsa4 and pAsa7) was rationalized on the copy number of gyrB. The CDNA synthesis was executed from 2 µg RNA using an iScript Advanced cDNA Synthesis kit by Bio-Rad. To normalize gene expression values according to each growth condition, the housekeeping gene gyrB was used. The CDNA copy numbers of the CHL resistance genes (cat-pAsa4 or cat-pAsa7) were weighted on the basis of the CDNA copy number of gyrB of their corresponding isolate (RS 534 and JF3791, respectively).

RESULTS

Discovery of pAsa7

Most isolates of the pathogen A. salmonicida subsp. salmonicida were shown to bear four small plasmids (pAsa1, pAsa2, pAsa3 and pAsa1) (Attéré et al., 2015; Boyd et al., 2003; Fehr et al., 2006). A recently published, large-scale study investigated the potential diversity of these plasmids through plasmid-DNA isolation, digestion by the EcoRI restriction enzyme and finally electrophoresis (Attéré et al., 2015). The effectiveness of this procedure resides in the fact that each plasmid has at least one EcoRI restriction site and produces fragments of different sizes, except for pAsa1 and...
pAsa2, to be resolved by a standard electrophoresis (pAsa1, 6371 bp; pAsa1, 5424 bp; pAsa2, 5247 bp; pAsa3, 4806 bp).

The plasmidic profile of a Swiss isolate named JF3791 and recovered in 2006 from a sick Arctic char (Burr & Frey, 2007) showed an unusual profile (Attéré et al., 2015). In fact, JF3791 lacked the characteristic pAsal1’s band and had two novel bands at approximately 4.0 kbp and 1.3 kbp (Fig. 1).

Isolate JF3791 was found to be resistant to CHL (data not shown). This resistance was first believed to be caused by a plasmid like pAsa4, pABB59b, pSN254b or pAr-32 (Reith et al., 2008; Særum et al., 2003; Vincent et al., 2014). To solve this point and determine the nature of the unusual plasmidic profile, the total DNA of isolate JF3791 was sequenced by NGS (see Methods). The de novo assembly of the sequencing reads revealed an unexpected result, a new plasmid, which is structurally similar to pAsa2, the only known ColE1-type replicon plasmid usually found in the bacterium A. salmonicida subsp. salmonicida. Compared with pAsa2, this new plasmid does not harbour the genes orf2 and orf3, which encode hypothetical proteins. Instead of these two genes, pAsa7 has a new region containing a cat gene encoding a CAT, which is the enzyme responsible for resistance to CHL (Fig. 2).

Since this plasmid has not been reported previously in the literature, it was named pAsa7. A blastp analysis against the non-redundant (nr) NCBI database revealed that the protein encoded by the cat gene of pAsa7 (named cat-pAsa7 in the present article) had previously been listed in other bacteria such as Enterobacter, Buttiauxella and Escherichia. However, a blastn analysis of the complete pAsa7 sequence against the nr/nt database and the whole genome shotgun (Gammaproteobacteria, taxid: 1236) (NCBI) resulted in partial results and consequently did not allow us to find the complete new sequence in other bacterial genomes. The pAsa7 sequence bears two EcoRI sites, compared with one for pAsa2 (Fig. 2), thus explaining the bands at ~1.3 and ~4.0 kbp in the plasmidic profile (Fig. 1).

pAsa7 characterization

Interestingly, pAsa2 and pAsa7 share a high level of similarity of their structure, with the exception of the region containing the cat gene in pAsa7 (Fig. 2). Knowing this high structural likelihood between both plasmids, we investigated if the shared region also has a high identity at the nucleotides level. Interestingly, the portion common to both plasmids exhibited 94.1 % identity. This relatively low value was unexpected knowing that pAsa2s are well known to be very stable with few mutations within their sequences (Attéré et al., 2015). For example, the study of Attéré et al. (2015) showed that the pAsa2 of JF3791 and the one of the reference strain A449 (GenBank: NC_004925) are 100 % identical. Analyses of the substitutions within the coding sequences between pAsa2 and pAsa7 revealed that no gene was more prone to accumulate mutations since all genes exhibited a relatively constant total substitution rate over the sequence length (Table 1). The analyses showed that only the mohB gene might have a bias to gain non-synonymous substitutions.

pAsa2 and pAsa7 copy number

The ColE1-type replicon plasmids are well known to be in high copy per cell (Camps, 2010). We used the high amount of sequencing reads provided by the Illumina technology and a qPCR approach to assay the pAsa2 and pAsa7 copy number per cell (see Methods). The pAsa2 plasmid, which is usually found in A. salmonicida subsp. salmonicida, is approximately in 13 to 19 copies per cell in strain JF3791 while pAsa7 is in 27 to 34 copies per cell. A paired t-test based on the qPCR data had found that pAsa7 is in significantly higher copies per cell than pAsa2 (P<0.01).

The ColE1-type plasmid replication involves an RNA (RNA II) that forms a hybrid RNA–DNA at oriV in order to initiate the replication. Plasmid replication is regulated stringently by a short RNA (RNA I), which is complementary to RNA II and transcribed constitutively from a strong promoter (Tomizawa, 1984). Both RNAs form three complementary stem–loops and consequently may interact in a ‘kissing complex’. This sequestration of RNA II by RNA I prevents RNA II from playing its preprimer role at oriV and then directly influences the plasmid’s copy number.

As reviewed elsewhere (Camps, 2010), two ColE1-type replicon plasmids rarely co-exist in the same cell since a contralateral regulation between plasmids creates interference with the ipsilateral regulation. However, it is also well described in the same review that mutations in the region containing the regulator RNAs may have a double effect: (1) a modification of the plasmid copy number and (2) a modification of its incompatibility group. In order to find an explanation for the copy number difference between pAsa2 and pAsa7, and since the respective rate of transcription for both RNAs will regulate the plasmid replication positively (in presence of more RNA II) and negatively (when there is more RNA I), we investigated the promoter

![Fig. 1. Plasmid profiles of isolates 01-B526 and JF3791. Isolate 01-B526 exhibits the standard plasmid profile: pAsa1, pAsa2, pAsa3 and pAsa1, while JF3791 does not bear the pAsa1 plasmid and shows two unusual bands (* and **).](http://mic.microbiologyresearch.org)
regions (−35 and −10 boxes) and the sps sequence (Wu & Liu, 2010) for both plasmids. We found no difference directly in the promoter regions (Fig. S2), which is interesting knowing the relatively high sequence divergence between both plasmids and thus shows the importance of conservation of these sequences through evolution.

The bioinformatics-predicted secondary structures of the regulator RNAs showed many differences between those of pAsa2 and pAsa7 (Fig. 3). However, even with several differences, the estimated free energy values (ΔG) of each secondary structure are low enough to be confident in the robustness of each of them. It is also interesting to note differences in the free energy values even for RNAs of the same strain. This is mainly due to differences caused by non-Watson–Crick pairing available for an RNA (I or II) but not for its reverse-complement (Leontis et al., 2002; Vendeix et al., 2009). Investigation by bioinformatics simulation of the ‘hug’ step between RNA I and II for both pAsa2 and pAsa7 resulted, respectively, in −295.04 and −305.81 kcal mol$^{-1}$ ($−1234.4$ and $−1279.5$ kJ).

**CHL resistance**

Knowing that pAsa7 bears a cat gene conferring CHL resistance and that it has a high copy number per cell, it was tempting to assay the CHL resistance level of the JF3791 isolate. The MIC of CHL was determined for some CHL-resistant *A. salmonicida* subsp. *salmonicida* isolates (Table 2).

### Table 1. Mutational comparison between gene sequences of pAsa2 and pAsa7

<table>
<thead>
<tr>
<th>Gene</th>
<th>$S^*$</th>
<th>NS†</th>
<th>NS/S</th>
<th>($S+NS$)/length‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1</td>
<td>13.00</td>
<td>7.00</td>
<td>0.54</td>
<td>0.07</td>
</tr>
<tr>
<td>mobC</td>
<td>16.00</td>
<td>8.00</td>
<td>0.50</td>
<td>0.08</td>
</tr>
<tr>
<td>mobA</td>
<td>35.00</td>
<td>39.00</td>
<td>1.11</td>
<td>0.05</td>
</tr>
<tr>
<td>mobB</td>
<td>8.00</td>
<td>19.00</td>
<td>2.37</td>
<td>0.06</td>
</tr>
<tr>
<td>mobD</td>
<td>6.00</td>
<td>6.00</td>
<td>1.00</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Synonymous substitution.
†Non-synonymous substitution.
‡The value used corresponds to the alignment length.
Isolates A449 and RS 534, known to bear a gene of the cat family since they contain the large plasmid pAsa4 (Reith et al., 2008; Vincent et al., 2016), were used for comparative purposes. Additionally, the 2004-05MF26 and 2009-144K3 CHL-resistant isolates, which harbour, respectively, the plasmids pSN254b and pAB559b known to bear a floR gene (Vincent et al., 2015), were also used to have a more complete panel of CHL resistance genes.

One striking result was the high MIC for the RS 534 and A449 isolates caused by the cat gene on the large plasmid pAsa4 (cat-pAsa4) (Table 2). In fact, both RS 534 and A449 have a MIC more than two times higher than the one of...
JF3791. Even though the MIC of CHL caused by pAsa4 was already evaluated to be high (Belland & Trust, 1989), this difference was unexpected since this large plasmid was found by the present study to be only in a single copy per cell while pAsa7 was at a much higher copy number per cell. In order to determine if the difference in resistance seen is plasmid or strain specific, pAsa7 was introduced by electroporation into two strains isolated in different geographical areas [01-B526 (Canada) and JF3224 (Switzerland)] and their MICs of CHL assessed before and after having the plasmid. In both cases, the MIC values obtained with 01-B526 and JF3224 bearing pAsa7 were identical and near that for the original isolate (JF3791) (Table 2). Since strain A449, which also contains pAsa4, had the same MIC value as RS 534, it appears that the high MIC value is due to the plasmid and is not strain specific. Moreover, a previous study showed that the cloning of the cat-pAsa4 gene into a vector transferred into E. coli gave a high MIC of CHL for the recipient strain (Belland & Trust, 1989).

The MICs of the isolates bearing the floR gene, encoding an efflux pump, were lower than those bearing a cat gene. This result was not unexpected since the two resistance mechanisms are different. However, interestingly, the MIC of isolate 2009-144K3, caused by the pAB559b plasmid, was 1.5 times higher than that for 2004-05MF26, caused by the pSN254b plasmid. In both cases, the gene upstream of the floR gene is virD2 and the intergenic DNA between these genes is 100% identical for pAB559b and pSN254b, meaning that the promoter regions are also the same. An investigation using the amount of sequencing reads showed that pSN254b and pAB559b were maintained in approximately one and two copies per cell, respectively. The difference in copy number between the two plasmids might help to explain why pAB559b gave a higher MIC than pSN254b.

**Transcription level of cat genes**

The transcription levels of cat-pAsa7 and cat-pAsa4 were assayed by qRT-PCR comparatively with the housekeeping gene gyrB. The results showed without any doubt that the transcription level of cat-pAsa4 was higher than that of cat-pAsa7 (Fig. 4) \( P<0.01 \) with an unpaired t-test. In fact, even if we take into account the copy number of the plasmid, the transcription of the cat gene found in JF3791 was only two times higher than that of the housekeeping gene gyrB. On the other hand, the cat gene found in pAsa4 showed an absolute transcription level more than 100 times higher than that of gyrB.

**Phylogenetic position of CAT proteins**

Since the cat-pAsa7 gene was never previously found in *A. salmonicida* subsp. *salmonicida* and knowing the high MIC difference caused by this gene and cat-pAsa4, it was interesting to establish their phylogenetic relationship with other

### Table 2. MIC of CHL for some known *A. salmonicida* subsp. *salmonicida* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg ml(^{-1}))</th>
<th>Plasmid</th>
<th>Resistance gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>01-B526</td>
<td>2</td>
<td>NA*</td>
<td>NA</td>
</tr>
<tr>
<td>JF3224</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2004-05MF26</td>
<td>64</td>
<td>pSN254b</td>
<td>floR</td>
</tr>
<tr>
<td>2009-144K3</td>
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</tr>
<tr>
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<td>pAsa7</td>
<td>cat-pAsa7</td>
</tr>
<tr>
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<td>pAsa4</td>
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<tr>
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*NA, not applicable.*
CAT proteins. The molecular phylogeny of 34 cat-translated sequences showed without any doubt that CAT-pAsa7 and CAT-pAsa4 were in different phylogenetic groups (Fig. 5). The pairwise alignments of the 22 important residues (Biswas et al., 2012) from the active site for the 34 CAT sequences were expressed as an identity matrix (Fig. 5). Interestingly, many phylogenetic groups were well defined by this matrix. Both the molecular phylogeny and the identity matrix showed that CAT-pAsa4 and CAT-pAsa7 belong to two different groups.

**Homology modelling**

Since there is a strong divergence between CAT-pAsa4 and CAT-pAsa7 based on 22 residues from the active site in addition to a difference in their biological efficiency (Table 2), it was interesting to further characterize the structural differences between the two proteins. Homology modelling revealed a structural reorganization of the interactions as shown in Fig. 6. The CHL substrate is bound mostly through similar hydrophobic contacts and by two polar interactions. However, as shown in Fig. 6, one of the polar interactions is not achieved by the same residue in CAT-pAsa4 as in CAT-pAsa7. The first H-bond, identical for both CAT-pAsa4 and CAT-pAsa7, is between CHL and the catalytic His194 (His187 for CAT-pAsa4). In most CATs, as in CAT-pAsa4, the second H-bond involves a Tyr at position 25 (Tyr21 for CAT-pAsa4). In CAT-pAsa7, this Tyr is replaced with a Phe25, conserving the aromatic character, but losing the H-bonding ability. Directed mutagenesis of Tyr/Phe at this position was reported to lead to a reduced catalytic activity for CATIII explained by the lost H-bond (Murray et al., 1991). However, for CAT-pAsa7 this mutation is compensated by the presence of a Tyr133 (Met126 for CAT-pAsa4), restoring the H-bond with CHL lost by Phe25. Thus, while having different catalytic site residues, CAT-pAsa4 and CAT-pAsa7 feature similar interactions with CHL.

**DISCUSSION**

The present study characterized a new ColE1-type replicon plasmid, named pAsa7, found in *A. salmonicida* subsp. *salmonicida*. The molecular architecture of this plasmid is interesting since it shares a high backbone similarity with pAsa2, another ColE1-type replicon, which is usually found...
in the normal plasmidome of this bacterium (Attérel et al., 2015). However, pAsa7 bears a gene encoding for a CAT. Even if both plasmids share a high structural similarity, it is still unclear if pAsa7 is a derivative of pAsa2 since there are many differences at the nucleotide level. This analysis also revealed that mobB might have a bias to gain non-synonymous mutations. This is surprising knowing that this gene is essential for plasmid mobilization and can interact with the primase and thus indirectly regulates plasmid replication (Meyer, 2011). Since pAsa2 also encodes MobB, it is possible that it can act in trans for pAsa7, making the gene of the latter more prone to a relaxation of the conservative pressure. This observation is congruent with another study, which reported that mobilization genes of the small-plasmids found in A. salmonicida subsp. salmonicida could be more prone to accumulating mutations (Attérel et al., 2015). The same study also reported that pAsa2 plasmids are very stable with especially few mutations between their sequences (Attérel et al., 2015).

Our study also found that pAsa7 was in significantly higher copy number per cell than pAsa2. We propose that it is reasonable to rule out the possibility that promoter regions of RNA I and II are involved in the copy number difference of pAsa2 and pAsa7, since there is no difference in these regions between the two plasmids (Fig. S2). Many host factors, such as RNA polymerase, DNA polymerase, RNaseH and topoisomerase I, are known to influence the copy number of ColE1-type plasmids (Camps, 2010). However, since both plasmids are present in the same isolate and consequently share the same environment, we also rule out the hypothesis that the host factors are determinant for the copy number difference. Finally, it was also demonstrated experimentally that uncharged tRNAs may interact with RNA I (Wróbel & Wegrzyn, 1998), resulting in an RNA I decay and consequently an increase in plasmid copy number (Wang et al., 2006). However, here again we can also reasonably rule out this scenario to explain the difference in copy number since both plasmids are in the same host.

Knowing that pAsa7 is in a higher copy number and consequently potentially with a replication less stringently regulated than pAsa2, we can also exclude a weaker ‘hug’ step

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**Fig. 6.** Divergent substrate binding between CAT-pAsa7 and CAT-pAsa4. Multiple sequence alignment for 22 important residues in the active site (a) (Biswas et al., 2012). Structural representations of CAT-pAsa7 (b) and CAT-pAsa4 (c) are in grey cartoon. Residues involved in the structural reorganization of H-bonds with CHL are highlighted in green (no H-bond) and red (H-bond) in the primary sequence alignment and are shown in purple and blue sticks for CAT-pAsa7 and CAT-pAsa4, respectively. In (b) and (c), distances are in Ångströms (Å).
for pAsa7 as a possible explanation since the simulation exhibited a lower free energy. However, it is not impossible that the initiation of the contact between both RNAs (i.e. RNA I and II) is less efficient for pAsa7, for example because the pAsa7 'T2' loop was predicted to be smaller than the one of pAsa2 (Fig. 3). Moreover, it is known that a single well-placed point mutation can disturb the complex and consequently the replication regulation. For example, the pUC18 plasmid, which is a derivative of pBR322, a ColE1-type replicon plasmid, replicates several-fold higher than its parental plasmid (i.e. pBR322). The reason for that is a single point mutation in RNA II, which produces a non-optimal folding for the interaction with RNA I (kissing complex), and the lack of the Rom protein which helps to stabilize the RNA I–II interaction (Lin-Chao et al., 1992). Interestingly, both pAsa2 and pAsa7 also lack a gene encoding a Rom protein and consequently their replication regulation might be more vulnerable to point mutations. We also suggest that mutations changed the incompatibility group of pAsa7 since both plasmids co-exist in the same isolate.

A large difference in the copy number between the small plasmids found in A. salmonicida subsp. salmonicida has already been demonstrated and was postulated to be caused by, among other things, the biological state of the cells during the DNA extraction (Attéré et al., 2015). However, this same study also showed that the ratios between plasmids were relatively constant from one isolate to another. Consequently, since both pAsa2 and pAsa7 were in the JF3791 isolate, we are confident that pAsa7 was in higher copy number than pAsa2.

The salient feature of pAsa7, compared with pAsa2, is that pAsa7 bears a cat gene, which allows CHL resistance. Since this gene is on a high-copy plasmid, it was reasonable to think that isolate JF3791 would be highly resistant to this drug. However, the comparison of the CHL resistance levels showed that isolates harbouring the large, single-copy plasmid pAsa4 were clearly more resistant to CHL (Table 2). This unexpected result was caused by the high transcription level of cat-pAsa4 compared with cat-pAsa7, regardless of the clear advantage of pAsa7 versus pAsa4 in copy number, as both CAT-pAsa4 and CAT-pAsa7 feature similar catalytic site residue interactions with CHL, suggesting a similar CAT activity. This supposes that the promoter region of cat-pAsa7 was less adapted to A. salmonicida subsp. salmonicida than the one found on pAsa4. It is also known that the high amount of mRNA of cat-pAsa4 is stable and translated since a previous study reported that CAT-pAsa4 was a predominant protein in whole-cell lysates of E. coli bearing a part of the pAsa4 plasmid containing cat-pAsa4 (Belland & Trust, 1989).

A phylogenetic analysis showed that both CAT-pAsa4 and CAT-pAsa7 were in different clusters. It is also important to note that the statistical values (bootstraps and posterior probabilities) were high overall within the tree. The clustering was also well supported by the identity of the catalytic residues. The correlation between the phylogenetic positions and the residues found in the catalytic core is interesting and showed that protein structures may help to define phylogenetic groups.

It was postulated in a recent study that small plasmids, shown by the derivation of pAsa3 to pAsa1 and pAsa1 to pAsa2, might be evolutionary templates for genomic innovations (Attéré et al., 2015). As stated above, it is actually not clear if pAsa7 is derived from pAsa2. The evolutionary scenario might be non-parsimonious including the acquisition of pAsa2 by various bacteria, thus blurring the evolutionary way by different mutational pressures. Even if this scenario might help to explain the mutation differences between pAsa7 and pAsa2, we cannot rule out a convergent evolution.

The ColE1-type replicon plasmid characterized by the present study, pAsa7, is interesting since it allows drug resistance. Other ColE-type replicon plasmids bearing drug-resistance genes have been identified in other species of Aeromonas, such as Aeromonas hydrophila and Aeromonas sobria (Han et al., 2012a, b; Piotrowska & Popowska, 2015). As stated elsewhere, the ColE1-like plasmids are generally maintained at high copy number (as is the case for pAsa7), which makes them more prone to being vectors of drug resistance, and, consequently, plasmids having this replicon should be closely monitored (Chen et al., 2010).

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**REFERENCES**


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