Génomique d'*Aeromonas salmonicida* et de ses phages

Thèse

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Résumé

Depuis la découverte de la pénicilline par Sir Alexander Fleming, les antibiotiques ont joué un rôle primordial et incontestable en médecine moderne en aidant à combattre les infections bactériennes. Cependant, les bactéries ont la capacité de se protéger par différents moyens des molécules antibiotiques. La surutilisation de ces molécules a accéléré le phénomène de résistance aux antibiotiques, rendant difficile, voire impossible, le traitement de certaines maladies infectieuses par cette approche. La résistance aux antibiotiques est une problématique d'envergure mondiale qui touche aussi négativement l'aquaculture, où les infections bactériennes peuvent causer d'importantes pertes économiques. L'une de ces bactéries est *Aeromonas salmonicida* subsp. *salmonicida*, l'agent étiologique de la furonculose. Bien qu'il fût déjà connu que plusieurs souches de cette bactérie étaient porteuses de plasmides conférant des résistances aux antibiotiques, l'ampleur de la problématique était encore inconnue.

Les bactériophages (phages) sont des virus infectant spécifiquement les bactéries. Cette capacité à lyser les bactéries leur a valu d'être utilisés dans un contexte thérapeutique presque dès leur découverte au début du 20^{e} siècle. Cependant, l'avènement des antibiotiques a fait en sorte que la thérapie par les phages a été oubliée dans plusieurs pays occidentaux. Maintenant que la résistance aux antibiotiques est devenue une inquiétude pour la pérennité de notre société, plusieurs études suggèrent que la thérapie par les phages pourrait être une alternative ou un complément aux traitements par antibiotiques.

La présente thèse avait comme objectifs : (1) d'explorer la diversité génomique causant une résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida* et (2) d'investiguer le potentiel d'un traitement par les phages pour contrer les infections causées par cette bactérie.

Il a été possible de mettre à jour et de caractériser cinq nouveaux plasmides avec des gènes de résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida*. De plus, la présence de deux de ces plasmides (pAB5S9b et pSN254b) causent une résistance à tous les antibiotiques approuvés par le gouvernement canadien pour une utilisation par l'industrie piscicole.

Avant d'investiguer la diversité des phages infectant *A. salmonicida* subsp. *salmonicida*, il était crucial de mieux connaître la bactérie d'intérêt. Plusieurs phages sont connus pour avoir un spectre lytique étroit, n'infectant ainsi que certaines souches ou certaines sous-espèces d'une bactérie. Or, la structure intra-espèce d'*A. salmonicida* était encore mal définie. De plus, l'une des sous-espèces d'*A. salmonicida*, *pectinolytica*, est considérée comme mésophile avec la capacité de croître à 37°C, alors que les autres sous-espèces, comme *salmonicida*, sont limitées à des températures d'environ 20°C et sont par conséquent qualifiées de psychrophiles. En caractérisant de nouvelles souches mésophiles, mes travaux ont mis en lumière que les séquences d'insertion peuvent être une raison pour expliquer cette dichotomie. De plus, il a été possible de démontrer une grande diversité génétique chez les souches mésophiles, comparativement à celles psychrophiles.

Afin de vérifier le potentiel d'un traitement par les phages contre la furonculose, trois phages spécifiques à *A. salmonicida* subsp. *salmonicida* ont été isolés de l'environnement. L'ADN de ces phages, en plus de celui de neuf autres disponibles à la collection Félix d'Hérelle, a été séquencé à haut-débit sur un appareil MiSeq d'Illumina. En comparant ces séquences génomiques à celles déjà disponibles publiquement, il a été possible de déterminer six groupes génomiques de phages contre *A. salmonicida* subsp. *salmonicida*. Les 12 phages disponibles pour la présente étude ont été testés sur 65 souches d'*A. salmonicida* (incluant des sous-espèces autres que *salmonicida*), permettant de dresser un portrait de la capacité lytique de chacun de ces virus. Cette analyse a mis en lumière trois groupes de phages ayant des capacités lytiques variables. De plus, il a été possible de montrer que d'autres sous-espèces d'*A. salmonicida* psychrophiles peuvent être infectées par les phages isolés à partir de la sous-espèce *salmonicida*. Cependant, les souches mésophiles d'*A. salmonicida* sont insensibles à ces phages.

Cette étude doctorale a montré que la résistance aux antibiotiques est un problème d'envergure dont l'ampleur était insoupçonnée chez *A. salmonicida* subsp. *salmonicida*. Elle a aussi permis d'investiguer le potentiel de la thérapie par les phages.

Abstract

Since the discovery of penicillin by Sir Alexander Fleming, antibiotics have played a paramount and indisputable role in modern medicine in helping to treat bacterial infections. However, bacteria have the ability to protect themselves against antibiotics by various mechanisms. The overuse of these molecules has accelerated the phenomenon of antibiotic resistance, making it difficult, if not impossible, to treat certain bacterial infections. Antibiotic resistance is a global problem that also negatively affects aquaculture, where bacterial infections can cause significant economic losses. One of these bacteria is *Aeromonas salmonicida* subsp. *salmonicida*, the etiologic agent of furunculosis. Although it was already known that several strains of this bacterium were carriers of plasmids conferring resistance to antibiotics, the extent of the problem was still unknown before this study.

Bacteriophages (phages) are viruses specifically infecting bacteria. Their ability to lyse bacteria has been used in a therapeutic context almost as soon as they were discovered at the beginning of the 20th century. However, the advent of antibiotics has meant that phage therapy was forgotten in several Western countries. Now that antibiotic resistance has become a significant concern for the sustainability of our society, several studies suggest that phage therapy could be an alternative or supplement to antibiotic treatments.

The objectives of this thesis were: (1) to explore the genomic diversity causing resistance to antibiotics in *A. salmonicida* subsp. *salmonicida* and (2) to investigate the potential of phage therapy to treat infections caused by this bacterium.

Five new plasmids conferring antibiotic resistance to *A. salmonicida* subsp. *salmonicida* were discovered and characterized. Two of these plasmids, pAB5S9b and pSN254b, cause resistance to all antibiotics approved by the Canadian government for use in the fish industry.

Before investigating the diversity of phages infecting *A. salmonicida* subsp. *salmonicida*, it was crucial to better know the bacterium of interest. Several phages are known to have a narrow host spectrum, infecting certain strains or subspecies. Until the present doctoral

study, the intra-species structure of *A. salmonicida* was poorly defined. In addition, one of the subspecies of *A. salmonicida*, *pectinolytica*, is considered mesophilic with the ability to grow at 37°C, while other subspecies, such as *salmonicida*, are limited to growth temperatures around 20°C and are therefore considered psychrophilic. By characterizing new mesophilic strains, we found that insertion sequences may be a reason for this dichotomy. In addition, it was possible to demonstrate a high genetic diversity in mesophilic strains compared to psychrophilic strains.

In order to verify the potential of phage treatment against furunculosis, three phages specific to *A. salmonicida* subsp. *salmonicida* were isolated from the environment. The genomic DNA of these phages, in addition to that of nine other phages available at the Felix d'Hérelle collection, was sequenced on an Illumina MiSeq device. By comparing these genomic sequences to those already available publicly, it was possible to determine six genomic groups of phages infecting *A. salmonicida* subsp. *salmonicida*. The 12 phages available were tested on 65 strains of *A. salmonicida* (including subspecies other than *salmonicida*), providing the host range of each virus. This analysis revealed three groups of phages with variable lytic capacities. In addition, it was possible to show that other psychrophilic subspecies of *A. salmonicida* can be infected by phages isolated from the subspecies *salmonicida*. However, the mesophilic strains of *A. salmonicida* are insensitive to these phages.

This doctoral study showed that resistance to antibiotics is a large-scale problem in *A. salmonicida* subsp. *salmonicida*, and that phage therapy may represent one of the solutions to the growing concern.

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Liste des abréviations

ADN (DNA)	Acide désoxyribonucléique (*Deoxyribonucleic acid*)
ANI	Identité nucléotidique moyenne (*Average nucleotide identity*)
ARN (RNA)	Acide ribonucléique (*Ribonucleic acid*)
ARNm (mRNA)	Acide ribonucléique messager (*Messenger ribonucleic acid*)
ARNr (rRNA)	Acide ribonucléique ribosomique (*Ribosomal ribonucleic acid*)
ARNt (tRNA)	Acide ribonucléique de transfert (*Transfer ribonucleic acid*)
CAT	Chloramphénicol acétyltransférase (*Chloramphenicol acetyltransferase*)
CDC	Centres pour le contrôle et la prévention des maladies (*Centers for Disease Control and Prevention*)
CHL	Chloramphénicol (*Chloramphenicol*)
DR	Répétition directe (*Direct repeat*)
FAO	Organisation des nations unies pour l'alimentation et l'agriculture (*Food and Agriculture Organization of the United Nations*)
ICNV	Comité international de la nomenclature des virus (*International Committee on Nomenclature of Viruses*)
ICTV	Comité international de taxonomie des virus (*International Committee on Taxonomy of Viruses*)
InDel	Insertions et délétions (*Insertions and deletions*)
IR	Répétition inversée (*Inverted repeat*)
IS	Séquence d'insertion (*Insertion sequence*)

LPS	Lipopolysaccharide
MIC	Concentration minimale inhibitrice (*Minimal inhibitory concentration*)
NGS	Séquençage de nouvelle génération (*Next-generation sequencing*)
OD	Densité optique (*Optical density*)
OMS (WHO)	Organisation mondiale de la santé (*World Health Organization*)
ORF	Cadre de lecture ouvert (*Open reading frame*)
PacBio	Pacific Biosciences
pb	Paire de base (*base pair*)
PCR	Réaction en chaîne par polymérase (*Polymerase chain reaction*)
RAPD	ADN polymorphe amplifié aléatoire (*Random amplified polymorphic DNA*)
RFLP	Polymorphisme de longueur de fragment de restriction (*Restriction fragment length polymorphism*)
RPM	Tours par minute (*Revolutions per minute*)
SNP	Polymorphisme d'un seul nucléotide (*Single-nucleotide polymorphism*)
subsp.	Sous-espèce (*Subspecies*)
TTSS	Système de sécrétion de type III (*Type III secretion system*)

« Seuls ceux qui sont assez fous pour penser qu'ils peuvent changer le monde y parviennent. »
Publicité d'Apple « Think different » (1997)

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Plusieurs personnes mériteraient de longs et chaleureux remerciements pour la réalisation de cette thèse de doctorat. Malheureusement, il est irréaliste de coucher sur papier l'ensemble de ceux-ci. J'ai cependant fait mon possible au quotidien pour faire savoir l'appréciation que j'avais des personnes qui m'ont aidé au fur et à mesure de mes études graduées.

Dans un premier temps, je veux remercier mon directeur Steve Charette. Steve a été un mentor extraordinaire. Surtout, il a cru en mes capacités à réaliser cette étude doctorale. Nous avons vécu, ensemble, cette grande et passionnante aventure scientifique. J'espère de tout cœur que celle-ci va continuer pour encore de nombreuses années. En plus du volet scientifique, Steve a su m'enseigner avec patience et confiance les différents aspects administratifs de la vie d'un chercheur. J'espère pouvoir faire honneur dans le futur à tout l'enseignement qu'il m'a donné.

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Il est primordial de remercier les professeurs Michel Frenette et Alexander Culley, tous deux sur mon comité d'encadrement. Leurs conseils avisés m'ont guidé à travers mes études doctorales. Un merci particulier à Michel de m'avoir impliqué dans le cours Génétique et biologie moléculaire microbiennes. Il est aussi important de remercier le professeur Louis-Charles Fortier (Université de Sherbrooke) d'avoir accepté de lire la présente thèse et d'agir à titre d'évaluateur externe pour son évaluation.

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avant tout une amie sincère en qui j'ai confiance. Elle a été présente tout au long de mon doctorat et a joué un rôle primordial dans l'ensemble de celui-ci.

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Un énorme merci à Sabrina, mon épouse, de m'avoir supporté au quotidien. La plus grande découverte de mon doctorat a été cette femme. Quoi de mieux pour un homme de science de pouvoir publier avec son épouse ? Je suis content d'avoir partagé certains des plus beaux moments de mes études graduées avec la femme que j'aime (congrès, publication, découvertes de nouveaux résultats, etc.). En plus de m'avoir assisté au niveau scientifique, sans la patience et la compréhension de mon épouse envers les longues heures passées à travailler, jamais mes études doctorales n'auraient été aussi fécondes. Il y a donc un peu de Sabrina à travers toute la présente thèse.

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Avant-propos

La présente thèse est divisée en huit chapitres. Le premier chapitre est une introduction générale aux différents concepts abordés : les antibiotiques et la résistance des bactéries à ceux-ci, les bactériophages, l'aquaculture et finalement la bactérie *Aeromonas salmonicida*. Les chapitres 2, 3 et 4 correspondent à des articles publiés sur de nouveaux plasmides avec des gènes de résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida*. Les chapitres 5 et 6 sont aussi des articles publiés, mais sur la diversité taxonomique et phylogénétique d'*A. salmonicida*. Le 7^{e} chapitre correspond au dernier article publié et inséré dans la présente thèse. Celui-ci explore les phages infectant *A. salmonicida*. Finalement, le 8^{e} chapitre est une discussion sur l'ensemble des travaux présentés.

Premier article (Chapitre 2)

Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug-resistance, interspecies exchanges, and plasmid reshaping.

Cet article a été publié en 2014 dans le 58^{e} volume du journal *Antimicrobial Agents and Chemotherapy* aux pages 7367-7374 (doi: 10.1128/AAC.03730-14).

La contribution des auteurs est la suivante : Antony T. Vincent (premier auteur) a participé à l'élaboration de l'étude, a réalisé l'ensemble des analyses bio-informatiques et a rédigé la majeure partie du manuscrit ; Mélanie V. Trudel (co-première auteure) a découvert les plasmides lors du criblage de la collection des souches et a réalisé les PCR pour fermer les séquences plasmidiques ; Valérie E. Paquet a réalisé les analyses pour déterminer les concentrations minimales inhibitrices de tétracycline pour les différentes souches étudiées ; Brian Boyle a supervisé le séquençage de l'ADN des souches ; Katherine H. Tanaka, Stéphanie Dallaire-Dufresne et Rana K. Daher ont participé aux criblages PCR des souches ; Michel Frenette a contribué à l'élaboration du projet ; Nicolas Derome a participé au financement et à la planification de l'étude et finalement Steve J. Charette a supervisé l'ensemble du travail et a participé activement à la rédaction du manuscrit.

Au moment de cette étude, Antony T. Vincent, Mélanie V. Trudel, Valérie E. Paquet, Katherine H. Tanaka, Stéphanie Dallaire-Dufresne, Rana K. Daher, Michel Frenette et Steve J. Charette étaient affiliés au Département de biochimie, de microbiologie et de bio-informatique de l'Université Laval. Brian Boyle et Nicolas Derome étaient respectivement affiliés à l'Institut de Biologie Intégrative et des Systèmes et au Département de biologie, tous deux de l'Université Laval.

Les numéros des figures et des tableaux ainsi que les références ont été modifiés pour respecter le style de la présente thèse. Le matériel supplémentaire de cet article est disponible sur le site web du journal (doi: 10.1128/AAC.03730-14).

Deuxième article (Chapitre 3)

Diversity of antibiotic-resistance genes in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida*: dominance of pSN254b and discovery of pAsa8.

Cet article a été publié en 2016 dans le 6^{e} volume du journal *Scientific Reports* à la page 35617 (doi: 10.1038/srep35617).

La contribution des auteurs est la suivante : Antony T. Vincent (co-premier auteur) a participé à l'élaboration du projet, fait l'ensemble des travaux bio-informatiques, a extrait l'ADN pour le séquençage MinION et a rédigé la deuxième version du manuscrit ; Mélanie V. Trudel a rédigé la première version du manuscrit et a fait la grande majorité des travaux expérimentaux ; Sabrina A. Attéré a découvert le plasmide pAsa8 lors d'un criblage de la collection de souches et a criblé celle-ci pour trouver le transposon Tn*1721* ; Myriam Labbé a fait la librairie d'ADN pour le séquençage MinION ; Nicolas Derome a participé au financement et à la planification de l'étude ; Alexander I. Culley a supervisé le séquençage MinION et a participé à la rédaction de l'article. Finalement Steve J. Charette a supervisé l'ensemble du travail et a participé activement à la rédaction du manuscrit.

Au moment de cette étude, Antony T. Vincent, Mélanie V. Trudel, Sabrina A. Attéré, Myriam Labbé, Alexander I. Culley et Steve J. Charette étaient affiliés au Département de biochimie, de microbiologie et de bio-informatique de l'Université Laval. Seul Nicolas Derome était affilié au Département de biologie de l'Université Laval.

Les numéros des figures et des tableaux ainsi que les références ont été modifiés pour respecter le style de la présente thèse. Le matériel supplémentaire de cet article est disponible sur le site web du journal (doi: 10.1038/srep35617).

Troisième article (Chapitre 4)

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

Cet article a été publié en 2016 dans le 162^{e} volume du journal *Microbiology* aux pages 942-953 (doi: 10.1099/mic.0.000286).

La contribution des auteurs est la suivante : Antony T. Vincent (premier auteur) a participé à l'élaboration de l'étude, a réalisé la majorité des analyses bio-informatiques, a rédigé la majeure partie du manuscrit et est responsable de la correspondance ; Jean-Guillaume Emond-Rheault a réalisé les analyses de qPCR et qRT-PCR ; Xavier Barbeau a été responsable de la modélisation de la protéine CAT ; Sabrina A. Attéré a découvert le plasmide pAsa7 et a participé aux premières analyses de biologie moléculaire ; Michel Frenette a participé à l'élaboration de l'étude ; Patrick Lagüe a supervisé les analyses structurales et finalement Steve J. Charette a supervisé l'ensemble de l'étude et participé à la rédaction du manuscrit.

Au moment de cette étude, Antony T. Vincent, Jean-Guillaume Emond-Rheault, Sabrina A. Attéré, Michel Frenette, Patrick Lagüe et Steve J. Charette étaient affiliés au Département de biochimie, de microbiologie et de bio-informatique de l'Université Laval. Seul Xavier Barbeau était affilié au Département de chimie de l'Université Laval.

Les numéros des figures et des tableaux ainsi que les références ont été modifiés pour respecter le style de la présente thèse. Le matériel supplémentaire de cet article est disponible sur le site web du journal (doi: 10.1099/mic.0.000286).

Quatrième article (Chapitre 5)

Increasing genomic diversity and evidence of constrained lifestyle evolution due to insertion sequences in *Aeromonas salmonicida*.

Cet article a été publié en 2016 dans le 17e volume du journal *BMC Genomics* à la page 44 (doi: 10.1186/s12864-016-2381-3).

La contribution des auteurs est la suivante : Antony T. Vincent (premier auteur) a participé à l'élaboration de l'étude, a réalisé l'ensemble des analyses bio-informatiques et a rédigé la majeure partie du manuscrit ; Mélanie V. Trudel a réalisé les analyses biochimiques ainsi que les courbes de croissance ; Luca Freschi a participé à l'analyse des gènes sous sélection ; Vandan Nagar a fourni les souches mésophiles d'*A. salmonicida* et a réalisé certaines analyses biochimiques ; Cynthia Gagné-Thivierge a fait les courbes de croissance à 7°C ; Roger C. Levesque a participé à la supervision de l'étude et finalement Steve J. Charette a supervisé l'ensemble de l'étude et participé à la rédaction du manuscrit.

Au moment de cette étude, Antony T. Vincent, Mélanie V. Trudel, Cynthia Gagné-Thivierge et Steve J. Charette étaient affiliés au Département de biochimie, de microbiologie et de bio-informatique de l'Université Laval. Vandan Nagar était affilié au Food Technology Division du Bhabha Atomic Research Centre (Mumbai, Inde). Finalement, Luca Freschi et Roger C. Levesque étaient affiliés à l'Institut de Biologie Intégrative et des Systèmes de l'Université Laval.

Les numéros des figures et des tableaux ainsi que les références ont été modifiés pour respecter le style de la présente thèse. Le matériel supplémentaire de cet article est disponible sur le site web du journal (doi: 10.1186/s12864-016-2381-3).

Cinquième article (Chapitre 6)

Study of mesophilic *Aeromonas salmonicida* A527 strain sheds light on the species lifestyles and taxonomic dilemma.

Cet article a été publié en 2017 dans le 364e volume du journal *FEMS Microbiology Letters* à la page fnx239 (doi: 10.1093/femsle/fnx239).

La contribution des auteurs est la suivante : Antony T. Vincent (premier auteur) a participé à l'élaboration de l'étude, a réalisé l'ensemble des analyses bio-informatiques, a rédigé le manuscrit et est responsable de la correspondance ; François D. Rouleau a fait les analyses

biochimiques ainsi que l'annotation des séquences d'insertion ; Sylvain Moineau et Steve J. Charette ont participé au financement de l'étude, à sa planification et ont révisé le manuscrit. Au moment de cette étude, tous les auteurs étaient affiliés au Département de biochimie, de microbiologie et de bio-informatique de l'Université Laval.

Les numéros des figures et des tableaux ainsi que les références ont été modifiés pour respecter le style de la présente thèse. Le matériel supplémentaire de cet article est disponible sur le site web du journal (doi: 10.1093/femsle/fnx239).

Sixième article (Chapitre 7)

Characterization and diversity of phages infecting *Aeromonas salmonicida* subsp. *salmonicida.*

Cet article a été publié en 2017 dans le 7^{e} volume du journal *Scientific Reports* à la page 7054 (doi: 10.1038/s41598-017-07401-7).

La contribution des auteurs est la suivante : Antony T. Vincent (premier auteur) a participé à l'élaboration de l'étude, a réalisé l'ensemble des analyses bio-informatiques, a participé à la campagne d'échantillonnage ainsi qu'à la réalisation expérimentale des analyses sur l'activité lytique des phages ; Valérie E. Paquet a participé à l'élaboration de l'étude, a réalisé la grande majorité des travaux expérimentaux en collaboration avec Alex Bernatchez et a aussi participé à la compagne d'échantillonnage ; Denise M. Tremblay a participé à l'élaboration de l'étude et a fait les analyses de microscopie ; Sylvain Moineau et Steve J. Charette ont obtenu le financement pour l'étude, ont participé à son élaboration et ont révisé le manuscrit. Au moment de cette étude, tous les auteurs étaient affiliés au Département de biochimie, de microbiologie et de bio-informatique de l'Université Laval.

Les numéros des figures et des tableaux ainsi que les références ont été modifiés pour respecter le style de la présente thèse. Le matériel supplémentaire de cet article est disponible sur le site web du journal (doi: 10.1038/s41598-017-07401-7).

Chapitre 1 - Introduction

Les antibiotiques

Les antibiotiques sont naturellement présents dans la nature et sont principalement produits par les microorganismes afin d'augmenter leur compétitivité pour les ressources (Berdy 2012; Raaijmakers & Mazzola 2012). Bien que l'utilisation des antibiotiques pourrait remonter à plusieurs centaines d'années (Aminov 2010), il est admis par la communauté scientifique que le début de l'ère des antibiotiques comme nous la connaissons aujourd'hui correspond à la découverte par Sir Alexander Fleming de la pénicilline, produite par l'ascomycète *Penicillium* (Fleming 1929). Il existe maintenant plusieurs classes d'antibiotiques pouvant être synthétisés et ayant des cibles diverses (Fair & Tor 2014). Par exemple, certains antibiotiques, comme la rifamycine, bloquent l'ARN polymérase, alors que d'autres perturbent ou inhibent les voies de synthèse des parois cellulaires et des LPS, des acides nucléiques, des protéines ainsi que de celle du folate (Kohanski *et al.* 2010; Brown 2015).

L'antibiorésistance

Malgré le fait qu'il soit indéniable que les antibiotiques aient participé à modeler la médecine moderne ainsi qu'à sauver un nombre incalculable de vies, le combat contre les bactéries pathogènes est loin d'être derrière nous (Holmes *et al.* 2016). En ce sens, les bactéries peuvent utiliser divers mécanismes de protection contre les molécules antibiotiques. Il est connu que des souches bactériennes résistantes aux antibiotiques sont présentes en absence de sélections extérieures (Martinez 2009). Cependant, un équilibre va exister entre ces souches résistantes et celles qui sont sensibles. Le problème est que les antibiotiques ont été surutilisés dans les contextes de médecines humaine (Goossens *et al.* 2005) et vétérinaire (Wayne *et al.* 2011) ainsi que dans la production alimentaire (animaux terrestres, poissons et agriculture) (Cabello 2006; Martin *et al.* 2015). Bien que certains pays aient banni l'utilisation d'antibiotiques comme facteurs de croissance pour les animaux, dont ceux de l'Union européenne en 2006 (Martin *et al.* 2015), il est estimé que 80 % des antibiotiques utilisés aux États-Unis sont pour l'agriculture et l'aquaculture, bien souvent pour stimuler la croissance du bétail ou pour prévenir les maladies (Hollis & Ahmed 2013). Bien qu'il soit difficile de clairement définir le secteur ayant le plus

contribué à l'amplification du phénomène de résistance aux antibiotiques (Chang *et al.* 2015), cette surutilisation a généré une pression de sélection sur les cellules bactériennes résistantes à ces molécules, les faisant devenir dominantes dans certains écosystèmes (Figure 1).

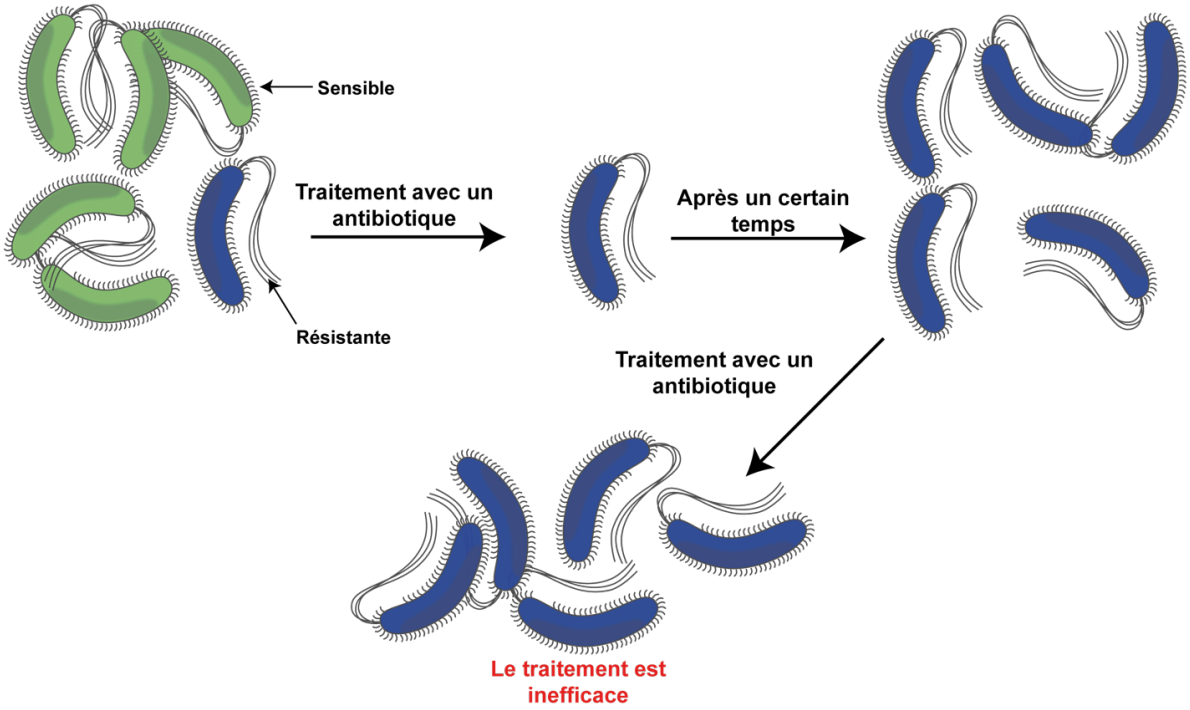

Figure 1. Schématisation de l'évolution d'une population bactérienne suite à l'utilisation d'un antibiotique.

Les bactéries peuvent se protéger des antibiotiques en utilisant principalement deux stratégies (Munita & Arias 2016): (I) en mutant des gènes clés et (II) en acquérant par transferts horizontaux des gènes causant une résistance aux différents composés antibiotiques. Un exemple bien connu de résistance produite par la mutation d'un gène est la résistance pour la rifampicine. Cet antibiotique se lie à la sous-unité β de l'ARN polymérase (encodée par le gène *rpoB*), bloquant ainsi la transcription (Campbell *et al.* 2001). Il est connu que certaines mutations non synonymes dans le gène *rpoB* peuvent diminuer l'affinité de rifampicine à sa cible, résultant ainsi en une résistance (Floss & Yu 2005).

Il y a principalement trois mécanismes permettant aux bactéries d'échanger du matériel génétique : la transformation (incorporation d'ADN environnemental), la transduction

(transfert par les phages) et finalement la conjugaison (contact entre les cellules) (Holmes *et al.* 2016). En général, la conjugaison, qui est l'un des principaux moyens pour le transfert de gènes de résistance aux antibiotiques (Holmes *et al.* 2016), utilise les plasmides comme vecteurs pour promouvoir le mouvement de ces gènes. Les plasmides sont définis comme des éléments génétiques extrachromosomiques autoréplicatifs (Actis *et al.* 1999). En fait, les fonctions biologiques encodées par les plasmides sont extrêmement diverses et peuvent aider à améliorer le *fitness* des cellules en induisant une résistance aux agents antimicrobiens, en augmentant les capacités métaboliques ainsi qu'en fournissant des facteurs de virulence (Srivastava 2013). Finalement, les bactéries peuvent aussi acquérir des gènes de résistance aux antibiotiques grâce à des intégrons qui sont des systèmes de recombinaison site-spécifique capables de recruter des gènes (notamment de résistance aux antibiotiques) sous la forme de cassettes mobiles (Deng *et al.* 2015).

Les gènes codant pour une résistance aux antibiotiques se divisent en deux groupes : ceux qui altèrent ou détruisent la molécule antibiotique et ceux qui diminuent la pénétration ou augmentent l'expulsion de cette molécule (principalement par des pompes à efflux) hors de la cellule (Munita & Arias 2016). Les deux mécanismes de résistance (modification de l'antibiotique ou diminution de son flux) peuvent exister pour une même molécule antibiotique. C'est le cas par exemple du chloramphénicol dont la résistance peut être apportée par une acétylation de la molécule par une chloramphénicol acétyltransférase ainsi que par une diminution de la concentration intracellulaire par une pompe à efflux (Schwarz *et al.* 2004).

Bien que de nouveaux antibiotiques soient développés ou en cours de développement, il est possible de constater une diminution des nouveaux composés antimicrobiens (Högberg et al. 2010; Piddock 2012). De plus, lorsque de nouvelles molécules antibiotiques sont commercialisées, des souches bactériennes résistantes sont souvent répertoriées peu de temps après leur arrivée sur le marché et parfois même avant (Tableau 1).

Nous constatons maintenant une augmentation drastique des souches bactériennes résistantes, voire multirésistantes, aux antibiotiques. Récemment, des cas de résistance à la colistine, un antibiotique de dernier recours, ont été répertoriés en Chine (Liu *et al.* 2016), en Amérique et en Europe (McGann *et al.* 2016). Il est évident pour la communauté

scientifique, mais aussi pour les agences gouvernementales comme Santé Canada (Public Health Agency of Canada 2016), le CDC (Centers for Diseases Control and Prevention 2013) et l'OMS (World Health Organization 2015) que la résistance aux antibiotiques est un problème d'envergure mondiale requérant des actions concrètes et immédiates. Selon un rapport de 2016 commissionné par le premier ministre du Royaume-Uni, il est estimé qu'en 2050, il pourrait il y avoir jusqu'à 10 millions de morts causés directement par les bactéries résistantes aux antibiotiques, un niveau aussi élevé que les décès par cancer, ce qui ferait d'elles la première source de décès à travers le monde (O'Neill 2016).

Tableau 1. Historique de l'introduction et de la résistance répertoriée pour les classes d'antibiotiques[a]

Classe	Introduction	Résistance	Activité	Cible
Sulfamidé	1936	1942	Inhibition dihydroptéroate synthase	Gram positif
β-lactame	1938	1945	Inhibition de la biosynthèse de la paroi cellulaire	Large spectre
Aminoside	1946	1946	Liaison à la sous-unité ribosomale 30S	Large spectre
Chloramphénicol	1948	1950	Liaison à la sous-unité ribosomale 50S	Large spectre
Macrolide	1951	1955	Liaison à la sous-unité ribosomale 50S	Large spectre
Tétracycline	1952	1950	Liaison à la sous-unité ribosomale 30S	Large spectre
Rifampicine	1958	1962	Liaison à la sous-unité β de l'ARN polymérase	Gram positif
Glycopeptide	1958	1960	Inhibition de la biosynthèse de la paroi cellulaire	Gram positif
Quinolones	1968	1968	Inhibition de la synthèse de l'ADN	Large spectre
Streptogramine	1998	1964	Liaison à la sous-unité ribosomale 50S	Gram positif
Oxazolidone	2000	2001	Liaison à la sous-unité ribosomale 50S	Gram positif
Lipopetide	2003	1987	Dépolarisation de la membrane cellulaire	Gram positif
Fidaxomicine	2011	1977	Inhibition de l'ARN polymérase	Gram positif
Diarylquinolines	2012	2006	Inhibition de F1FO-ATPase	*M. tuberculosis*

a. Les données sont dérivées de Lewis 2013.

Alternatives aux antibiotiques et concept d'« une seule santé »

Il est maintenant clair qu'en plus de meilleurs moyens diagnostiques, nous devons nous doter de nouveaux traitements pour lutter contre les bactéries résistantes aux antibiotiques (Allen *et al.* 2014; Reardon 2015; Czaplewski *et al.* 2016). Un groupe de 24 scientifiques du milieu académique et de l'industrie a relevé 10 alternatives aux antibiotiques avec suffisamment de données cliniques et d'études indépendantes pour croire en leur homologation d'ici 2025 (Tableau 2) (Czaplewski *et al.* 2016). Cependant, cette même équipe indique qu'un budget d'un milliard et demi de livres sterling devrait être nécessaire pour tester et développer ces 10 alternatives aux antibiotiques. De plus, les essais cliniques

sont principalement axés sur les bactéries *Clostridium difficile*, *Pseudomonas aeruginosa* et *Staphylococcus aureus*. Par conséquent, il est très difficile de prédire le succès de ces alternatives contre d'autres bactéries pathogènes et résistantes aux antibiotiques, comme *Enterococcus*, *Klebsiella*, *Acinetobacter* ou *Enterobacter* (Czaplewski *et al.* 2016).

Tableau 2. Liste de 10 alternatives aux antibiotiques considérées comme prometteuses

Alternative	Description	*P*(Homologation[%])[a]
Anticorps	Protéines utilisées par le système immunitaire afin de cibler spécifiquement un agent pathogène	183[b]
Probiotiques	Microorganismes conférant une résistance à l'hôte contre d'autres microorganismes	124
Lysines	Enzymes utilisées par les phages pour détruire les parois cellulaires bactériennes	26
Phages	Virus infectant spécifiquement les bactéries	9[c]
Phages modifiés	Phages modifiés génétiquement afin d'augmenter leur potentiel antimicrobien	9[c]
Stimulation immunologique	Augmentation de la réponse immunitaire de l'hôte	43
Vaccins	Injection de fragments ou de l'agent pathogène dans un hôte afin de créer une réaction immunitaire	188
Peptides antimicrobiens	Fragments d'acides aminés ciblant les agents pathogènes	52[d]
Peptides de défense de l'hôte et innés	Fragments d'acides aminés qui stimulent le système immunitaire	52[d]
Peptides antibiofilms	Fragments d'acides aminés qui ciblent les biofilms (communauté multicellulaire vivant dans une matrice composée de substances polymères)	52[d]

a : Probabilité d'être homologuée d'ici 2025
b : Les probabilités supérieures à 100 % indiquent qu'avec l'expertise et le financement nécessaires, ces projets ont réalistement beaucoup de chance d'être homologués d'ici 2025
c : Les phages « sauvages » et ceux génétiquement modifiés ont été regroupés ensemble pour le calcul de la probabilité de leur homologation
d : Les peptides ont été regroupés ensemble pour le calcul de la probabilité de leur homologation

En plus d'alternatives aux antibiotiques, les experts sont consensuels sur le fait qu'il faut considérer la résistance aux antibiotiques dans le concept de l'initiative d'une seule santé (*One health*). Le concept d'une seule santé stipule qu'il faut considérer les humains, les animaux et l'environnement comme formant un tout (Queenan *et al.* 2016). Une défaillance dans l'une de ces trois catégories aura forcément des conséquences sur les deux autres. De plus, il existe certains environnements clés étroitement liés avec les animaux et les humains et dont, par conséquent, la « santé » doit être surveillée. Comme indiqué dans une revue récente de la littérature sur le sujet, les environnements aquatiques sont particulièrement importants à prendre en considération puisqu'ils produisent une part non négligeable de

l'alimentation et fournissent un milieu propice à la dissémination des gènes de résistance aux antibiotiques entre les différentes bactéries (Cabello *et al.* 2016).

Les bactériophages

La découverte et l'importance des bactériophages

Les bactériophages (phages) sont des virus infectant spécifiquement et exclusivement les bactéries. Ces entités biologiques ont été codécouvertes indépendamment par le Britannique Frederick Twort (Twort 1915) et le Franco-québécois Félix d'Hérelle (d'Herelle 1917) et sont connues pour être les plus abondantes sur la planète, dépassant même leurs hôtes (les bactéries) d'un facteur dix (Dublanchet & Bourne 2007). Bien qu'il soit maintenant impensable de remettre en question la nature virale des phages, il n'en a pas toujours été ainsi. En fait, après la publication faite par d'Hérelle, il s'en est suivi plusieurs années de débats afin de savoir s'ils étaient des virus ou des enzymes lytiques (Summers 2012). Ce n'est qu'en 1940 que la preuve de la nature virale des phages est arrivée avec la photo d'un coliphage (un phage infectant la bactérie *Escherichia coli*) par microscopie électronique (Ackermann 2011).

En 1962, l'élaboration d'un système de classification des virus basé sur leur type d'acides nucléiques (ADN ou ARN) ainsi que sur leur structure morphologique a vu le jour (Lwoff *et al.* 1962). L'*International Committee on Nomenclature of Viruses* (ICNV) a été formé en 1966 afin de baliser et d'officialiser la taxonomie et la classification des virus. Encore de nos jours, ce comité, appelé *International Committee on Taxonomy of Viruses* (ICTV) depuis 1975, joue un rôle de premier plan dans la classification des virus (Adams *et al.* 2017). Bien que la classification basée sur les caractères morphologiques et le type d'acides nucléiques soit encore couramment utilisée (Ackermann 2009), l'avènement de l'ère du séquençage et la facilité d'accès aux nouvelles technologies (Vincent *et al.* 2017) a permis l'élaboration d'outils utilisant les informations génomiques afin d'affiner la classification et la taxonomie des phages, et même des virus en général (Adriaenssens & Brister 2017). Cependant, encore de nos jours et malgré les technologies récentes, les virus, du fait du manque d'un marqueur commun (comme le gène de l'ARNr 16S bactérien, par exemple), d'un taux de substitutions élevé et de leur grande plasticité génomique, demeurent un défi à définir au niveau taxonomique (Gao & Qi 2007). Cette grande difficulté à introduire les

virus dans l'arbre de la vie au niveau phylogénétique est, pour certains chercheurs, justement un point clé pour démontrer qu'ils ne sont pas vivants (Moreira & López-García 2009).

Très tôt, l'utilisation des phages en laboratoire a permis de faire des découvertes importantes, comme le fait que l'ADN est la molécule portant le bagage génétique (Hershey & Chase 1952) et que des mutations peuvent survenir en absence de sélection (Luria & Delbrück 1943). Les phages ont aussi joué un rôle primordial dans la découverte d'enzymes clés utilisées en biologie moléculaire, comme les enzymes de restriction (Smith & Welcox 1970) ainsi que les ligases (Weiss & Richardson 1967). Finalement, les premiers génomes à avoir été séquencés sont ceux des phages MS2 (ARN simple brin) (Fiers *et al.* 1976), ΦX174 (ADN simple brin) (Sanger *et al.* 1977) et λ (ADN double brin) (Sanger *et al.* 1982).

Bien que le mode de multiplication d'une population de phages ait été très rapidement caractérisé à l'aide de la célèbre méthode du « *one-step growth* » (Ellis & Delbrück 1939) et que les phages aient été largement utilisés en biologie moléculaire, les connaissances sur leur importance écologique sont plutôt récentes. Ce n'est qu'en 1989 qu'on a pris conscience que les phages sont particulièrement abondants avec une concentration pouvant aller jusqu'à 2,5 x 10^8 particules virales par millilitre d'eau en milieux naturels (Bergh *et al.* 1989). Il est maintenant bien connu que les phages jouent d'importants rôles écologiques en contrôlant les populations bactériennes et en étant impliqués dans plusieurs cycles biogéochimiques (Bratbak *et al.* 1990; Proctor & Fuhrman 1990; Suttle 2007; Sime-Ngando 2014).

La biologie des phages

Les phages peuvent être lytiques ou lysogènes (Figure 2). Un cycle lytique comporte cinq grandes étapes (Sulakvelidze & Alavidze 2001; Drulis-Kawa *et al.* 2012): (I) l'adsorption du phage à la surface de la bactérie, (II) l'injection du matériel génétique phagique à l'intérieur de la bactérie hôte, (III) la réplication du génome phagique et la redirection de la machinerie cellulaire de l'hôte pour la production de particules virales, (IV) l'assemblage des phages et (V) la lyse de la cellule hôte. Pour un phage tempéré, les étapes I et II se

produisent comme pour un phage lytique, cependant, par la suite, il est possible que l'ADN du phage s'insère dans le chromosome bactérien (Sulakvelidze & Alavidze 2001). À cette étape, l'ADN du phage est répliqué en même temps que l'ADN bactérien et se nomme prophage. Un évènement, tel un stress, peut causer l'excision du prophage, lui permettant ainsi de poursuivre son cycle aux étapes III, IV et V comme pour un phage lytique. Il est à noter que certains prophages, dont ceux trouvés chez la bactérie *Leptospira*, peuvent répliquer leur ADN de façon extrachromosomique similaire à celle des plasmides (Girons *et al.* 2000; Zhu *et al.* 2015).

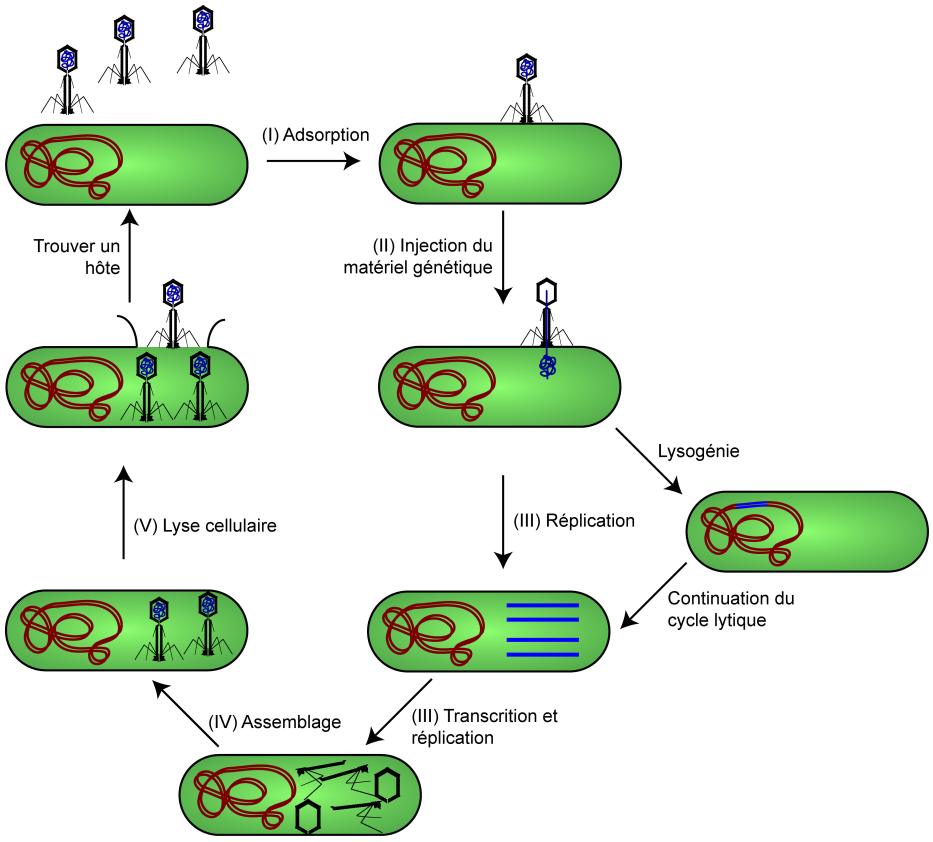

Figure 2. Schématisation des cycles lytique et lysogénique.

La thérapie par les phages

Rapidement, des études sur le potentiel thérapeutique des phages, aussi appelé phagothérapie, ont été publiées (McKinley 1923). C'est Félix d'Hérelle qui a été le pionnier dans la phagothérapie, en utilisant des phages contre la dysenterie, mais aussi contre le choléra et la peste bubonique (Chanishvili 2012). Cependant, le manque de matériel, de méthodes standardisées, de contrôles statistiques et de vérifications en aveugles a fait en

sorte que plusieurs études étaient contradictoires sur le potentiel des phages dans un contexte thérapeutique (Summers 2012). De plus, les phages ont un spectre lytique habituellement étroit, ce qui rendait complexe leur utilisation, contrairement aux antibiotiques qui ont un spectre d'action souvent bien plus large (Summers 2012).

Malgré tout, aujourd'hui encore, la thérapie par les phages est couramment utilisée avec succès en République de Géorgie, en Pologne et en Russie (Abedon *et al.* 2011). Avec la crise grandissante de la résistance aux antibiotiques chez les bactéries, l'utilisation des bactériophages dans un contexte thérapeutique ou de biocontrôle a été explorée à nouveau par de nombreuses études (Abedon 2014). Il y a d'ailleurs un nombre croissant d'articles chaque année mentionnant l'expression « *phage therapy* » (Figure 3).

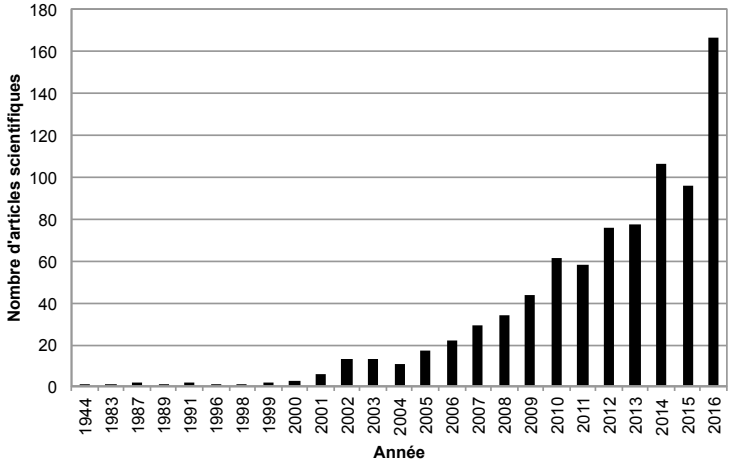

Figure 3. Nombre d'articles scientifiques indexés par Web of Science qui contiennent l'expression exacte « phage therapy ».

Une récente étude clinique a montré d'excellents taux de succès pour traiter des cas d'otites chroniques causés par *P. aeruginosa* (Wright *et al.* 2009). De plus, une initiative européenne, PhagoBurn (http://www.phagoburn.eu), est actuellement dans la première phase clinique pour élaborer une thérapie par les phages contre *E. coli* (110 patients) et *P. aeruginosa* (110 patients) chez les grands brulés. D'un point de vue commercial, plusieurs compagnies s'intéressent à une application thérapeutique des phages et certains produits à base de ces virus sont déjà homologués et commercialisés (Sarhan & Azzazy 2015). Par exemple, au Canada, il est possible d'utiliser le produit AgriPhage-CMM, qui contient des phages contre *Clavibacter michiganensis* subsp. *michiganensis*, l'agent étiologique causant

le chancre de la tomate. Des produits à base de phages sont aussi homologués aux États-Unis pour une application alimentaire (Bai *et al.* 2016): ListShield (*Listeria monocytogenes*), EcoShield (*E. coli* O157:H7) et SalmoFresh (*Salmonella enterica*).

Considérations à prendre pour la phagothérapie

Bien que la thérapie par les phages comporte plusieurs avantages tels un effet antibactérien, un potentiel d'autodosage, une faible toxicité et un faible cout de développement (Loc-Carrillo & Abedon 2011), certaines considérations sont nécessaires dans le choix d'un phage à des fins thérapeutiques. Les considérations les plus courantes sont : l'utilisation d'un phage exclusivement lytique (Brüssow 2012), ne faisant pas de transduction (Sulakvelidze & Alavidze 2001) et ne portant pas de gènes conférant de résistance aux antibiotiques, toxines ou autres facteurs de virulence bactériens (Sulakvelidze & Alavidze 2001). En effet, les phages peuvent être des vecteurs importants pour les transferts horizontaux de gènes et ainsi produire des bactéries plus virulentes ou résistantes par l'acquisition de gènes de toxines ou de résistance aux antibiotiques (Verheust *et al.* 2010; Abedon *et al.* 2011).

Un phage tempéré peut éventuellement s'insérer dans le génome de la bactérie cible et ainsi devenir un prophage. Dans cet état, l'ADN du phage devient une partie intégrante du génome bactérien et peut donc rendre ses gènes utilisables par la bactérie (De Paepe *et al.* 2014). Plusieurs génomes bactériens sont connus pour contenir des prophages et dans certains cas le pourcentage d'ADN phagique peut atteindre plus de 20 % du génome total (Casjens 2003; Hatfull & Hendrix 2011). Les prophages sont reconnus pour potentiellement conférer certaines caractéristiques causant une augmentation du *fitness* de la bactérie comme : (I) un point d'ancrage pour les réarrangements, (II) une protection contre les infections ultérieures de phages, (III) la lyse de souches compétitives par une induction des prophages et (IV) des facteurs de conversion lysogénique (Brüssow *et al.* 2004). *Escherichia coli* O157:H7 est un exemple connu de bactéries ayant des gènes de toxines (les toxines Shiga) provenant d'un prophage (Plunkett *et al.* 1999). De plus, la présence d'un prophage dans le chromosome de la bactérie hôte peut induire du transfert de matériel génétique avec un phage lytique par recombinaison homologue (Bouchard &

Moineau 2000; Brüssow 2006). Une connaissance du génome de la bactérie cible est donc primordiale.

Deux autres considérations importantes sont l'étroitesse du spectre lytique des phages ainsi que le développement de souches bactériennes résistantes aux phages. Les phages ont tendance à être spécifiques à quelques espèces bactériennes, voire même qu'à seulement quelques souches (Hyman & Abedon 2010). Ceci peut représenter un problème dans un contexte thérapeutique puisque cela exige de connaitre avec précision la bactérie causant l'infection. Cependant, il est souvent suggéré qu'un mélange de phages, sous la forme d'un cocktail, pourrait augmenter la largeur du spectre lytique (Chan & Abedon 2012; Chan *et al.* 2013). Plusieurs mécanismes bactériens de résistance contre les phages sont maintenant connus. Parmi les plus fréquents, nous pouvons penser à l'empêchement de l'adsorption du phage, le blocage de l'entrée du matériel génétique phagique, la dégradation des acides nucléiques phagiques et la mort cellulaire (systèmes Abi - *Bacterial abortive infection*) (Labrie *et al.* 2010). Cependant, les phages, contrairement aux antibiotiques, sont des entités biologiques dynamiques capables d'évoluer afin de contrecarrer les mécanismes de protection bactériens (Samson *et al.* 2013). De plus, encore cette fois, il est proposé que l'utilisation d'un cocktail de phages serait en mesure de diminuer la probabilité de résistance à ceux-ci (Abuladze *et al.* 2008; Lu & Koeris 2011). Il est cependant recommandé de minimiser le nombre de virus et de tester rigoureusement le cocktail pour vérifier que les phages n'ont pas d'effets antagonistes et qu'il n'y a pas de recombinaison entre les génomes des phages (Klumpp & Loessner 2013; Mateus *et al.* 2014).

L'aquaculture

L'aquaculture canadienne

L'aquaculture est une industrie clé dans l'alimentation où les productions de poissons et de plantes aquatiques étaient estimées en 2013 à 43,1 % et 95,5 % respectivement, de l'apport alimentaire mondial (FAO 2015). Le Canada est un petit producteur dans l'industrie aquacole où il n'a produit que 0,18 % des poissons d'élevage en 2015 (Figure 4A), alors que la Chine en a produit à elle seule 58 %. Malgré tout, l'aquaculture est en expansion au Canada (Figure 4B), passant de 3 566 tonnes en 1980 à 187 374 en 2015, soit une augmentation 5 254 %. L'industrie aquacole canadienne est principalement axée sur

l'élevage du saumon de l'atlantique (*Salmo salar*) avec 65 % de la production en 2015 tandis que la 2e espèce la plus élevée au Canada, la même année, était la moule commune (*Mytilus edulis*) avec 12 % (Figure 4C).

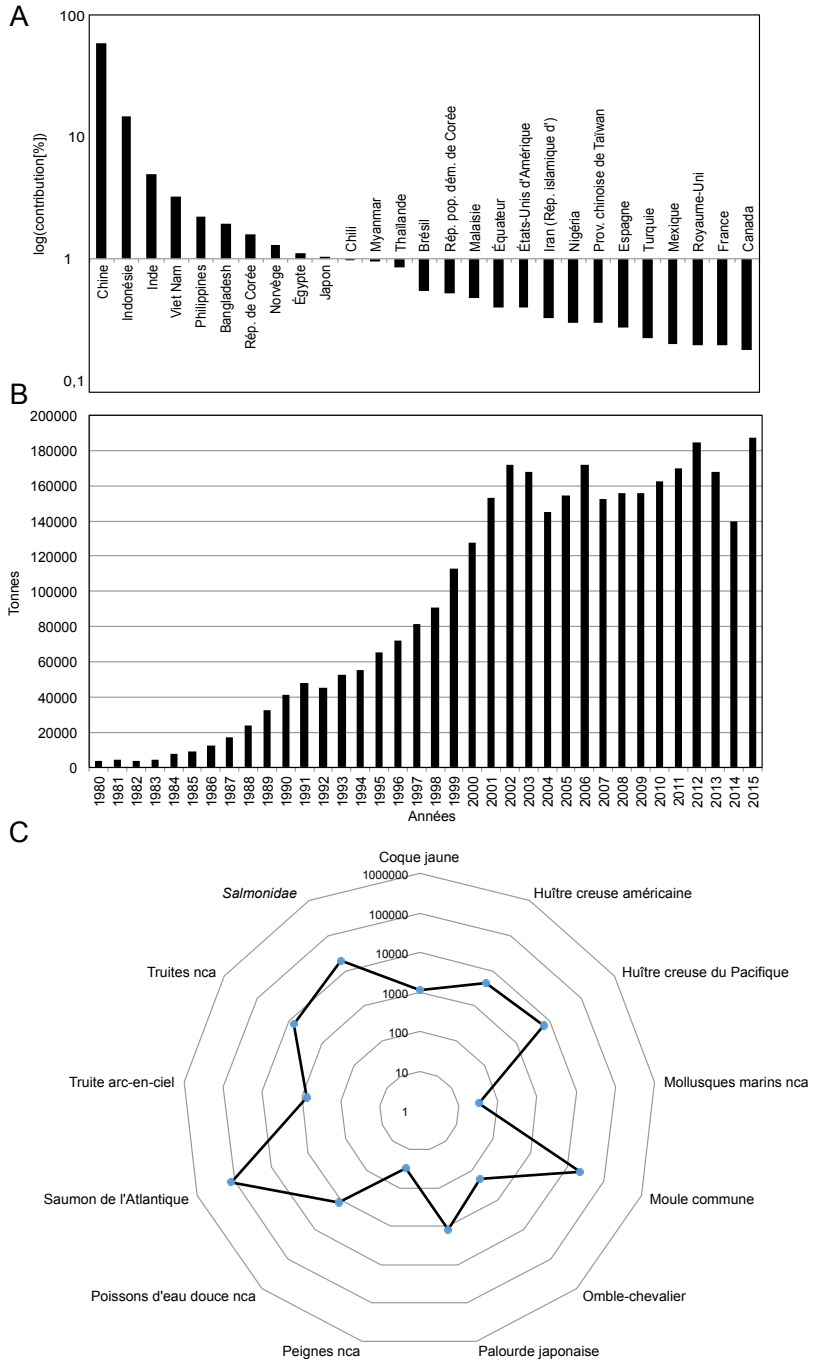

Figure 4. Statistiques sur l'aquaculture.

(A) Contribution par pays à la quantité d'animaux élevés en aquaculture pour l'année 2015 au niveau mondial. (B) Productions annuelles du Canada en tonnes d'animaux élevés en aquaculture de 1980 à 2015. (C) Nombre de tonnes d'animaux élevés en aquaculture au Canada en 2015. Les données ont été récupérées de la FAO (*Food and Agriculture Organization of the United Nations*) par le logiciel FishStatJ (FAO 2017).

L'aquaculture québécoise

Au Québec, l'aquaculture est principalement dulcicole (aquaculture en eaux douces) et est dominée par l'élevage de l'omble de fontaine (*Salvelinus fontinalis*) et de la truite arc-en-ciel (*Oncorhynchus mykiss*) où 475 et 555 tonnes ont été produites, respectivement, en 2014 (MAPAQ 2016). L'aquaculture dulcicole au Québec avait rapporté 9,4 millions de dollars en 2014 (MAPAQ 2016).

Les poissons d'élevage, comme tous les animaux, sont sensibles aux maladies. Au Québec, c'est principalement le service de diagnostic en ichtyopathologie de la faculté de médecine vétérinaire de l'Université de Montréal (http://servicedediagnostic.com) qui s'occupe de répertorier les cas de maladies aquicoles. Les clients de ce service sont majoritairement les pisciculteurs et les vétérinaires. Les deux principales espèces soumises sont l'omble de fontaine ainsi que la truite arc-en-ciel, les deux espèces dominantes au niveau de l'élevage (Lafaille 2016). Les maladies les plus répertoriées sont la furonculose (causée par la bactérie *Aeromonas salmonicida* subsp. *salmonicida*), les mycoses, les parasitoses, l'hyperplasie branchiale ainsi que la maladie de la selle (causée par la bactérie *Flavobacterium columnare*) (Figure 5) (Lafaille 2016). Il est possible d'observer une importante concentration d'éclosions de maladies durant les chauds mois d'été, où les poissons sont stressés et leur système immunitaire affaibli (Morin 2010; Lafaille 2016).

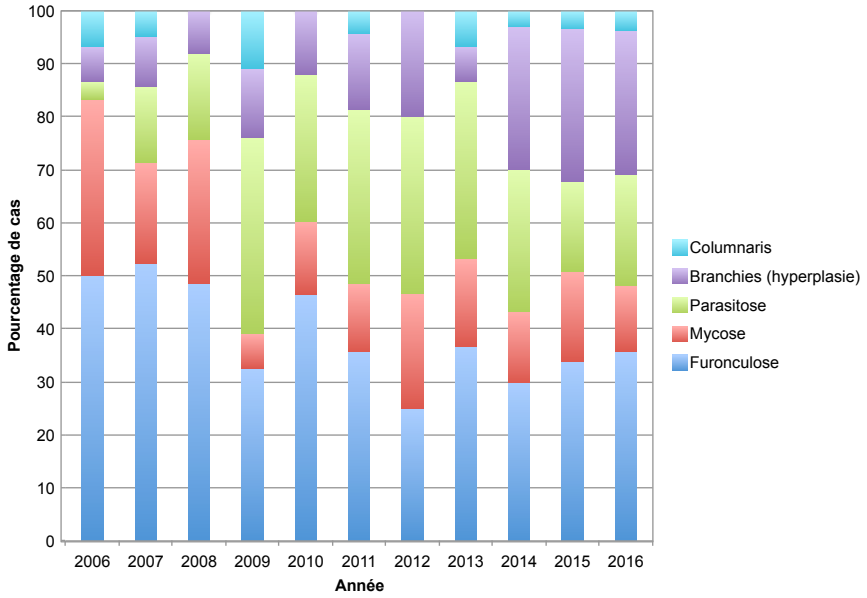

Figure 5. Cas de maladies aquicoles répertoriés au Québec.
Ces résultats sont dérivés des données produites par le service de diagnostic en ichtyopathologie de la faculté de médecine vétérinaire de l'Université de Montréal.

Un problème associé à certaines de ces maladies est l'antibiorésistance. Plusieurs cas de résistance aux antibiotiques sont répertoriés chaque année par le service de diagnostic en ichtyopathologie (Lafaille 2016, 2017). Selon ce service, l'évolution des souches résistantes aux antibiotiques peut venir d'une surutilisation ou d'un sous-dosage des antibiotiques. Sinon, les souches résistantes elles-mêmes peuvent provenir d'une contamination extérieure par l'achat de poissons ou par transmission d'autres espèces si la ferme est située dans un milieu agricole (Lafaille 2016). En 2015, il y a eu une diminution de 12,5 % des prescriptions d'antibiotiques émises par le service ambulatoire de la faculté de médecine vétérinaire comparativement à 2014. Malheureusement, le nombre de prescriptions a augmenté drastiquement de 42,9% en 2016 par rapport à 2015 (Lafaille 2017). Cette augmentation peut être expliquée par le fait que neuf fermes ont eu des récidives de furonculose. De ces neuf fermes, trois à elles seules ont reçu 22 ordonnances d'antibiotiques. Toujours en 2016, à l'exception d'un seul cas, les antibiotiques ont été prescrits pour des épisodes de furonculose. L'antibiotique le plus utilisé au Québec est l'Aquaflor (florfénicol) (75-96 %) étant donné sa courte période de retrait suivi par l'Oxysol (tétracycline) (4-22 %).

Aeromonas salmonicida subsp. *salmonicida*

La furonculose

La furonculose est une maladie aquicole d'envergure mondiale, retrouvée majoritairement chez les salmonidés d'élevage (Dallaire-Dufresne *et al.* 2014). Le terme furonculose est reconnu comme un abus de langage qui est tiré d'une pathologie humaine et qui a persisté dans la littérature jusqu'à devenir un terme admis pour désigner la présente maladie aquicole (McCarthy 1975b). Il est possible de reconnaître deux formes à la maladie : chronique et aigüe. La forme chronique affecte principalement les poissons en âge avancé et se caractérise par une léthargie, l'apparition de furoncles, des hémorragies de plusieurs tissus ainsi que par le mauvais fonctionnement de certains organes (foie, rate et reins) (Austin & Austin 2016). Il est cependant important de noter que dans certains cas il n'y a pas de signes apparents de la maladie et que la furonculose peut se déclarer suite à un stress (McCarthy 1975a). De plus, la forme chronique ne cause généralement pas un taux de mortalité très élevé (McCarthy 1975b; Austin & Austin 2016).

La forme aigüe, quant à elle, cause une septicémie générale et se déclare principalement chez les poissons en croissance (Austin & Austin 2016) (Figure 6). Cette forme de la furonculose cause notamment un haut taux de mortalité et les poissons meurent en général en deux à trois jours suivant le début de l'infection (McCarthy 1975b; Austin & Austin 2016).

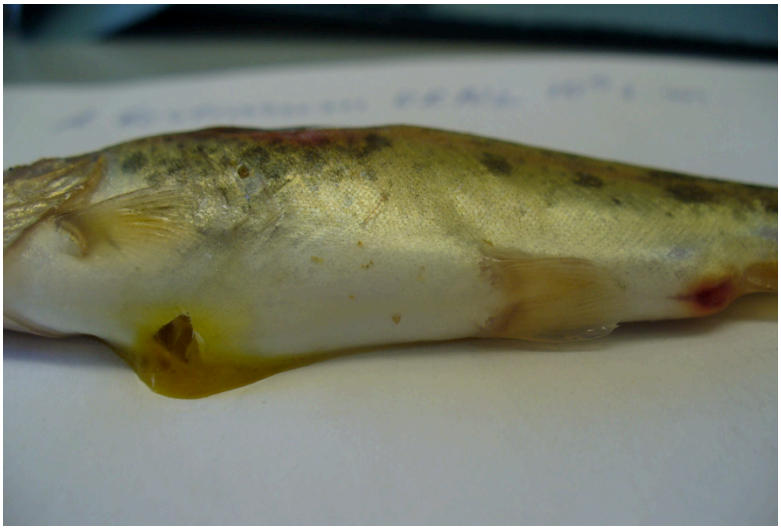

Figure 6. Infection aigüe de furonculose d'une truite arc-en-ciel.
Le poisson montre une septicémie hémorragique et une liquéfaction des organes internes. Cette photo est une gracieuseté du professeur Brian Austin de l'Université Stirling (Écosse).

Le contrôle de la furonculose

En 1925, il a été démontré qu'il était possible de sélectionner des lignées de poissons plus résistantes à la furonculose (Embody & Hayford 1925). Ce n'est qu'en 1959 qu'une étude démontra qu'il existait certains déterminants génétiques pouvant favoriser une résistance à cette maladie (Snieszko *et al.* 1959). Il y a principalement deux traitements contre la furonculose, l'antibiothérapie et la vaccination. L'antibiothérapie est l'approche actuellement préconisée, mais il existe de nombreux déterminants de résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida* (Piotrowska & Popowska 2015), il est donc difficile de traiter efficacement et durablement la furonculose avec cette méthode. En fait, l'émergence de souches d'*A. salmonicida* subsp. *salmonicida* antibiorésistantes est connue depuis les années 1950 (Snieszko & Bullock 1957).

Les premiers tests de vaccination contre la furonculose ont été effectués en 1942 avec l'administration d'un vaccin par voie orale (Duff 1942). Cependant, la première campagne

de vaccination bien documentée dans la littérature a été réalisée en 1964 (Krantz *et al.* 1964). Il existe maintenant une littérature abondante sur le sujet et plusieurs produits commerciaux (Midtlyng 2014; Austin & Austin 2016). Malheureusement, la vaccination des poissons est couteuse, demande une importante manipulation des animaux et produit plusieurs effets secondaires indésirables (Aunsmo *et al.* 2008a, 2008b; Dallaire-Dufresne *et al.* 2014).

La taxonomie d'*A. salmonicida*

La furonculose a été répertoriée la première fois dans la littérature scientifique en 1890 dans un journal allemand (Emmerich & Weibel 1890). À ce moment-là, la bactérie causant cette maladie était appelée *Bacillus der Forellenseuche* (bacille de la maladie de la truite). Par la suite, elle a eu de multiples noms et a subi plusieurs remaniements taxonomiques (Austin & Austin 2016). Ce n'est que récemment que la taxonomie de cette bactérie, maintenant appelé *Aeromonas salmonicida* subsp. *salmonicida*, s'est stabilisée avec la création de la classe *Aeromonadaceae* (Colwell *et al.* 1986). Cependant, il y a encore un débat au niveau du classement des sous-espèces (Austin & Austin 2016), ce qui soulève même à nouveau la vieille question à savoir si *A. salmonicida* devrait rester dans le genre *Aeromonas*, ou dans un nouveau appelé *Necromonas* (Smith 1963).

Dans la dernière édition du *Bergey's Manual of Systematic Bacteriology*, il y a cinq sous-espèces officiellement reconnues : *salmonicida*, *smithia*, *achromogenes*, *masoucida* et *pectinolytica* (Martin-Carnahan & Joseph 2005). La sous-espèce *salmonicida* est souvent considérée comme « typique » et l'agent étiologique de la furonculose chez les salmonidés. Les quatre autres sous-espèces sont souvent dites « atypiques » en référence à leurs différences phénotypiques, biochimiques et dans leur capacité à infecter une vaste gamme de poissons (Dallaire-Dufresne *et al.* 2014; Austin & Austin 2016) (Figure 7). Les souches des sous-espèces *salmonicida*, *smithia*, *achromogenes* et *masoucida* sont connues pour être psychrophiles et conséquemment ne pouvant pas croître à 37°C (Austin & Austin 2016). Cependant, en 2000, la sous-espèce *pectinolytica* a été isolée d'une rivière polluée (rivière Matanza) près de Buenos Aires en Argentine (Pavan *et al.* 2000). Les souches de cette sous-espèce, ayant la capacité de bien croître à 37°C, vinrent bouleverser les connaissances

établies sur *A. salmonicida* et ouvrirent la porte à une diversité beaucoup plus grande que soupçonnée précédemment.

Figure 7. Infection d'une carpe koï par une souche atypique d'*A. salmonicida*.
La carpe montre des signes d'ulcérations (flèches). Cette photo est une gracieuseté du professeur Brian Austin de l'Université Stirling (Écosse).

La diversité d'*A. salmonicida* subsp. *salmonicida*

La sous-espèce *salmonicida* a été longtemps considérée comme très homogène avec des structures populationnelle et génétique clonales (Belland & Trust, 1988; García *et al.*, 2000; McCormick *et al.*, 1990; O'hIci *et al.*, 2000; Umelo & Trust, 1998). Cependant, une étude avait déjà rapporté un regroupement génétique de cette bactérie basée selon les régions géographiques (Finlande, Danemark, Suède, Norvège et Canada) utilisant le ribotypage et le profil de l'ADN polymorphe amplifié au hasard (RAPD), deux méthodes à faible résolution (Hänninen *et al.* 1995). Avec la facilité d'accès au séquençage de nouvelle-génération, une vision neuve a permis de mettre en évidence un îlot génomique nommé *AsaGEI* qui se décline en cinq variants : 1a, 2a, 1b, 2b et 2c (Emond-Rheault *et al.* 2015a, 2015b; Long *et al.* 2016). Il a été estimé qu'environ 30 % des gènes d'*AsaGEI* auraient une origine phagique (Emond-Rheault *et al.* 2015a). Cependant, aucun gène n'encodant des protéines structurales (capside, queue, etc.) n'a été répertorié. Bien qu'aucune fonction connue n'ait encore été associée à *AsaGEI*, chaque variant est corrélé aux origines géographiques des souches (Figure 8).

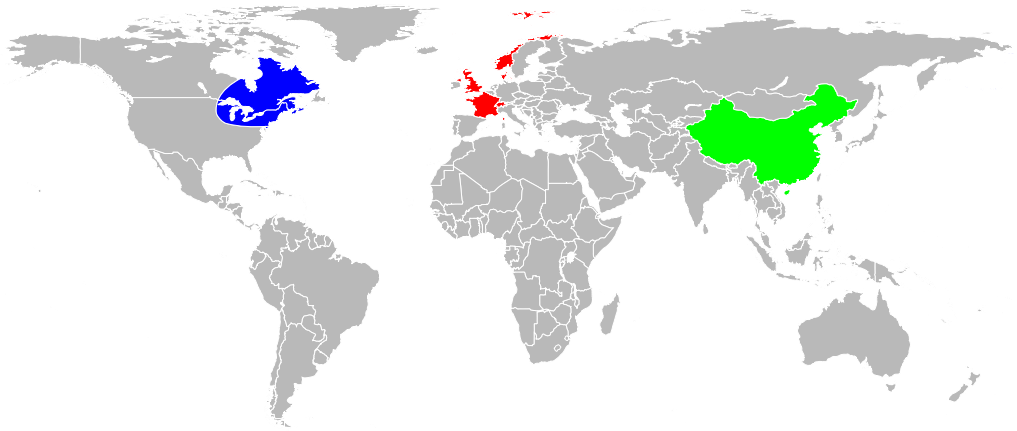

Figure 8. Répartition géographique des variants d'*AsaGEI.*
Les souches avec des *AsaGEI1a* et *2a* sont trouvées dans les pays en bleu et celles avec des *AsaGEI1b* et *2b* dans les pays en rouge. Des souches avec le variant *AsaGEI*2c n'ont pour l'instant été trouvées qu'en Chine (en vert).

Le plasmidome d'*A. salmonicida* subsp. *salmonicida*

Ce fut 81 ans après que la furonculose ait été répertoriée que le premier plasmide chez *A. salmonicida* a été publié (Aoki *et al.* 1971). Ce plasmide, isolé d'une souche japonaise, confère une résistance au chloramphénicol, à la streptomycine et au sulfathiazole. Après la publication d'Aoki et al., il s'ensuivit trois décennies de caractérisation du plasmidome d'*A. salmonicida* en utilisant principalement des outils classiques de biologie moléculaire. Plusieurs études ont porté sur les plasmides causant une résistance aux antibiotiques (Aoki *et al.* 1971, 1972, 1983, 1986; Inglis *et al.* 1993; Sandaa & Enger 1994, 1996; Adams *et al.* 1998; Sorum 1998; L'Abée-Lund & Sørum 2000, 2002) et quelques-unes sur l'implication de ceux-ci dans la virulence de la bactérie, mais sans grand succès (Ishiguro *et al.* 1981; Hackett *et al.* 1984). Certaines études ont aussi investigué le potentiel des plasmides comme marqueurs épidémiologiques. La majorité des études a trouvé que le plasmidome des souches typiques était trop conservé pour être utilisé dans un contexte épidémiologique (Bast *et al.* 1988; Belland & Trust 1989; Toranzo *et al.* 1991). Cependant, Nielson et collaborateurs ont trouvé certaines variations au sein de ce plasmidome et ne fermaient pas la porte à ce que les plasmides puissent donner quelques indications épidémiologiques (Nielsen *et al.* 1993).

L'année 1977 a été un point tournant décisif dans la science moderne avec la publication de la séquence génomique du phage ΦX174 par Frederick Sanger (Sanger *et al.* 1977). Le

premier plasmide séquencé fut pBR322 retrouvé chez *E. coli* et fréquemment utilisé en biologie moléculaire (Sutcliffe 1979). Les premiers plasmides d'*A. salmonicida* à avoir été séquencés sont pRAS3.1 et pRAS3.2, deux variants conférant une résistance à la tétracycline (L'Abée-Lund & Sørum 2002). Depuis, plusieurs plasmides de cette bactérie ont été caractérisés et séquencés (Piotrowska & Popowska 2015). Il est maintenant possible de catégoriser le plasmidome d'*A. salmonicida* subsp. *salmonicida* selon trois groupes : les plasmides cryptiques, ceux causant une résistance aux antibiotiques et finalement ceux impliqués dans la virulence (Figure 9).

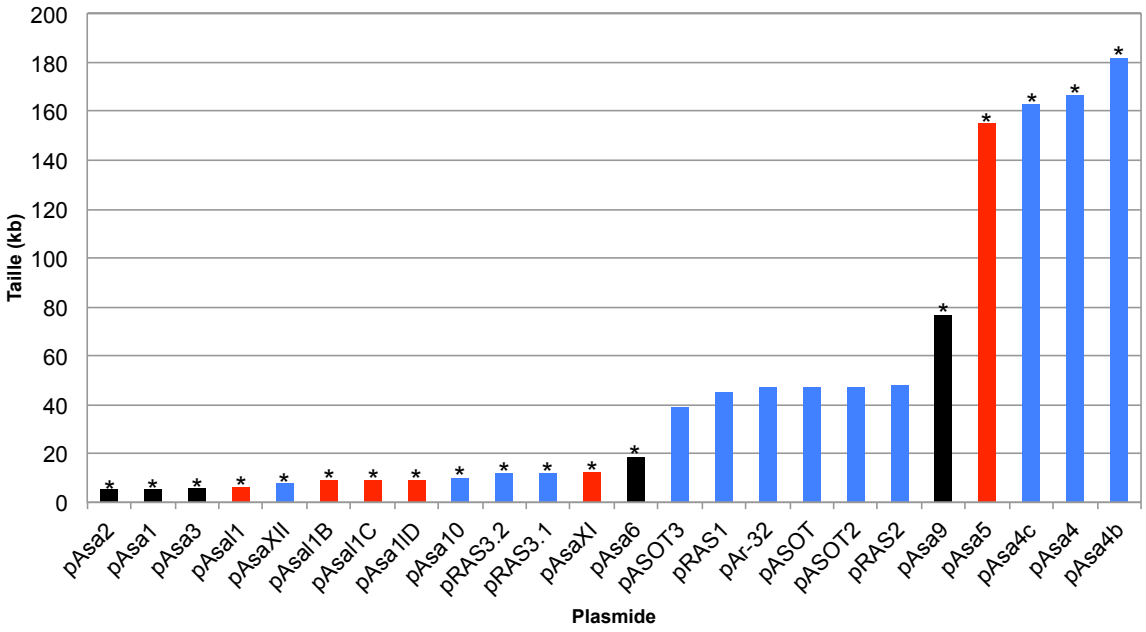

Figure 9. Distributions des plasmides d'*A. salmonicida* subsp. *salmonicida* actuellement connus dans la littérature et non directement reliés à la présente thèse.
Les plasmides cryptiques, impliqués dans la virulence et dans la résistance aux antibiotiques sont représentés par des barres noires, rouges et bleues, respectivement. Il est à noter que le plasmide pAsaXII ne confère pas une résistance à un antibiotique, mais au formaldéhyde. Les étoiles indiquent les plasmides dont l'ADN a été séquencé.

Les plasmides cryptiques

Les plasmides cryptiques, sans fonction connue, ont été largement étudiés avant l'ère du séquençage et sont au nombre de cinq : pAsa1, pAsa2, pAsa3, pAsa6 et pAsa9. La présence des plasmides pAsa1, pAsa2 et pAsa3 était connue depuis 1983 (Toranzo *et al.* 1983) et c'est en 1989 qu'ils ont été nommés (Belland & Trust 1989) pour finalement être séquencés en 2003 (Boyd *et al.* 2003). Le séquençage de ces plasmides a permis, entre autres, de mettre en lumière que pAsa1 et pAsa3 possèdent un mécanisme de réplication de type

ColE2, tandis que pAsa2 a un mécanisme de type ColE1. Plusieurs études avaient démontré que ces plasmides n'avaient aucun intérêt épidémiologique, ou minimalement limité (Bast *et al.* 1988; Belland & Trust 1989; Toranzo *et al.* 1991; Nielsen *et al.* 1993). Cependant, une étude récente, comprenant 153 souches, a montré que les souches européennes ont une plus grande propension à perdre les plasmides pAsa3 ou pAsal1 que celles canadiennes (Attéré *et al.* 2015). Une autre étude a suggéré que les plasmides cryptiques pAsa1, pAsa2 et pAsa3 peuvent être perçus comme des réceptacles permettant d'acquérir de nouveaux gènes pour conférer des avantages aux cellules hôtes, comme une résistance à certains composés antimicrobiens ou des facteurs de virulence (Attéré *et al.* 2017). Cette affirmation découle de la mise en évidence de deux plasmides dérivés de plasmides cryptiques : pAsaXI (dérivé de pAsa3) et pAsaXII (dérivé de pAsa2). Les différences entre pAsaXI et pAsaXII par rapport aux plasmides « parents » sont que pAsaXI possède un gène encodant un facteur de virulence putatif alors que pAsaXII possède un opéron conférant une résistance au formaldéhyde (Attéré *et al.* 2017).

De leur côté, les plasmides pAsa6 (Najimi *et al.* 2009) et pAsa9 (Tanaka *et al.* 2017), aussi sans fonction connue, ont une ressemblance structurale avec le plasmide pAsa5 impliqué dans la virulence et sont par conséquent discutés plus en détail dans la section « Les plasmides impliqués dans la virulence ».

Les plasmides portant des gènes de résistance aux antibiotiques

Tel que mentionné ci-haut, le premier plasmide à avoir été identifié chez *A. salmonicida* subsp. *salmonicida* confère une résistance aux antibiotiques. En fait, il s'agit de pAr-32, un plasmide d'environ 47 kb et du groupe d'incompatibilité IncU (Aoki *et al.* 1971, 1986). Il a été inféré que pAr-32 est identique au plasmide pRA3 qui est la référence des plasmides IncU (Bradley *et al.* 1982) et trouvé chez l'agent pathogène humain *Aeromonas hydrophila*. Malheureusement, pAr-32 n'est pas encore séquencé, il est donc impossible de vérifier l'identité de pAr-32 par rapport à pRA3.

Un autre plasmide non séquencé est pRAS1, un plasmide conjugatif d'environ 45 kb, également du groupe IncU (Sandaa & Enger 1994). Ce plasmide possède un intégron de classe 1 (In4) et un transposon Tn*1721* fragmenté. La combinaison de ces deux éléments apporte plusieurs gènes de résistance à des agents antimicrobiens : *dfrA16*, *qacEΔ1*, *sul1*,

tetA et *tetR* (Sørum *et al.* 2003). Il est intéressant de noter que pRAS1 a été trouvé dans des souches atypiques et typiques d'*A. salmonicida* (Sørum *et al.* 2003). Un autre plasmide conférant une résistance aux antibiotiques et encore non séquencé est pRAS2 (~ 48 kb). Ce plasmide trouvé dans la souche 1682/92 d'*A. salmonicida* subsp. *salmonicida* porte un transposon Tn*5393c* qui permet une résistance à la streptomycine, aux sulfonamides et à la tétracycline (L'Abée-Lund & Sørum 2000).

Trois autres plasmides, liés au groupe d'incompatibilité IncU et qui confèrent une résistance à l'oxytétracycline, ont été rapportés dans la littérature scientifique : pASOT (~ 47kb), pASOT2 (~ 47kb) et pASOT3 (~ 39 kb) (Adams *et al.* 1998). Les profils plasmidiques ont montré que pASOT et pASOT2 ont une grande ressemblance avec pRAS1, tandis que pASOT3 est plus éloigné. Ces plasmides portent un intégron de classe 1, qui peut différer au niveau des cassettes intégrées (L'Abée-Lund & Sørum 2001).

Tel que mentionné précédemment, les premiers plasmides chez *A. salmonicida* a avoir été séquencés sont pRAS3.1 et pRAS3.2 du groupe d'incompatibilité IncQ (L'Abée-Lund & Sørum 2002). Ces deux plasmides portent les gènes *tetA* et *tetR*, conférant ainsi une résistance à la tétracycline. En fait, les deux plasmides ne diffèrent que par de courtes répétitions dans deux régions : (1) dans la région promotrice des gènes *mobB-mobA* / *repB* et (2) près d'*oriV*. Le nombre d'unités répétées peut faire varier le nombre de copies des plasmides (Loftie-Eaton & Rawlings 2009). Une étude réalisée sur *E. coli* a révélé que les pRAS3 ayant un faible nombre de copies imposaient aux cellules un fardeau métabolique inférieur à celui des plasmides en haut nombre de copies et augmentaient par conséquent le *fitness* de la population bactérienne (Loftie-Eaton & Rawlings 2010).

Un plasmide important au niveau de la résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida* est pAsa4. En fait, ce plasmide est connu depuis 1989 pour avoir un gène *cat*, codant pour une chloramphénicol acetyltransferase (CAT) (Belland & Trust 1989). Il avait été répertorié dans cette même étude que la protéine CAT était prédominante dans les lysats d'*E. coli* portant une partie du plasmide pAsa4 contenant le gène *cat* et que les cellules receveuses étaient également très résistantes au chloramphénicol (Belland & Trust 1989).

Le séquençage complet de pAsa4 a été réalisé 19 ans après les observations de Belland et Trust (Reith *et al.* 2008). Ce grand plasmide de 166,749 kb est porteur d'un transposon Tn*21* avec un intégron In2, conférant une résistance à la streptomycine/spectinomycine (*aadA*), aux sulfonamides (*sul1*) et au chloramphénicol (*cat*). En plus de ces gènes apportés par Tn*21*, pAsa4 a également des gènes de résistance à la tétracyline: *tetA* et *tetR*.

Même si pAsa4 n'est pas un plasmide commun, deux variants appelées pAsa4b et pAsa4c ont été trouvés, séquencés et caractérisés (Tanaka *et al.* 2016). Les trois variants de pAsa4 présentent une identité élevée au niveau nucléotidique (94 à 99 %). Cependant, l'architecture des plasmides s'est révélée divergente entre eux. En fait, les pAsa4 contiennent une forte proportion de séquences d'insertion (IS), qui favorisent, entre autres, les réarrangements à grande échelle. Cette instabilité du génome causée par les IS a également été signalée pour d'autres plasmides de la bactérie *A. salmonicida* subsp. *salmonicida* (voir la section « Les plasmides impliqués dans la virulence »). Les variations structurelles affectent le répertoire de gènes de résistance aux antibiotiques, avec le pAsa4 original portant la plupart de ceux-ci (*tetA*(E)*, sul1, aadA, cat*). Le variant pAsa4c ne possède que le gène conférant une résistance à la tétracycline, *tetA*(E), tandis que pAsa4b ne porte pas les gènes *aadA* et *cat*, donnant respectivement une résistance à la streptomycine/spectinomycine et au chloramphénicol.

Les plasmides impliqués dans la virulence

Il est maintenant bien connu que la bactérie *A. salmonicida* possède plusieurs facteurs de virulence comme des polysaccarides capsulaires, des lipopolysaccharides (LPS), la couche de surface A, un système d'acquisition du fer ainsi qu'un système de sécrétion de type trois (TTSS) (Tomás 2012). Ce dernier est considéré comme crucial pour la virulence de la bactérie (Vanden Bergh & Frey 2014). Le TTSS est un ensemble protéique complexe qui exporte les effecteurs du cytoplasme bactérien à la zone extracellulaire ou même à l'intérieur d'une cellule cible (Costa *et al.* 2015). L'ensemble des gènes codant pour les protéines structurales du TTSS d'*A. salmonicida* subsp. *salmonicida* est situé sur un grand plasmide (~ 140 kb), appelé pASvirA (Stuber *et al.* 2003). Ce plasmide code également pour trois effecteurs et leurs chaperonnes (AopH/SycH, Ati2/Ati1 et AopO/SycO) et un putatif, AopX, pour lequel le gène est tronqué en raison d'une duplication de 20 pb

(Dallaire-Dufresne *et al.* 2014; Vanden Bergh & Frey 2014). D'autres effecteurs, AexT (Burr *et al.* 2003) et AopP (Fehr *et al.* 2006) sont codés par des gènes situés respectivement sur le chromosome et le petit plasmide pAsal1. Il existe également un second gène associé au chromosome et qui code pour un effecteur du TTSS, AopS, qui est tronqué chez *A. salmonicida* subsp. *salmonicida* mais intact chez *A. salmonicida* subsp. *achromogenes* (Vanden Bergh & Frey 2014).

Le plasmide pASvirA, renommé pAsa5 après son séquençage complet en 2008 (Reith *et al.* 2008), a été rapporté comme étant thermosensible, entrainant sa perte lorsque la bactérie le portant est cultivée à une température de 25°C ou plus, rendant la souche non virulente (Stuber *et al.* 2003). Cependant, à ce moment-là, le mécanisme de son instabilité était inconnu. Une étude a été réalisée en génotypant par PCR trois souches d'*A. salmonicida* subsp. *salmonicida* après exposition contrôlée à un stress thermique (Daher *et al.* 2011). Cette étude a montré que le locus du TTSS était préférentiellement perdu après ce stress et non le plasmide complet, comme soupçonné précédemment. Enfin, une autre étude a montré que la délétion du locus du TTSS était causée par la recombinaison de séquences d'insertion IS*AS11*, selon deux scénarios possibles (Figure 10), à une température de 25°C ou plus, expliquant ainsi une partie des réarrangements de pAsa5 qui avaient été observés (Tanaka *et al.* 2012). Une seconde étude a montré que certaines souches possèdent une séquence d'insertion IS*AS5* supplémentaire sur leur pAsa5 et que celle-ci peut recombiner avec une autre IS*AS5*, aussi présente sur le plasmide (Tanaka *et al.* 2017). Il y a donc trois scénarios de réarrangements actuellement connus pour pAsa5 : deux impliquant les IS*AS11* et un avec les IS*AS5* (Figure 10).

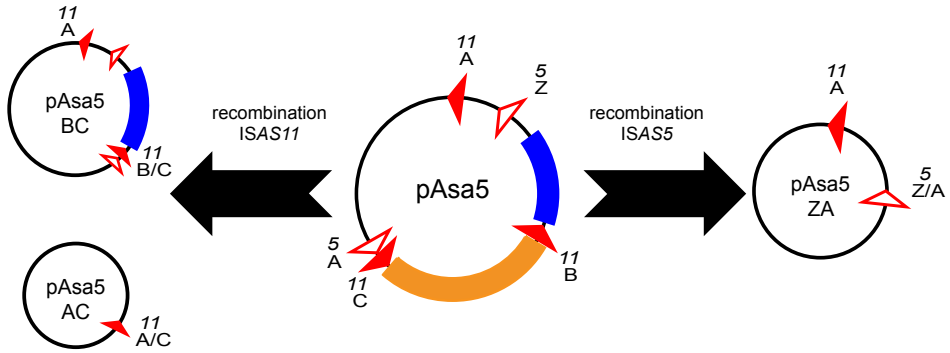

Figure 10. Schématisation des scénarios de réarrangements du plasmide pAsa5 causés par recombinaisons des séquences d'insertion IS*AS5* et IS*AS11*.

À gauche, la représentation des deux scénarios impliquant l'IS*AS11* et à droite celui avec l'IS*AS5*. La figure est adaptée de Tanaka *et al.* 2017.

Une autre étude concernant des réarrangements putatifs de pAsa5 a été publiée en 2009 (Najimi *et al.* 2009). Ces travaux ont montré la présence d'un nouveau plasmide de 18 536 pb, nommé pAsa6 et évoqué plus tôt dans ce texte. Ce plasmide a été identifié dans la souche RSP74.1 isolée d'un Turbot (*Psetta maxima*) au Portugal en 2002 (Najimi *et al.* 2008). Fait intéressant, pAsa6 présente une forte similitude avec pAsa5 et il n'est toujours pas clair si pAsa6 est un dérivé direct de pAsa5 provenant d'un processus de réduction de gènes ou s'il s'agit d'une fusion chimérique entre pAsa5 et un autre plasmide inconnu. Le plasmide pAsa6 possède le gène *aopH*, tout comme pAsa5, qui code pour un effecteur du TTSS. Cependant, contrairement à pAsa5, le locus avec les gènes structuraux du TTSS est absent chez pAsa6. Cette souche a montré avoir un profil plasmidique divergent de ce qui était attendu pour un membre de la sous-espèce *salmonicida* (Najimi *et al.* 2008; Attéré *et al.* 2015), suggérant ainsi qu'elle pourrait peut-être appartenir à une autre sous-espèce.

Un second plasmide présentant une forte similarité structurale avec pAsa5 a été récemment caractérisé (Tanaka *et al.* 2017). Ce plasmide, pAsa9, est cependant de plus grande taille (76 724 pb) que pAsa6 (18 536 pb), et presque la moitié de celle de pAsa5 (155 098 pb). Tout comme pour pAsa6, le locus et les effecteurs du TTSS sont absents de pAsa9. De plus, contrairement à pAsa6 qui n'a été retrouvé que dans une seule souche jusqu'à présent, pAsa9 est bien plus fréquent (Tanaka *et al.* 2017).

Comme discuté ci-dessus, pAsa5 héberge les gènes pour produire un TTSS fonctionnel, y compris trois effecteurs : AopH, Ati2 et AopO. Cependant, un autre plasmide, pAsal1 (6 371 pb), porte également un gène codant pour un effecteur du TTSS : AopP (Fehr *et al.* 2006). Cet effecteur est de la famille YopJ et inhibe la voie de signalisation NF-κB, qui joue un rôle important au niveau du système immunitaire et de plusieurs autres fonctions physiologiques (Hayden & Ghosh 2012). Le plasmide pAsal1 possède un réplicon de type ColE2 et a une forte similitude structurelle avec d'autres plasmides ayant le même réplicon chez *A. salmonicida* subsp. *salmonicida*, tels pAsa1 et pAsa3 (Attéré *et al.* 2015). Cependant, comparativement aux autres plasmides avec un réplicon de type ColE1 connus chez cette bactérie, pAsal1 porte une séquence d'insertion IS*AS11*, le même type d'IS qui favorise la perte du locus du TTSS par réarrangements dans pAsa5 (Tanaka *et al.* 2012).

L'étude de Tanaka et al., qui avait démontré le mécanisme causant les réarrangements de pAsa5, avait suggéré que l'IS*AS11* de pAsal1 pourrait également être impliquée dans un processus de réarrangement dans des conditions stressantes telles qu'une température de croissance égale ou supérieure à 25°C, entrainant ainsi la perte du plasmide (Tanaka *et al.* 2012). Cette hypothèse a été renforcée par une autre étude qui a démontré une corrélation non systématique entre la présence de pAsa5 réarrangés, révélant ainsi que les souches pourraient avoir subi un stress thermique, et l'absence de pAsal1 (Attéré *et al.* 2015).

Trois variants de pAsal1 ont été répertoriés : pAsal1B (Trudel *et al.* 2013), pAsal1C (Attéré *et al.* 2015) et pAsal1D (Attéré *et al.* 2015), tous codant putativement pour une protéine AopP fonctionnelle. Ils ont tous en commun une IS supplémentaire de la famille IS*21* : IS*AS5*. L'IS*AS5* s'est insérée dans *mobA* pour pAsal1B alors qu'elle a été retrouvée dans l'IS*AS11* pour pAsal1C et pAsal1D, montrant encore une fois que les IS sont des vecteurs d'innovations génomiques chez *A. salmonicida* subsp. *salmonicida*.

Les phages d'*A. salmonicida*

Les prophages

Le génome de la souche française A449 d'*A. salmonicida* subsp. *salmonicida* a été séquencé et annoté en 2008 (Reith *et al.* 2008). Le chromosome de cette bactérie comprend deux prophages de 32 114 pb et 34 204 pb. Une étude de génomique comparative suggère que ces prophages seraient de la famille des *Myoviridae* (Beilstein & Dreiseikelmann 2008a). Une seconde étude laisse entrevoir la présence d'un 3^{e} prophage dans la souche A449, mais incomplet, de 18 624 pb (Paul 2008). Aucun gène codant pour des toxines n'a été trouvé dans ces prophages. Il est aussi maintenant connu qu'un troisième prophage, nommé *Prophage 3*, est co-retrouvé à 94 % avec l'ilot génomique *AsaGEI2a* (Emond-Rheault *et al.* 2015a).

Les phages lytiques et tempérés

L'existence de phages infectant *A. salmonicida* a été répertoriée pour la première fois en 1933 (Todd 1933). Depuis, des phages d'*A. salmonicida* tempérés (lysogènes) et lytiques ont été découverts (Paterson *et al.* 1969; Popoff 1971; Ishiguro *et al.* 1980, 1984; Ackermann *et al.* 1985). Il a été, entre autres, montré que la majorité des phages infectant

A. salmonicida sont de la famille *Myoviridae* et inféré comme étant très hétérogènes, basé sur leur morphologie (Ackermann *et al.* 1985).

Les phages, afin d'infecter une cellule bactérienne, ont besoin de s'attacher à la surface de celle-ci en reconnaissant une structure servant de récepteur (Samson *et al.* 2013). Dans le cas d'*A. salmonicida*, le seul récepteur phagique actuellement connu est VapA (Ishiguro *et al.* 1983), une protéine impliquée dans la couche de surface A (Kay *et al.* 1981). Comme mentionné ci-haut, les IS peuvent induire des réarrangements génomiques chez *A. salmonicida*. En fait, le gène *vapA*, encodant la protéine VapA, est une autre des cibles pouvant être altérées par des IS (IS*AS1* et IS*AS2*) (Gustafson *et al.* 1994).

Avant la présente thèse, il y avait seize génomes de phages infectant le genre *Aeromonas* de déposés dans la base de données publique GenBank. De ces seize génomes, dix proviennent de phages infectant l'espèce *salmonicida* (Tableau 3). La majorité des phages sont de la famille des *Myoviridae*. Certains de ces phages ont aussi été classifiés en genre par l'ICTV (https://talk.ictvonline.org) : le genre *Secunda5virus* de la famille *Myoviridae* comprend les espèces *Aeromonas virus 25*, *Aeromonas virus 31*, *Aeromonas virus Aes508* et *Aeromonas virus AS4* tandis que le genre *Biquartavirus* de la famille *Myoviridae* comprend l'espèce *Aeromonas virus 44RR2*. Seul le phage ΦO18P infectant *Aeromonas media* est lysogène et a son génome séquencé (Beilstein & Dreiseikelmann 2008b).

Il existe une grande diversité dans la taille des génomes de phages connus pour infecter *Aeromonas* (Petrov *et al.* 2010) (Tableau 3). Une étude de génomique comparative de plusieurs phages de la famille des *Myoviridae* a révélé que les génomes des myophages d'*Aeromonas* ont une topologie très divergente des génomes de *Myoviridae* typiques (Petrov *et al.* 2010). Cette caractéristique était attendue puisque les grands génomes viraux ont tendance à avoir une grande plasticité (Hatfull 2008). De plus, il est notable que certains génomes ont des hauts pourcentages en G+C alors que d'autres ont un faible pourcentage. Une étude sur la coévolution des codons a montré que les génomes de phages d'*A. salmonicida* avec un petit pourcentage en G+C encodent des gènes d'ARNt car le répertoire de l'hôte est probablement inadéquat pour traduire efficacement les ARNm de ceux-ci (Prabhakaran *et al.* 2014).

Tableau 3. Caractéristiques générales des génomes de phages infectants *Aeromonas* et actuellement disponibles dans GenBank

Nom	Bactérie hôte	Taille (pb)	% GC	Protéines	Famille	No. Accession
phiO18P	*A. media*	33 985	61,7	45	*Myoviridae*	NC_009542
phiAS7	*A. salmonicida* subsp. *salmonicida*	41 572	56,9	51	*Podoviridae*	NC_019528
vB_AsaM-56	*A. salmonicida*	43 551	55,4	83	*Myoviridae*	NC_019527
pIS4-A	*Aeromonas* sp.	47 624	47,0	78	Inconnue	JF974294
pAh6-C	*A. hydrophila*	53 744	52,8	86	*Myoviridae*	KJ858521
Aes508	*A. salmonicida*	160 646	41,2	230	*Myoviridae*	NC_019543
25	*A. salmonicida*	161 475	41,0	242	*Myoviridae*	NC_008208
Aes012	*Aeromonas* sp.	161 978	41,3	243	*Myoviridae*	NC_020879
phiAS4	*A. salmonicida*	163 875	41,3	271	*Myoviridae*	NC_014635
31	*A. salmonicida*	172 963	43,9	247	*Myoviridae*	NC_007022
44RR2	*A. salmonicida*	173 591	43,9	252	*Myoviridae*	NC_005135
PX29	*A. salmonicida*	222 006	42,0	330	*Myoviridae*	NC_023688
phiAS5	*A. salmonicida*	225 268	43,0	343	*Myoviridae*	NC_014636
CC2	*A. hydrophila*	231 743	38,8	427	*Myoviridae*	NC_019538
Aeh1	*A. hydrophila*	233 234	42,8	352	*Myoviridae*	NC_005260
65	*A. salmonicida*	235 229	37,2	437	*Myoviridae*	NC_015251

La thérapie par les phages contre la furonculose

Comme pour d'autres domaines, l'utilisation des phages dans un contexte thérapeutique en aquaculture a été explorée (Mateus *et al.* 2014; Silva *et al.* 2014). La première mention d'utilisation des phages dans un contexte d'aquaculture a été faite dans le cadre d'une étude sur le contrôle d'*Aeromonas hydrophila* par le phage AH 1 (Wu *et al.* 1981). Des travaux sur l'utilisation des phages pour le contrôle d'*A. salmonicida* subsp. *salmonicida* montrent des résultats prometteurs laissant croire qu'ils pourraient être employés pour un traitement contre la furonculose (Imbeault *et al.* 2006a; Pereira *et al.* 2011; Kim *et al.* 2013). Il a aussi été observé que certains phages infectant *Aeromonas* conservent un pouvoir infectieux pendant 90 jours dans de l'eau d'aquaculture contre seulement 16 jours pour les phages infectant le genre bactérien *Vibrio* (Silva *et al.* 2014).

Les phages génèrent une importante pression évolutive sur les bactéries, qui doivent développer des mécanismes pour se protéger (Labrie *et al.* 2010). L'une des premières études aillant investigué l'utilisation des phages contre *A. salmonicida* subsp. *salmonicida* a rapporté qu'il était possible de générer des mutants bactériens insensibles aux phages (Imbeault *et al.* 2006b), bien que le mécanisme utilisé soit inconnu. Cette même étude a trouvé qu'environ la moitié des bactéries mutantes perdait l'insensibilité aux phages pour retrouver le phénotype sauvage. Sinon, les bactéries conservant la résistance poussaient très

lentement et mourraient après quelques générations, suggérant que le fait d'acquérir une résistance aux phages diminuait significativement leur *fitness*.

Le présent projet

Problématique 1 : l'antibiorésistance

Le travail de la présente thèse porte sur *A. salmonicida* subp. *salmonicida*. Cette bactérie, qui cause la furonculose chez les salmonidés, est particulièrement un fléau en aquaculture puisqu'elle cause d'importes pertes économiques (Dallaire-Dufresne *et al.* 2014). Elle est d'autant plus problématique que plusieurs souches sont résistantes aux antibiotiques, il est donc difficile de traiter efficacement et durablement les poissons atteints de furonculose. Bien que le phénomène d'antibiorésistance chez *A. salmonicida* subsp. *salmonicida* était connue avant le présent projet, l'ampleur de la problématique restait à être déterminée. Il était, et demeure encore aujourd'hui, crucial d'étudier la diversité, les causes et les mécanismes de propagation de résistance aux antibiotiques chez la bactérie à l'étude. Bien sûr, comme pour d'autres bactéries pathogènes, il était aussi essentiel d'investiguer le potentiel d'alternatives aux antibiotiques.

Problématique 2 : la taxonomie

Une seconde problématique chez *A. salmonicida* est sa taxonomie, qui possède officiellement cinq sous-espèces : *salmonicida*, *achromogenes*, *smithia*, *masoucida* et *pectinolytica*. Comme il a été clairement indiqué par le professeur émérite Brian Austin, spécialiste du genre *Aeromonas* à l'*University of Stirling* en Écosse, la structure intra-espèce d'*A. salmonicida* est complexe, sous-étudiée et sujette à remaniement (Austin 2011; Austin & Austin 2016). Un survol de la littérature au début du projet a permis de constater qu'il existait une multitude de phylogénies d'*A. salmonicida*, la grande majorité avec des topologies divergentes. Il était impossible d'étudier avec précision et robustesse la bactérie à l'étude sans avoir une vision plus précise de son évolution.

Hypothèses et objectifs

Les deux buts principaux du présent projet étaient (1) d'investiguer l'ampleur de l'antibiorésistance chez *A. salmonicida* subsp. *salmonicida* et (2) d'étudier le potentiel

thérapeutique des phages contre la furonculose. Afin de réaliser ces buts, deux hypothèses devaient être vérifiées et plusieurs objectifs réalisés.

Hypothèse 1

La première hypothèse est que la bactérie *A. salmonicida* subsp. *salmonicida* possède une importante diversité génomique pouvant influencer sa réponse à des traitements thérapeutiques classiques (antibiotiques) ou alternatifs (phages). Quatre objectifs spécifiques étaient reliés à cette hypothèse.

Objectif 1 : Établir le résistome d'*A. salmonicida* subsp. *salmonicida*

Le laboratoire Charette possède une collection de plus de 250 isolats d'*A. salmonicida* subsp. *salmonicida*, isolés dans différentes régions du monde et à différents moments. Au fil des années, ces souches ont été criblées par PCR et par antibiogrammes afin d'identifier leurs profils de résistance aux antibiotiques. Les souches présentant des profils inhabituels de résistance aux antibiotiques ont été analysées en détail, principalement en faisant séquencer leur ADN à haut débit. Les diverses analyses subséquentes ont permis de mettre en lumière plusieurs nouveaux plasmides encodant des gènes de résistance à divers antibiotiques.

Objectif 2 : Isoler des bactériophages infectant *A. salmonicida* subsp. *salmonicida*

Bien que la collection de phages Félix d'Hérelle de l'Université Laval (http://www.phage.ulaval.ca/en/home/) possédait déjà huit phages infectant *A. salmonicida* subsp. *salmonicida*, des cours d'eau au Québec, dont certains passant par des pisciculture, ainsi que des poissons morts ont été échantillonnés afin de tenter d'isoler de nouveaux phages infectant la bactérie à l'étude. Trois nouveaux phages spécifiques à *A. salmonicida* ont été isolés : SW69-9, L9-6 et Riv-10.

Objectif 3 : Faire la caractérisation génomique des phages

Comme indiqué précédemment, il est primordial de bien connaitre les phages, tant au niveau microbiologique que génomique, avant d'envisager de les utiliser dans un contexte thérapeutique. C'est pourquoi les génomes des 12 phages disponibles ont été séquencés et analysés. Ces analyses ont permis de mettre en lumière une classification des phages infectant *A. salmonicida*, de valider qu'il existe une grande variabilité dans la taille des génomes ainsi qu'une dichotomie dans le pourcentage en G+C, de trouver certains gènes

sous pression Darwinienne (en tenant compte des évènements putatifs de recombinaisons), d'identifier dans certains génomes la présence d'un gène codant pour une intégrase et finalement de valider qu'aucun génome des phages à l'étude ne possède de gènes de résistance aux antibiotiques ou de facteurs de virulence.

Objectif 4 : Étudier le pouvoir lytique des phages contre *A. salmonicida*

Bien que certains phages aient été déterminés comme étant de potentiels candidats intéressants pour être utilisés dans un contexte thérapeutique contre la furonculose, il était aussi crucial d'étudier leur capacité à lyser différents isolats d'*A. salmonicida* subsp. *salmonicida*. Les 12 phages à l'étude ont donc été testés sur 59 isolats d'*A. salmonicida* subsp. *salmonicida* et 6 isolats provenant d'autres sous-espèces (psychrophiles et mésophiles) par la méthode de *spot-tests*. L'utilisation d'un regroupement hiérarchique a permis de déterminer trois groupes de phages selon leur spectre lytique.

Hypothèse 2

La seconde hypothèse de la présente thèse est qu'il existe des relations évolutives entre *A. salmonicida* et les bactériophages l'infectant. Sachant que la majorité des phages est très spécifique à leur hôte, il était raisonnable de croire que des souches d'*A. salmonicida* ayant des caractéristiques divergentes répondraient aussi différemment au niveau de la sensibilité aux phages. La validation ou l'invalidation de cette hypothèse exigeait la réalisation de deux objectifs spécifiques.

Objectif 1 : Faire une phylogénie robuste d'*A. salmonicida*

Cet objectif est clé pour mieux comprendre les liens évolutifs entre les différentes souches et sous-espèces d'*A. salmonicida*. Deux phylogénies robustes ont été réalisées. Afin d'avoir une bonne résolution phylogénétique au niveau des isolats d'une même sous-espèce, plusieurs milliers de séquences orthologues ont été utilisés. Dans un premier temps, il a été possible de déterminer une topologie robuste montrant les liens entre les différentes sous-espèces. Cette étude a établi que les souches mésophiles sont plus basales que celles psychrophiles et que les séquences d'insertion pourraient être des déterminants majeurs pour expliquer cette dichotomie intra espèce (mésophiles et psychrophiles). Cette étude a aussi démontré que les souches européennes et canadiennes se regroupent en deux clades distincts et même que celles canadiennes peuvent être sous-groupées selon les provinces du

Québec et du Nouveau-Brunswick. Par conséquent, les regroupements géographiques et phylogénétiques corrèlent aussi avec la présence des différents variants de l'ilot génomique *AsaGEI*, qui est connu pour être un fort marqueur de la provenance géographique des isolats (Emond-Rheault *et al.* 2015a, 2015b). Finalement, une seconde étude a permis de caractériser le génome complet et fermé d'une souche mésophile : A527. Ce génome est le premier d'une souche d'*A. salmonicida* mésophile à être complètement fermé et a permis de confirmer que les séquences d'insertion seraient des éléments déterminants pour expliquer la dichotomie mésophile/psychrophile de cette bactérie.

Objectif 2 : Vérifier s'il y a une concordance entre l'emplacement phylogénétique des isolats d'*A. salmonicida* et la sensibilité aux phages

Avec une phylogénie robuste d'*A. salmonicida* (objectif 1 de l'hypothèse 2) ainsi qu'une analyse du spectre lytique des phages à l'étude contre 65 isolats (objectif 4 de l'hypothèse 1), il était possible de vérifier la présence ou l'absence de corrélation entre l'emplacement phylogénétique des souches et leur sensibilité aux phages. L'une des observations est que l'ensemble des isolats provenant de sous-espèces psychrophiles peut être infecté par les phages isolés à partir de souches de la sous-espèce *salmonicida.* À l'inverse, les souches mésophiles sont insensibles à ces virus. Bien qu'il existe une très robuste relation phylogénétique et géographique entre les souches, aucune corrélation géographique ou même temporelle (année d'isolation des souches) ne peut être faite avec le profil de sensibilité aux phages. La présence ou l'absence de l'ilot génomique *AsaGEI* (et du prophage 3) n'a aussi aucune incidence claire sur la sensibilité aux phages.

Chapitre 2 – Article 1

Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug-resistance, interspecies exchanges, and plasmid reshaping

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Running title: Plasmid variants in *A. salmonicida*

Résumé

La bactérie *Aeromonas salmonicida* subsp. *salmonicida* est l'agent étiologique de la furonculose. Il était connu que certaines souches de cette bactérie possédaient des plasmides avec des gènes de résistance aux antibiotiques. L'objectif était de caractériser de nouveaux plasmides porteurs de tels gènes. Nous avons séquencé l'ADN de plasmides présents dans deux isolats multirésistants aux antibiotiques et étudié par génotypage PCR 19 autres isolats avec différents profils de résistance. Il a été possible d'identifier des variants des plasmides pAB5S9 et pSN254 qui avaient déjà été répertoriés dans d'autres bactéries, suggérant la possibilité d'échanges de matériel génétique entre différentes espèces. Les analyses soutiennent l'idée que plusieurs variants de pAB5S9 et pSN254 existent. Nous avons également identifié des variants du plasmide pRAS3. La présente étude a révélé qu'*A. salmonicida* subsp. *salmonicida* possède une grande variété de plasmides et que cette bactérie peut contribuer à la propagation de gènes de résistance aux antibiotiques.

Abstract

The ubiquitous waterborne Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is the causative agent of furunculosis, a worldwide disease in fish farms. Plasmids encoding antibiotic resistance genes have been already described for this bacterium. The aim of the present study was to identify and characterize additional multidrug-resistance plasmids in *A. salmonicida* subsp. *salmonicida*. We sequenced the plasmids present in two multiple antibiotic resistant isolates using high-throughput technologies. We also investigated 19 other isolates with various multidrug-resistance profiles by genotyping PCR and assessed their resistance to tetracycline. We identified variants of the pAB5S9 and pSN254 plasmids that carry several antibiotic resistance genes and that have been previously reported in bacteria other than *A. salmonicida* subsp. *salmonicida*, which suggests a high level of interspecies exchange. Genotyping analyses and the antibiotic resistance profiles of the 19 other isolates support the idea that multiple versions of pAB5S9 and pSN254 exist in *A. salmonicida* subsp. *salmonicida.* We also identified variants of the pRAS3 plasmid. The present study revealed that *A. salmonicida* subsp. *salmonicida* harbors a wide variety of plasmids, which suggests that this ubiquitous bacterium may contribute to the spread of antibiotic resistance genes in the environment.

Introduction

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is an opportunistic fish pathogen (Derome *et al.* 2014). It is the etiological agent of furunculosis, a disease that especially affects salmonids in fish farms (Dallaire-Dufresne *et al.* 2014). While antibiotics are commonly used to treat *A. salmonicida* subsp. *salmonicida* infections, multi-drug resistant isolates have been frequently detected (Sandaa & Enger 1994; Sørum *et al.* 2003; McIntosh *et al.* 2008), preventing the effective treatment of furunculosis.

Many fully characterized plasmids from *A. salmonicida* subsp. *salmonicida* have been shown to provide antibiotic resistance to this species (Dallaire-Dufresne *et al.* 2014). All the known plasmids in *A. salmonicida* subsp. *salmonicida* harboring antibiotic resistance genes include at least a tetracycline resistance gene. The vast majority of the plasmids bearing antibiotic resistance genes confer multiple types of resistance on *A. salmonicida* subsp. *salmonicida*, including the large plasmid pAsa4 (167 kb), which provides resistance against chloramphenicol, spectinomycin, streptomycin, sulfonamides, tetracycline, mercury, and quaternary ammonium compounds (Reith *et al.* 2008). A plasmid bearing multiple resistance genes that is similar to the large pSN254 plasmid in *Salmonella enterica* (Welch *et al.* 2007) has been partially described in *A. salmonicida* subsp. *salmonicida* (McIntosh *et al.* 2008). This pSN254-like plasmid can be transferred via conjugation from *A. salmonicida* subsp. *salmonicida* to multiple receivers, including *Escherichia coli*, *Edwardsiella tarda*, and *Aeromonas hydrophila* (McIntosh *et al.* 2008).

Plasmid variants appear to be relatively frequent in *A. salmonicida* subsp. *salmonicida.* The best example is the pRAS3 plasmid. Two variants of this plasmid (pRAS3.1 and pRAS3.2) have been described to date (L'Abée-Lund & Sørum 2002). The differences between them are very subtle and consist of two additional repetition units in pRAS3.1, one 22 nucleotides in length and the other 6 nucleotides in length. pAsal1B is another example of a plasmid variant. In this case, pAsal1B differs from the parental plasmid (pAsal1) by the presence of an *AS5* insertion sequence (IS) as well as a fragment of the same IS (Trudel *et al.* 2013).

Given these observations regarding plasmids in *A. salmonicida* subsp. *salmonicida*, it is possible that additional multidrug-resistant plasmids remain to be identified in this bacterium. We thus analyzed 78 *A. salmonicida* subsp. *salmonicida* isolates to identify those displaying multidrug resistance. By using next-generation sequencing methods, we detected pRAS3, pAB5S9, and pSN254 plasmid variants. We showed that *A. salmonicida* subsp. *salmonicida* displays marked heterogeneity in terms of the repertoire of plasmids bearing antibiotic resistance genes. This plasmid diversity may have major consequences in terms of treatments for furunculosis and highlights the fact that there is a significant flow of antibiotic resistance genes between different waterborne bacteria.

Methods

Bacterial isolates and growth conditions

The *A. salmonicida* subsp. *salmonicida* isolates used in the present study were the same as those analyzed by Trudel et al. (Trudel *et al.* 2013) (Table S1). They were grown on furunculosis agar (Hänninen & Hirvelä-Koski 1997) for two or three days at 18°C.

Antibiogram analyses

One colony of each strain was resuspended in 2 ml of furunculosis broth, which was shaken at 200 rpm overnight at 18°C. The cultures were adjusted to an optical density (OD) of 0.9 at 595 nm and were spread on Mueller-Hinton agar medium (Oxoid, Canada) to determine their antibiotic resistance with the following antibiotics using the disc diffusion method: ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), sulfamethoxazole/trimethoprim (23.75 µg/1.25 µg), streptomycin (10 µg), tetracycline (5 µg), and trimethoprim (5 µg) (Becton Dickinson, Sparks, MD, USA). The plates were incubated at 18°C to prevent possible instability of the plasmids bearing antibiotic resistance genes. This kind of instability has been already described for pAsa5, a large plasmid bearing the type three secretion system (Stuber *et al.* 2003; Daher *et al.* 2011; Tanaka *et al.* 2012). Bacterial growth was recorded after 48 h. Since there is, to our knowledge, no control chart for the diameter of the zones of antibiotic-driven growth inhibition at this temperature with this bacterium, antibiotic resistance was determined based on the relative sizes of the growth inhibition zones of the isolates compared to the sensitive strain 01-B526, which has been sequenced and has no antibiotic resistance genes

(Charette *et al.* 2012), and the resistant strain m15879-11 (see below and data not shown). Every assay was performed at least in duplicate.

Assessment of the minimum inhibitory concentration (MIC) of tetracycline

The MIC of tetracycline was assessed for 21 *A. salmonicida* subsp. *salmonicida* isolates (Table 4). The isolates were inoculated on furunculosis agar from frozen stocks and were grown at 18°C for 48 h before each experiment. Several colonies of each isolate were suspended in fresh LB medium (EMD). The optical densities (O.D.) of the bacterial suspensions were measured at 595 nm, diluted to 0.1 of O.D. and $3x10^7$ bacteria (30 µl) were deposited in the wells of 48-well microplates. Tetracycline (Calbiochem) was serially diluted in LB, and an aliquot of each dilution was placed in the wells of the 48-well microplates to obtain antibiotic concentrations ranging from 0 to 256 µg/ml in a final volume of 300 µl. The plates were incubated at 18°C for 48 h with shaking at 200 rpm in a Tecan Infinite® F200 PRO microplate reader (Tecan, Morrisville, USA). Growth was assessed both during and at the end of the incubation period. Every assay was performed at least in duplicate.

DNA extraction and genomic sequencing

The total genomic DNA of the isolates was extracted using DNeasy blood and tissue kits (Qiagen, Canada). A 5-kb mate-pair library was prepared using isolate 2009-144K3 and was sequenced on a GS-FLX+ instrument (Roche, Branford, USA). A TruSeq shotgun library prepared with isolate 2004-05MF26 was sequenced using the Illumina MiSeq sequencing system (Illumina, San Diego, USA). Both isolates were sequenced at the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval). The resulting reads of the 2009-144K3 isolate were *de novo* assembled with Newbler version 2.5.3 (Margulies *et al.* 2005). In the case of 2004-05MF26, the reads were filtered using Trimmomatic version 0.32 (Bolger *et al.* 2014) using the parameters suggested in the manual and were *de novo* assembled with Ray version 2.3.1 (Boisvert *et al.* 2010) with a K-mer length of 75.

Sequence analyses

Contigs resulting from the *de novo* assemblies were first mapped on the chromosome and the A449 plasmid sequence using the CONTIGuator webserver (Galardini *et al.* 2011) with default parameters. The large unmapped contigs were investigated by conducting BLASTx and BLASTn searches of the NCBI databases nr/nt. For the 2009-144K3 isolate, two contigs exhibited high degrees of identities with the pAB5S9 plasmid (Gordon *et al.* 2008), while one contig exhibited a high degree of identity with the pRAS3 plasmid (L'Abée-Lund & Sørum 2002). The two pAB5S9-like contigs were assembled using CONSED version 35 (Gordon & Green 2013), and the junctions were verified by PCR amplifications. The resulting plasmid was named pAB5S9b. A third party annotation was produced for the original pAB5S9 plasmid (accession number EF495198). Since the pRAS3-like plasmid was in a single contig, no subsequent assembly was required, and it was named pRAS3.3 (see Results and Discussion section).

In the case of the 2004-05MF26 isolate, a single unmapped contig was found. This contig corresponded to a pSN254-like plasmid, which differed markedly from the pSN254 plasmid originally found in *S. enterica* (Welch *et al.* 2007). As such, it was named pSN254b (see Results and Discussion section).

Annotations for the pAB5S9, pAB5S9b, and pRAS3.3 plasmids were performed manually. ORFs were found by a BLASTx search of the NCBI non-redundant database, and each ORF was verified. Given the size of the pSN254b plasmid, it was annotated using a custom Perl script. ORFs superior or equal to thirty amino acids were found using the GETORF function of EMBOSS (Rice *et al.* 2000). Their products were then identified using a similarity search of a local formatted database with the heuristic fasta35 algorithm (Pearson & Lipman 1988). The coordinates of each ORF-encoded protein were determined using a similarity search with tfasty35 (Pearson *et al.* 1997) of the main sequence, which in this case was pSN254b. The search generated output files compatible with the Artemis genome viewer (Rutherford *et al.* 2000) and the Sequin annotation tool. Each ORF from the resulting annotation was manually verified.

The genomic maps and GC skews of the plasmids were generated using DNAPlotter version 1.10 (Carver *et al.* 2009) and were visualized using genome viewer Artemis version 15 (Rutherford *et al.* 2000).

PCR analyses

The DNA templates, PCR mixtures and program cycles were done as previously described (Trudel *et al.* 2013). The PCR assays were performed at least twice, and suitable positive and negative controls were included in each assay.

The PCR primers used in the present study are listed in Table S2. We designed primers that were specific for each multidrug-resistance plasmid (pAsa4, pAB5S9, pSN254). In the case of pAB5S9, two primer pairs detected both pAB5S9 and pAB5S9b with the same amplicon size while the third primer pair detected different amplicon sizes for the two plasmids (Table S2). For pSN254, two primer pairs detected pSN254 from *S. enterica* and pSN254b from *A. salmonicida* subsp. *salmonicida* with the same amplicon size while the third primer pair was specific to pSN254b (Table S2).

The two regions in pRAS3-like plasmids which exhibit variations among repetition units were sequenced for all pRAS3-like positive isolates other than 2009-144K3 (2010-47K18, 2009-157K5, and 2009-195K29) using the sanger method on a 3130XL apparatus (Applied Biosystems) at the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval).

The sequences of pAB5S9, pAB5S9b, pRAS3.3, and pSN254b were deposited in GenBank under accession numbers BK008853, KJ909292, KJ909291, and KJ909290, respectively.

Results and Discussion

We tested the antibiotic resistance of 78 *A. salmonicida* subsp. *salmonicida* isolates, of which 30 were resistant to at least two antibiotics (Table S1). To investigate the putative presence of new plasmids carrying multidrug-resistance genes, we used high-throughput methods to determine the sequences of the 2009-144K3 and 2004-05MF26 isolates, which were resistant to a large number of antibiotics, but displayed differences in their antibiotic

resistance profiles (Table 4). These bacteria were isolated from New Brunswick and Quebec (Canada), respectively.

Tableau 4. Characteristics of multidrug-resistant *A. salmonicida* subsp. *salmonicida* isolates harboring a tetracycline resistance gene

Name[a]	Antibiotic resistance[b]	MIC of tetracycline (µg/ml)[c]	Plasmids found[d]
01-B522	SXT, TET, TMP	128	Cryptic, pAsal1, pAsa5, pAsa4-like
07-9324	STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
07-7817	STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
08-2647	SXT, STR, AMP, TET	16	Cryptic, pAsal1, pAsa5, pSN254-like
07-7287	STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
08-2783	STR, AMP, TET	16	Cryptic, pAsal1, pAsa5, pSN254-like
08-4188	STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
2009-157K5	STR, TET, ERY	128	Cryptic, pAsal1, pAsa5, pRas3.3
2010-47K18	STR, TET, CHL	128	Cryptic, pAsal1, pAsa5, pRas3.4, pAB5S9-like
2004-05MF26	NAL, SXT, STR, AMP, TET, ERY, GEN, CHL	16	Cryptic, pAsal1, pAsa5, pSN254b
2004-68K52	NAL, SXT, STR, AMP, TET, CHL	64	Cryptic, pAsal1, pAsa5, pSN254-like
2009-195K29	STR, TET, ERY	32	Cryptic, pAsal1, pAsa5, pRas3.3,
2009-144K3	SXT, STR, TET, ERY, CHL	256	Cryptic, pAsal1, pAsa5, pRas3.3, pAB5S9b
M15448-11	STR, AMP, TET, CHL	32	Cryptic, pAsal1, pAsa5
M16474-11	STR, AMP, TET, ERY, CHL	32	Cryptic, pAsal1, pAsa5
M14481-11	SXT, STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
M15879-11	SXT, STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
M17739-11	SXT, STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
M13732-11	STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
M17053-11	STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like
M15469-11	STR, AMP, TET, CHL	16	Cryptic, pAsal1, pAsa5, pSN254-like

a. The origin and source of these isolates are given in Supplemental Table 1.
b. NAL = nalidixic acid, SXT = sulfamethoxazole/trimethoprim, STR = streptomycin, AMP = ampicillin, TET = tetracycline, ERY = erythromycin, GEN = gentamycin, TMP = trimethoprim, and CHL = chloramphenicol.
c. The MIC of the sensitive 01-B526 isolate was 0.5 µg/ml.
d. The three cryptic plasmids pAsa1, pAsa2, and pAsa3 as well as pAsal1 and pRAS3 were identified in these isolates in a previous study Trudel *et al.* 2013. The pAsa5, pAsa4-like, pAB5S9-like, and pSN254-like plasmids were found by PCR genotyping in the present study (see Table S2).

The sequencing of the *A. salmonicida* subsp. *salmonicida* 2009-144K3 isolate identified a large contig sharing no identity with the chromosome or known plasmids sequences associated with *A. salmonicida* subsp. *salmonicida*. Further analyses revealed a high level of homology with pAB5S9, a previously published plasmid from *Aeromonas bestiarum* (Gordon *et al.* 2008), which carries multiple antibiotic resistance genes. pAB5S9b, the 2009-144K3 variant, exhibited a highly conserved gene distribution and gene order with its homolog except for a transposition of two regions (Figure 11). One of these regions, part A of Figure 11, had a complete putatively functional transposase as well as a truncated transposase (delta-tnp). These transposases have been reported to be part of an ISCR2 element in *A. bestiarum* (Gordon *et al.* 2008). The other region, part B, contained two genes involved in tetracycline resistance. Surprisingly, one of these genes in the pAB5S9 plasmid, *tet*(Y) did not appear to have a homologous gene in the pAB5S9b plasmid. However, another gene involved in tetracycline resistance *tet*(H) was found at the same position. Moreover, the pAB5S9b variant contained genes that should confer resistance to sulphonamide (*sulII*), streptomycin (*strA* and *strB*), florfenicol (*floR*), and chloramphenicol (*floR*).

A region in the original pAB5S9 (Gordon *et al.* 2008) has been shown to share a high degree of homology with a part of the *SXT* element of *Vibrio cholera* that contains the *floR*, *strB*, *strA*, and *sulII* genes (Beaber *et al.* 2002). The region is cut in half in pAB5S9 by the *tetR* and *tet*(Y) genes (Gordon *et al.* 2008). Interestingly, the *SXT* region in pAB5S9b in *A. salmonicida* subsp. *salmonicida* is not altered (Figure 11), suggesting that this plasmid might be closer to an ancestral *SXT*-like element than the original pAB5S9.

pAB5S9b is 824 bp larger than the pAB5S9 plasmid in *A. bestiarum*. This difference was mainly due to an insertion between parts A' and B' of the variant in *A. salmonicida* subsp. *salmonicida*. The insertion bears a single ORF that encodes a short hypothetical protein. An ortholog of this ORF has only been annotated in *Acinetobacter baumannii* (accession number EXS20171), an opportunistic Gram-negative human pathogen.

The pAB5S9 plasmid was first discovered in France (Gordon *et al.* 2008). Our study identified, for the first time, a plasmid in the pAB5S9 family isolated in North America from a different *Aeromonas* species. This discovery showed that the distribution of

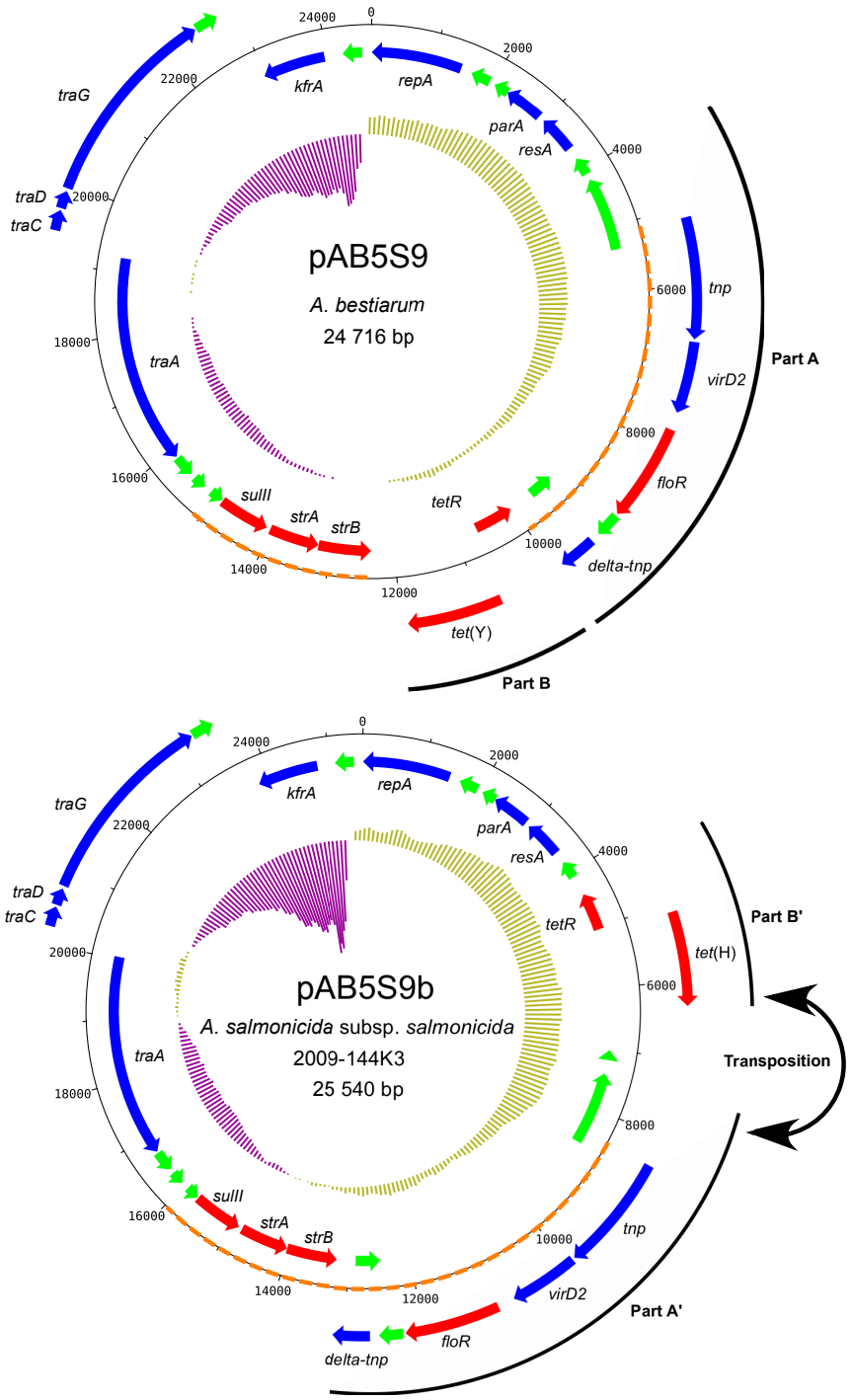

Figure 11. Maps of pAB5S9 and pAB5S9b.

Genes are represented by arrows. Genes on the outside are transcribed clockwise, whereas genes on the inside are transcribed counterclockwise. Colors represent gene function: red, green, and blue represent genes coding for antibiotic resistance, hypothetical proteins, and other functions, respectively. The inner ring represents the GC skew calculated by DNAPlotter with a window and step size of 10 000 bp and 100 bp, respectively. Part A and part B in pAB5S9 have been transposed in pAB5S9b and are indicated as part A' and part B'. The orange dotted line represents the *SXT* region in both pAB5S9 and pAB5S9

pAB5S9 may be geographically independent and may be involved in the propagation of antibiotic resistance in many regions of the world.

The pSN254 variant of the 2004-05MF26 isolate differed significantly from the original plasmid and was named pSN254b (Figure 12). The variant in *A. salmonicida* subsp. *salmonicida* was smaller than the replicon identified in *S. enterica* by approximately 24 kbp. There were three sections in pSN254b missing, one approximately 11.6 kbp in size, one 6.4 kpb in size, and one 6.0 kbp in size. The 11.6-kbp section corresponded to a duplicated fragment previously reported in *S. enterica* (Welch *et al.* 2007) that contains genes encoding hypothetical proteins and some are involved in multidrug resistance, including the quaternary ammonium compound-resistance (SugE1) and CMY-2 beta-lactamase proteins (Fig. 13-A). This section in the pSN254 plasmid in *S. enterica* is bordered by IS*Ec9*, suggesting that it may be a putative duplicated composite transposon. The pSN254b variant contains a single copy of this IS. However, we cannot discriminate between the possible insertion of this putative transposon in pSN254 or its deletion from pSN254b by conservative transposition. This deletion is interesting because it has been previously reported in another plasmid, pAR060302, which is in the same incompatibility plasmid group (IncA/C) (Call *et al.* 2010). This suggests that the two plasmids share a high degree of homology.

Interestingly, the 6.4-kbp and 6.0-kbp fragments, which are present in pSN254 but absent in pSN254b, are located in the transposon Tn*21* (Fig. 13-B), which is also present in the large plasmid pAsa4 (Reith *et al.* 2008). The first cassette located immediately downstream from the *attI* site is *aadA*, which has been previously identified in this transposon (Liebert *et al.* 1999) and is known to confer resistance to streptomycin and spectinomycin by adenylylation (Sandvang 1999). However, there are five other cassettes following the *aadA* cassette in the pSN254 plasmid of *S. enterica*. These cassettes consist of the 6.4-kbp section and are not present in the pSN254b plasmid of *A. salmonicida* subsp. *salmonicida*.

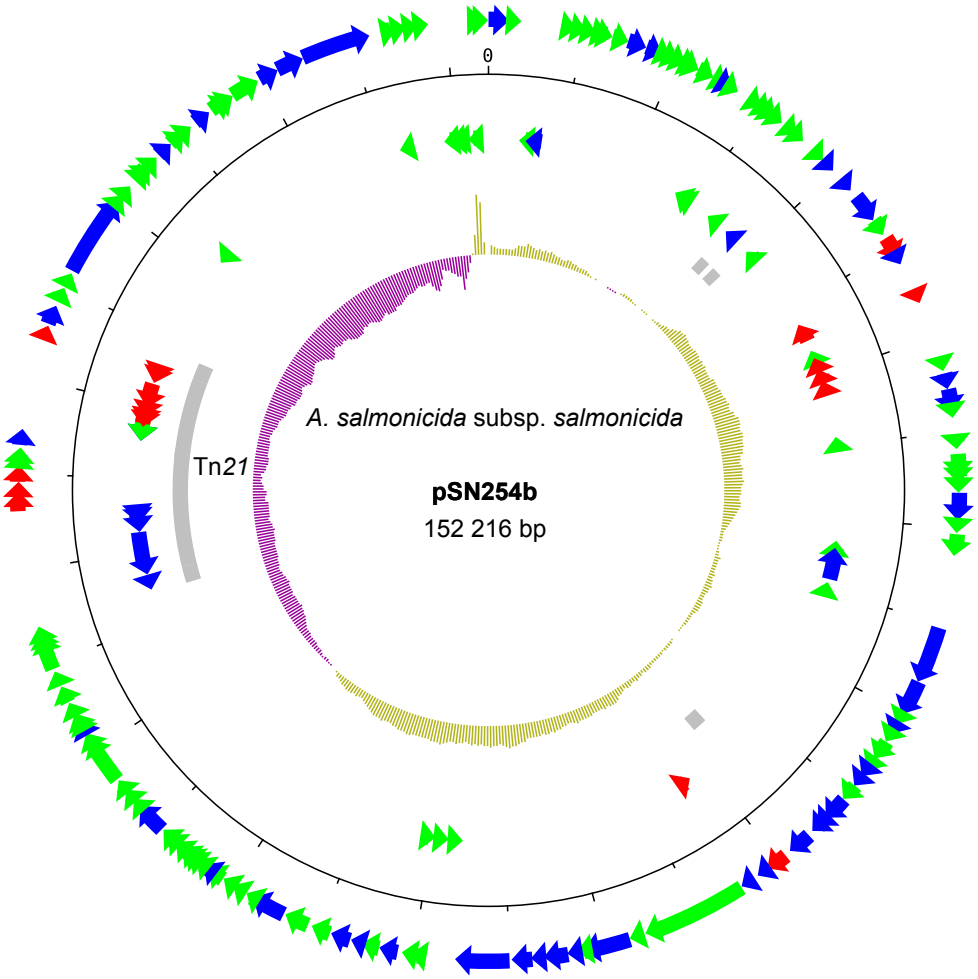

Figure 12. Map of pSN254b.
Genes are represented by arrows. Genes on the outside are transcribed clockwise, whereas genes on the inside are transcribed counterclockwise. Colors represent gene functions: red, green, and blue represent genes coding for antibiotic resistance, hypothetical proteins, and other functions, respectively. Gray rectangles represent mobile elements such as transposons and IS. The inner ring represents the GC skew calculated by DNAPlotter with a window and step size of 20 000 bp and 300 bp, respectively. Each tick mark represents a 5 000-bp step.

The other section absent from pSN254b (6.0 kbp) corresponds to the cassettes for the *istB* and *istA* genes, the insertion sequence IS*1353*, and the *tni* module, which encodes an ATPase and a transposase. Another interesting characteristic of the Tn*21* of *A. salmonicida* subsp. *salmonicida* is the replacement of IS*26* by IS*6100*, which has been shown to be present in a wide range of hosts (Dogra *et al.* 2004). Overall, pSN254b provides resistance to florfenicol, chloramphenicol, tetracycline, streptomycin, spectinomycin, sulphonamide, quaternary ammonium compounds, beta-lactam antibiotics, and mercury.

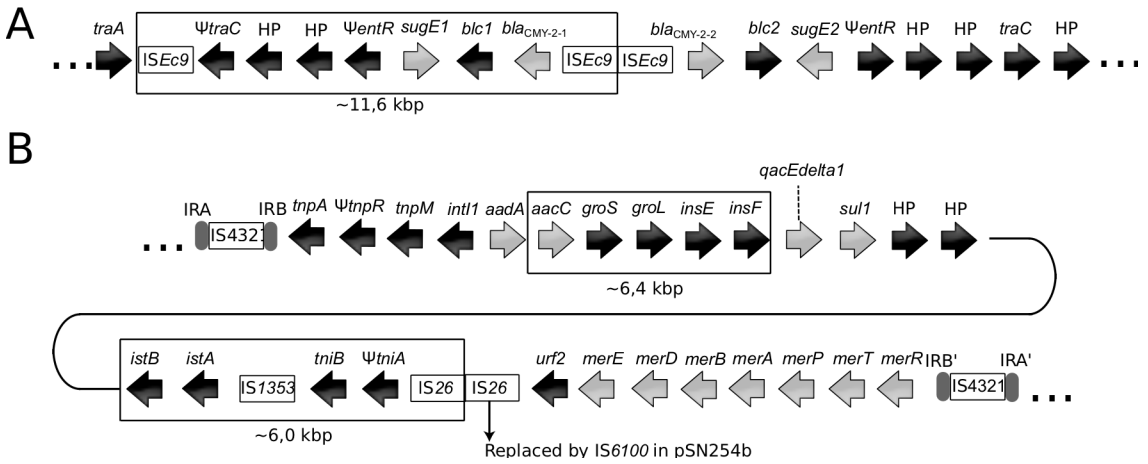

Figure 13. Differences between pSN254 and pSN254b

A. Putative composite transposon. The genes and the IS in the box are present in the pSN254 of *S. enterica* but are absent from the pSN254b of *A. salmonicida* subsp. *salmonicida*. **B.** Transposon Tn*21*. The genes and the IS in the boxes are present in the pSN254 of *S. enterica* but are absent from the pSN254b of *A. salmonicida* subsp. *salmonicida*. Grey arrows are genes coding for antibiotic and mercury resistance.

pSN254b is related to pSN254, a bla_{CMY-2} plasmid, and shares a typical backbone with other plasmids from the A/C incompatibility plasmid group (IncA/C). The majority of the genes with known functions code for the replication/maintenance of the plasmid and for a type IV conjugative transfer system (Welch *et al.* 2007). Putative hypothetical protein-coding genes make up approximately half of the gene repertoire of the plasmid, suggesting that additional functions for this plasmid may be discovered in the future.

Variations among bla_{CMY-2} plasmids have been reported (Call *et al.* 2010) and it was already proposed that if a bla_{CMY-2} plasmid is discovered in *A. salmonicida*, this would be the first time that the spread of a bla_{CMY-2} plasmid would be associated with a marine disease.

Two variants of pRAS3, an IncQ-related plasmid that carries genes for tetracycline resistance, have been described previously (L'Abée-Lund & Sørum 2002). They display minor differences, mainly in repetition units at two sites. We identified two additional variants, pRAS3.3 and pRAS3.4. They differed in terms of the repetition units located at the same sites in pRAS3.1 and pRAS3.2 (Fig. 14). Four isolates, which are known to bear pRAS3 (Trudel *et al.* 2013), were included in our analysis. In addition to 2009-144K3, these isolates included 2010-47K18, 2009-157K5, and 2009-195K29. Of these four isolates, three bore pRAS3.3 and one, pRAS3.4 (Table 4). Tandem repeated sequences

ended with a highly similar repeated unit but with a point mutation at the last nucleotide for both repetition spots. The repetition spot identified as "Reg A" (for Region A) ended with a repeated unit with a transversion (Fig. 14-B) while the repeated unit of "Reg B" had a transition (Fig. 14-C). In both cases, the repetitions were close to the regulation sequence involved in mobilization or replication (*oriT* or *oriV*, respectively). We propose that these repeated sequences may be involved in the regulation of plasmid transfer or copy number, as previously suggested for IncQ plasmids (Gardner *et al.* 2001; L'Abée-Lund & Sørum 2002; Loftie-Eaton & Rawlings 2009), but further studies will be required to confirm this.

Like pRAS3.1 and pRAS3.2, which have two ORFs that code for an active toxin-antitoxin system related to the PemK/Mazf family (Loftie-Eaton & Rawlings 2009, 2010), pRAS3.3 has the same ORFs.

After identifying pAB5S9b and pSN254b, we PCR genotyped the multidrug-resistant *A. salmonicida* subsp. *salmonicida* isolates harboring a tetracycline resistance gene in order to identify other isolates that potentially bear these plasmids or pAsa4. Only the tetracycline-resistant isolates were tested because pAB5S9b, pSN254b, pAsa4 and pRAS3 shared resistance to this antibiotic. Nineteen isolates in addition to 2009-144K3 and 2004-05MF26 were tested (Table 4). The genotyping primers were designed to detect pAB5S9 and pSN254. Other primers were also used to detect the presence of pAsa4 and pAsa5 (Table S1). When combined with our previous study on small plasmids from the same isolates (Trudel *et al.* 2013), the genotyping provided a more complete view of the plasmid profiles.

As expected for pathogenic isolates, all displayed positive PCR results for pAsa5 (Table 4), which encodes the type three secretion system essential for the virulence of *A. salmonicida* subsp. *salmonicida* (Dacanay *et al.* 2006). One isolate (01-B522) was positive for pAsa4. However, this isolate displayed less antibiotic resistance than expected for a bacterium bearing pAsa4 (Reith *et al.* 2008). Given this, the pAsa4 plasmid in the 01-B522 isolate is likely a variant of the pAsa4 plasmid (i.e., pAsa4-like) in the A449 reference strain. Further analyses of this plasmid will be required once its complete sequence has been determined in order to evaluate its relationship to pAsa4.

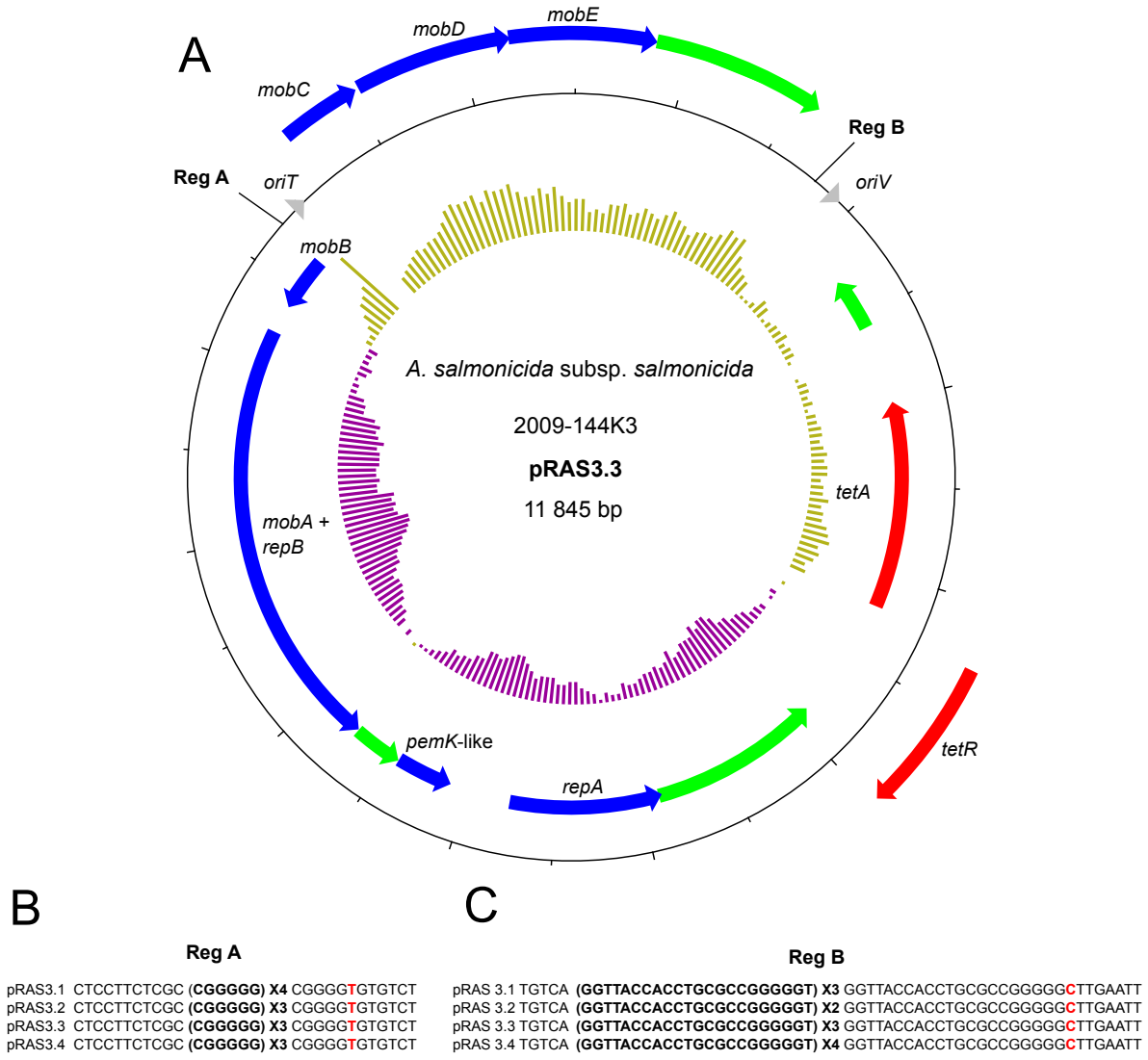

Figure 14. Map of pRAS3.3 and differences between variants.
A. The map of pRAS3.3 in the 2009-144K3 isolate. Genes are represented by arrows. Genes on the outside are transcribed clockwise, whereas genes on the inside are transcribed counterclockwise. Colors represent gene functions: red, green, and blue represent genes coding for antibiotic resistance, hypothetical proteins, and other functions, respectively. The inner ring represents the GC skew calculated by DNAPlotter with a window and step size of 1000 bp and 50 bp, respectively. Each tick mark represents a 500-bp step. **B. and C.** Representation of the differences in the number of repeat units between all pRAS3 variants for the "Reg A" and "Reg B" spots. The red nucleotides are point mutations in the last repetition unit, whether a transversion (B) or a transition (C).

In addition to 2009-144K3, only the 2010-47K18 isolate contained a pAB5S9 plasmid (Table 4). The three pAB5S9 primer pairs gave positive PCR signals for 2010-47K18, suggesting that this isolate bears a pAB5S9b plasmid. However, the 2010-47K18 isolate was not resistant to sulfamethoxazole, unlike 2009-144K3, whose resistance is provided by the *sulII* gene on pAB5S9 and pAB5S9b. As such, the pAB5S9b-like plasmid in 2010-

47K18 was probably another variant of the pAB5S9 family. Interestingly, these two isolates harboring the pAB5S9 variants also bore a pRAS3 plasmid. Both plasmid types encoded resistance genes for tetracycline. As expected, these isolates, as well as the isolate containing the pAsa4-like plasmid and an isolate likely bearing an unknown plasmid (see below), had the highest MIC for tetracycline.

Lastly, in addition to 2004-05MF26, 13 isolates exhibited positive PCR signals for all the pSN254 primer pairs (data not shown and Table 4). This is surprising since these isolates had four different antibiotic profiles with respect to sulfamethoxazole, streptomycin, ampicillin, tetracycline, and chloramphenicol resistance. These results indicated that multiple variants of pSN254 (pSN254-like) may exist in various populations of *A. salmonicida* subsp. *salmonicida*. Further analyses will be required to confirm this possibility.

Only four of the isolates did not bear one of the multidrug-resistance plasmids (pAB5S9, pSN254, pAsa4). Isolates 2009-157K5 and 2009-195K29 displayed resistance to tetracycline, erythromycin, and streptomycin. Since these two isolates possessed a pRAS3 plasmid, which confers tetracycline resistance, and since resistance to erythromycin and streptomycin is frequently due to point mutations on chromosomal genes (Coculescu 2009), it is plausible that these isolates contained no other plasmids than those found by genotyping and plasmid profiling (Trudel *et al.* 2013). However, the MIC of tetracycline for 2009-157K5 was higher than for 2009-195K29. This may be due to a mutation in the pRAS3 plasmid of one of the isolates that increases its copy number, the expression of a *tet* gene, or another parameter related to the level of resistance to tetracycline. Another scenario is that 2009-157K5 bears another, as yet unidentified plasmid that also codes for tetracycline resistance. The two other isolates that lacked a known multidrug-resistance plasmid (M15448-11 and M16474-11) displayed potentially chromosome-encoded resistance to streptomycin and erythromycin (Coculescu 2009) as well as resistance to ampicillin, tetracycline, and chloramphenicol. This suggested that these isolates may possess other multidrug-resistance plasmids that have not yet been identified.

Overall, the present study showed that *A. salmonicida* subsp. *salmonicida* harbors a high number of plasmid variants. Two such variants (pAB5S9 and pSN254) have been identified

in other bacterial genera or species, including *S. enterica*, a well-documented human pathogen. Moreover, pSN254 plasmids have been shown to be transferable by conjugation from *A. salmonicida* subsp. *salmonicida* to *E. coli*, *A. hydrophila*, and *E. tarda* (McIntosh *et al.* 2008). In fact, DNA transfer between non-human and human pathogenic bacteria is probably frequent in some conditions. It is possible that the genomic rearrangement activity seen in *A. salmonicida* subsp. *salmonicida* (Tanaka *et al.* 2013) coupled with a DNA flow between this bacterium and human pathogens may play a role in spreading antibiotic resistance among human pathogens.

The marked heterogeneity in the composition and distribution of plasmids in *A. salmonicida* subsp. *salmonicida*, as revealed in the present study, confirmed the importance of studying the genome of this bacterium. Our results suggested that multiple variants still uncharacterized of pSN254, pAB5S9, pRAS3, and pAsa4 exist and indicated that additional multidrug-resistance plasmids may be identified in the future. High-throughput sequencing technologies will likely increase documented cases of DNA transfer between *A. salmonicida* subsp. *salmonicida* and other bacteria. Given that *A. salmonicida* subsp. *salmonicida* is a ubiquitous bacterium in aquatic environments (Janda & Abbott 2010) and considering the constantly growing number of multidrug resistance plasmids that are being found in this bacterium, it is reasonable to consider *A. salmonicida* subsp. *salmonicida* as a potential reservoir of antibiotic resistance genes that can be transferred to human pathogens.

This study revealed additional elements of a growing worldwide problem, which is the prevalence of multidrug-resistant bacteria. It is now clear that environments polluted by human activities, such as contamination by heavy metals, have contributed to selecting for multidrug-resistant bacteria (Martínez 2008). It is also known that the use of antibiotics in veterinary medicine has led to an increase in antibiotic levels in water (Tello *et al.* 2012). Our study provides a better understanding of *A. salmonicida* subsp. *salmonicida*, which is a model for bacteria in environments such as fish farms that have been significantly altered by human activities. We hope that this will prompt more research on this issue given its health and economic impacts.

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References

Beaber JW, Hochhut B, Waldor MK. Genomic and functional analyses of SXT, an integrating antibiotic resistance gene transfer element derived from *Vibrio cholerae*. *J Bacteriol* 2002;**184**:4259–69.

Boisvert S, Laviolette F, Corbeil J. Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. *J Comput Biol* 2010;**17**:1519–33.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;**30**:2114–20.

Call DR, Singer RS, Meng D *et al.* blaCMY-2-positive IncA/C plasmids from *Escherichia coli* and *Salmonella enterica* are a distinct component of a larger lineage of plasmids. *Antimicrob Agents Chemother* 2010;**54**:590–6.

Carver T, Thomson N, Bleasby A *et al.* DNAPlotter: circular and linear interactive genome

visualization. *Bioinformatics* 2009;**25**:119–20.

Charette SJ, Brochu F, Boyle B *et al.* Draft genome sequence of the virulent strain 01-B526 of the fish pathogen *Aeromonas salmonicida*. *J Bacteriol* 2012;**194**:722–3.

Coculescu B-I. Antimicrobial resistance induced by genetic changes. *J Med Life* 2009;**2**:114–23.

Dacanay A, Knickle L, Solanky KS *et al.* Contribution of the type III secretion system (TTSS) to virulence of *Aeromonas salmonicida* subsp. *salmonicida*. *Microbiology* 2006;**152**:1847–56.

Daher RK, Filion G, Tan SGE *et al.* Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions. *Vet Microbiol* 2011;**152**:353–60.

Dallaire-Dufresne S, Tanaka KH, Trudel MV *et al.* Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* 2014;**169**:1–7.

Derome N, Boutin S, Llewellyn M *et al.* Chapter 4: Opportunistic pathogens of fish. In: Hurst CJ (ed.). *The Rasputin effect: When commensals and symbionts become parasitic*. Advances in Environmental Microbiology, Springer, 2014.

Dogra C, Raina V, Pal R *et al.* Organization of *lin* genes and IS*6100* among different strains of hexachlorocyclohexane-degrading *Sphingomonas paucimobilis*: Evidence for horizontal gene transfer. *J Bacteriol* 2004;**186**:2225–35.

Galardini M, Biondi EG, Bazzicalupo M *et al.* CONTIGuator: a bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol Med* 2011;**6**:11.

Gardner MN, Deane SM, Rawlings DE. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. *J Bacteriol* 2001;**183**:3303–9.

Gordon D, Green P. Consed: a graphical editor for next-generation sequencing. *Bioinformatics* 2013;**29**:2936–7.

Gordon L, Cloeckaert A, Doublet B *et al.* Complete sequence of the *floR*-carrying multiresistance plasmid pAB5S9 from freshwater *Aeromonas bestiarum*. *J Antimicrob Chemother* 2008;**62**:65–71.

Hänninen ML, Hirvelä-Koski V. Molecular and phenotypic methods for the characterization of atypical *Aeromonas salmonicida*. *Vet Microbiol* 1997;**56**:147–58.

Janda JM, Abbott SL. The genus *Aeromonas*: Taxonomy, pathogenicity, and infection. *Clin Microbiol Rev* 2010;**23**:35–73.

L'Abée-Lund TM, Sørum H. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. *Plasmid* 2002;**47**:172–81.

Liebert CA, Hall RM, Summers AO. Transposon Tn*21*, flagship of the floating genome. *Microbiol Mol Biol Rev* 1999;**63**:507–22.

Loftie-Eaton W, Rawlings DE. Comparative biology of two natural variants of the IncQ-2 family plasmids, pRAS3.1 and pRAS3.2. *J Bacteriol* 2009;**191**:6436–46.

Loftie-Eaton W, Rawlings DE. Evolutionary competitiveness of two natural variants of the IncQ-like plasmids, pRAS3.1 and pRAS3.2. *J Bacteriol* 2010;**192**:6182–90.

Margulies M, Egholm M, Altman WE *et al.* Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005;**437**:376–80.

Martínez JL. Antibiotics and antibiotic resistance genes in natural environments. *Sci* 2008;**321**:365–7.

McIntosh D, Cunningham M, Ji B *et al.* Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *J Antimicrob Chemother* 2008;**61**:1221–8.

Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* 1988;**85**:2444–8.

Pearson WR, Wood T, Zhang Z *et al.* Comparison of DNA sequences with protein sequences. *Genomics* 1997;**46**:24–36.

Reith ME, Singh RK, Curtis B *et al.* The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* 2008;**9**:427.

Rice P, Longden I, Bleasby A. EMBOSS: The european molecular biology open software suite. *Trends Genet* 2000;**16**:276–7.

Rutherford K, Parkhill J, Crook J *et al.* Artemis: sequence visualization and annotation. *Bioinformatics* 2000;**16**:944–5.

Sandaa RA, Enger O. Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. *Appl Environ Microbiol* 1994;**60**:4234–8.

Sandvang D. Novel streptomycin and spectinomycin resistance gene as a gene cassette within a class 1 integron isolated from *Escherichia coli*. *Antimicrob Agents Chemother* 1999;**43**:3036–8.

Sørum H, L'Abée-Lund TM, Solberg A *et al.* Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 2003;**47**:1285–90.

Stuber K, Burr SE, Braun M *et al.* Type III secretion genes in *Aeromonas salmonicida* subsp. *salmonicida* are located on a large thermolabile virulence plasmid. *J Clin Microbiol* 2003;**41**:3854–6.

Tanaka KH, Dallaire-Dufresne S, Daher RK *et al.* An insertion sequence-dependent plasmid rearrangement in *Aeromonas salmonicida* causes the loss of the type three secretion system. *PLoS One* 2012;**7**:e33725.

Tanaka KH, Frenette M, Charette SJ. IS-mediated loss of virulence by *Aeromonas salmonicida*: A tangible piece of an evolutionary puzzle. *Mob Genet Elements* 2013;**3**:e23498.

Tello A, Austin B, Telfer TC. Selective pressure of antibiotic pollution on bacteria of importance to public health. *Environ Health Perspect* 2012;**120**:1100–6.

Trudel MV, Tanaka KH, Filion G *et al.* Insertion sequence *AS5* (IS*AS5*) is involved in the genomic plasticity of *Aeromonas salmonicida*. *Mob Genet Elements* 2013;**5**:1–7.

Welch TJ, Fricke WF, McDermott PF *et al.* Multiple antimicrobial resistance in plague: An emerging public health risk. *PLoS One* 2007;**2**:e309.

Chapitre 3 – Article 2

Diversity of antibiotic-resistance genes in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida*: dominance of pSN254b and discovery of pAsa8

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Résumé

Dans cette étude, nous avons investigué dans 100 isolats d'*A. salmonicida* subsp. *salmonicida* la présence de plasmides et de gènes de résistance aux antibiotiques par PCR multiplex. Il a été possible de mettre en évidence la dominance du plasmide conjugatif pSN254b et la présence d'un nouveau plasmide, pAsa8, qui confère lui aussi une résistance a de nombreux antibiotiques. En fait, ce dernier possède un intégron similaire à celui de l'ilot génomique *Salmonella Genomic Island 1* (SGI) présent chez *Salmonella enterica* et *Proteus mirabilis*. Cette étude a par conséquent démontré qu'*A. salmonicida* subsp. *salmonicida* a une grande propension à acquérir du matériel génétique d'autres bactéries, par exemple de l'agent pathogène humain *S. enterica*. Dans le concept d'une seule santé «*One Health*», il va être important de surveiller *A. salmonicida* subp. *salmonicida* puisque cette bactérie pourrait être un important réservoir de gènes de résistance aux antibiotiques.

Abstract

The bacterium *Aeromonas salmonicida* subsp. *salmonicida* is a common pathogen in fish farms worldwide. Since the antibiotic resistance of this bacterial species is on the increase, it is important to have a broader view on this issue. In the present study, we tested the presence of known plasmids conferring multi-drug resistance as well as antibiotic resistance genes by a PCR approach in 100 Canadian *A. salmonicida* subsp. *salmonicida* isolates. Our study highlighted the dominance of the conjugative pSN254b plasmid, which confers multi-drug resistance. We also identified a new multi-drug plasmid named pAsa8, which has been characterized by a combination of sequencing technologies (Illumina and Oxford nanopore). This new plasmid harbors a complex class 1 integron similar to the one of the *Salmonella genomic island 1* (SGI) found in *Salmonella enterica* and *Proteus mirabilis*. Consequently, in addition to providing an update on the *A. salmonicida* subsp. *salmonicida* isolates that are resistant to antibiotics, our data suggest that this bacterium is potentially an important reservoir of drug resistance genes and should consequently be monitored more extensively. In addition, we describe a screening method that has the potential to become a diagnostic tool that is complementary to other methods currently in use.

Introduction

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is a fish pathogen that causes furunculosis worldwide (Dallaire-Dufresne *et al.* 2014; Hiney & Olivier 1999). In Quebec (Canada), furunculosis is the most common infection encountered in fish farms, especially brook trout farms, and causes between 30 and 60% of reported outbreaks every year (Morin 2010). Antibiotics are the most widely used treatment for furunculosis. In Canada, four antibiotics (oxytetracycline, florfenicol [a chloramphenicol analog], sulfadimethoxine/ormetoprim, and sulfadiazine/trimethoprim) are approved by the Veterinary Drugs Directorate (VDD) of Health Canada to treat infected fish. In Quebec, florfenicol is the antibiotic of choice because of its short withdrawal period (12 days) compared to antibiotics such as oxytetracycline and sulfadimethoxine/ormetoprim (42 days) (Morin 2010). However, intensive use of these four antibiotics has been correlated with growing number of antibiotic-resistant isolates (Morin 2010).

Antibiotic resistance genes in *A. salmonicida* subsp. *salmonicida* are mostly located on plasmids (Piotrowska & Popowska 2015). Among them, pSN254b and pAB5S9b plasmids, which bear multi-drug resistance genes, as well as variants of pRAS3 have been recently described in isolates from Canada (Vincent *et al.* 2014b). To date, pSN254b has been identified in isolates from Quebec, New Brunswick (Canada) and Nova Scotia (Canada), while pAB5S9b and pRAS3 variants have been identified only in New Brunswick. pAB5S9b and pSN254b bear genes coding for resistance to the three antibiotics used in veterinary medicine in Quebec. There are currently no government-approved antibiotics that are effective against bacterial isolates that host either one of these two plasmids. For their part, pRAS3 variants contain a gene coding for tetracycline resistance (Vincent *et al.* 2014b). In addition to these plasmids conferring drug resistance (R-plasmids), many others were found in *A. salmonicida*, some of them having a large-panel of genes causing resistance to antibiotics (Table 5).

Even if the plasmidome (i.e., the total plasmid content) of *A. salmonicida* subsp. *salmonicida* harbor multiple R-plasmids, the dispersion of these plasmids and the gravity of the situation are presently unknown. In order to determine the fraction of isolates bearing antibiotic-resistance genes and to infer the geographic distribution of the R-plasmids, we

investigated a collection of 100 *A. salmonicida* subsp. *salmonicida* Canadian isolates, using, among other methods, optimized multiplex PCR assays. This approach was also done to assess the presence of new R-plasmids.

This screening showed that pSN254b is the most common R-plasmid in tested Canadian isolates of *A. salmonicida* subsp. *salmonicida*. A new R-plasmid, pAsa8, was also found, which had genetic features that provided additional evidence that *A. salmonicida* subsp. *salmonicida* is an important reservoir for mobile genetic elements as well as antibiotic resistance genes.

Tableau 5. R-plasmids found in *A. salmonicida*

Plasmid	Length (kb)	R-genes	Found in Canada	Reference
pAsa7	5,276	*cat*	No	(Vincent *et al*. 2016)
pRAS3.2	11,823	*tetA*	No	(L'Abee-Lund & Sorum 2002)
pRAS3.3	11,845	*tetA*	Yes	(Vincent *et al*. 2014b)
pRAS3.1	11,851	*tetA*	No	(L'Abee-Lund & Sorum 2002)
pAB5S9b	25,540	*tet*(H), *floR, sul2, strA, strB*	Yes	(Vincent *et al*. 2014b)
pASOT3	~ 39	*aadA2, tetA*	No	(Adams *et al*. 1998; L'Abee-Lund & Sorum 2001)
pRAS1	~ 45	*dfrA16, tetA, sul1*	No	(Sorum *et al*. 2003)
pAr-32	~ 47	*aadA2, sul1, catA2*	No	(Aoki *et al*. 1971a; Sorum *et al*. 2003)
pASOT	~ 47	*aadA2 or dfrIIc, tetA*	No	(Adams *et al*. 1998; L'Abee-Lund & Sorum 2001)
pASOT2	~ 47	*aadA2, tetA*	No	(Adams *et al*. 1998; L'Abee-Lund & Sorum 2001)
pRAS2	~48	*sul2, tet 31, strA, strB*	No	(L'Abee-Lund & Sorum 2000; Sorum *et al*. 2003)
pSN254b	152,216	*tetA, floR, sul1, sul2, blaCMY, aadA, strA, strB, sugE2, qacEΔ1*	Yes	(Vincent *et al*. 2014b)
pAsa4	166,749	*tet*(E), *sul1, aadA, cat*	Yes (pAsa4-like)	(Reith *et al*. 2008; Vincent *et al*. 2014b)

Results

Plasmid content determined by PCR genotyping

The presence of plasmids containing known antibiotic resistance genes (pAsa4, pAB5S9b, pRAS3.3, pSN254b) has been tested in 100 Canadian *A. salmonicida* subsp. *salmonicida* isolates (Vincent *et al*. 2014b) using the primers listed in Supplementary Table S1 online. The results of the genotyping for the 100 isolates are given in Supplementary Table S2

online. The pSN254b plasmid was the most prevalent (25/100), while the other plasmids were detected in two to four isolates each.

Multiplex PCR to detect antibiotic resistance genes

We subsequently developed four multiplex PCR assays (see the Methods section) to detect the antibiotic resistance genes known to be present in *A. salmonicida* subsp. *salmonicida* isolates: *floR*, *cat*, *sul1*, *sul2*, *tetA*, *tet*(C), *tet*(E), and *tet*(H). The results of these tests for the 100 *A. salmonicida* subsp. *salmonicida* isolates are presented in Supplementary Table S2 online.

The *floR* gene was detected in 29 of the 100 isolates, while the *cat* gene was not detected in any of the isolates excepted in A449, which was the positive control. The *floR* gene, which has been detected in isolates with the pSN254b and pAB5S9b plasmids (Vincent *et al*. 2014b), was also detected in the M15448-11 and M16474-11 isolates, despite the fact that they do not host either of the two plasmids. This result suggests that an unknown genomic entity might be responsible for the presence of the *floR* gene in these two isolates.

The *sul1* and *sul2* genes were detected in 30 and 28 isolates, respectively. Both genes were detected in isolates that contained pSN254b, while only *sul2* was detected in isolates carrying pAB5S9b, as reported previously (Vincent *et al.* 2014b). The *sul1* gene was detected in four isolates (three from Quebec and one from Ontario (Canada)) with a pAsa4-like plasmid. This multiplex PCR experiment also revealed that the *sul1* gene was present in M15448-11 and M16474-11.

The *tet*(C) gene was detected in four isolates with the pRAS3 plasmid while *tet*(H) was detected in two isolates harboring the pAB5S9b plasmid (Vincent *et al.* 2014b). The *tet*(E) gene was present in four isolates with a pAsa4-like plasmid. The *tetA* gene was detected in isolates with the pSN254b plasmid (Vincent *et al*. 2014b) as well as in the M15448-11 and M16474-11 isolates.

Characterization of pAsa8

The presence of multiple closely related antibiotic resistance genes in M15448-11 and M16474-11 isolates without the detection of any recognized plasmids in these isolates

prompted us to sequence the complete DNA of M16474-11 by Illumina and Oxford nanopore (MinION) technologies. Only the DNA of the M16474-11 isolate was sequenced since both isolates (i.e., M15448-11 and M16474-11) exhibited the same profile.

The sequencing revealed the presence of a new plasmid, that we named pAsa8, of 110 577 bp and which has a region containing many drug resistance genes. This region is the product of multiple integration events involving mobile genetic elements (Figure 15). One of them is a transposon Tn*1721* (Allmeier *et al*. 1992), which is integrated in the gene *umuC*, encoding for a UV mutagenesis and repair protein, of the ancestral pAsa8. This transposon brings a *tetA* gene, causing resistance to tetracycline, which explains the positive result of the multiplex PCR.

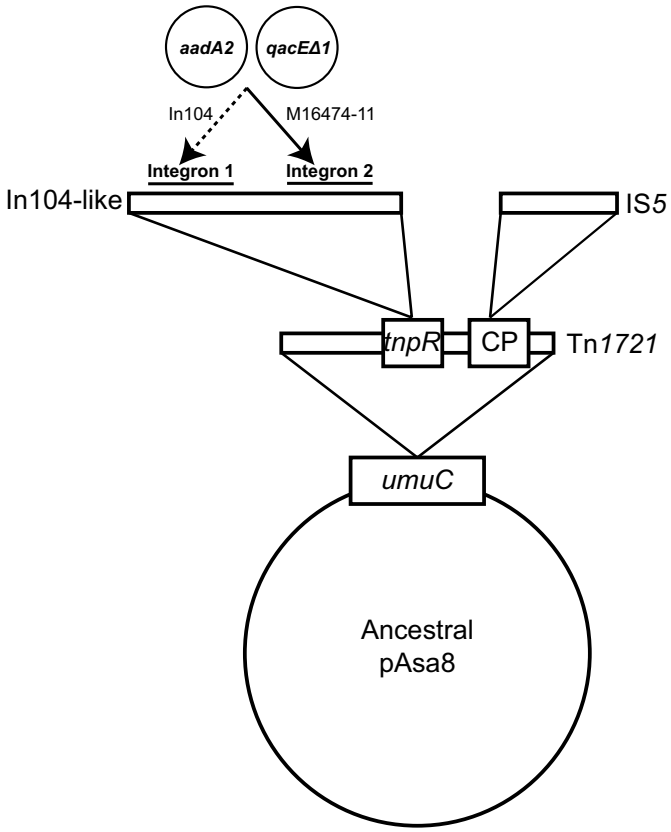

Figure 15. Putative evolutionary scenario of the multidrug region of pAsa8.
Only the elements involved are shown: the gene *umuC* of ancestral pAsa8, the genes *tnpR* and the one encoding for the chemotaxis protein (CP) located on the transposon Tn*1721*, the insertion sequence IS*5* and finally the integron In104-like. Cassettes *aadA2* and *qacEΔ1* are located within integron 1 of In104 (dashed arrow), and also within integron 2 of In104-like found on pAsa8 (solid line arrow) (isolate M16474-11).

A second integration event involved a complex class 1 integron In104-like (In4-group) previously found in a 43-kb integrated element known as the *Salmonella genomic island 1*

(SGI1) found in *Salmonella enterica* (Boyd *et al*. 2000) and *Proteus mirabilis* (Bi *et al*. 2011; Lei *et al*. 2014; Qin *et al*. 2015; Siebor & Neuwirth 2013) and for which numerous variants were found (Kiss *et al*. 2012). The In104-like integron allows resistance to florfenicol/chloramphenicol (*floR*), tetracyclines (*tet*(G)), sulfonamides (*sul1*), ampicillin/carbenicillin (bla_{PSE-1}) and streptomycin/spectinomycin (*aadA2*) (Figure 16) in the previously described M16474-11 *A. salmonicida* subsp. *salmonicida* isolate. The complex In104 integron contains two functional *IntI1* integron regions (Kiss *et al*. 2012; Michael *et al.* 2013) with many variants reported (Mulvey *et al*. 2006). The integron regions found in the In104-like of *A. salmonicida* differs from those of the original In104 found in *S. enterica* serovar *Typhimurium* DT104 in the sense that the cassettes *aadA2* and *qacEΔ1* found in the first integron (*IntI1*) in *S. enterica* are in the second (*groEL/IntI1*) for *A. salmonicida* subsp. *salmonicida* (Figure 16). The evolutionary scenario that would account for the cassette swap is not clear, but a BLASTn revealed that the second integron region (*groEL/IntI1*) is identical to one of the transposon, Tn*2610*, which is formed by parts of Tn*1721*, Tn*21* and SGI1 (Takaya *et al*. 2006). The third and last integration event involves the integration of an IS*5* into the gene that encodes the chemotaxis protein of Tn*1721*.

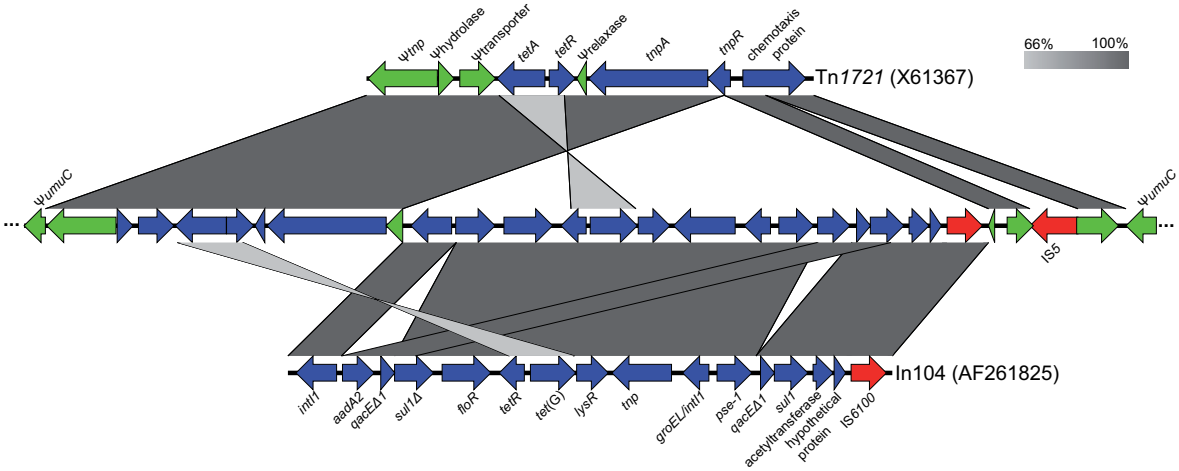

Figure 16. Comparisons of the multi-drug region of pAsa8 with the transposon Tn*1721* and the integron In104 found in SGI1.

Interestingly, the IncU plasmid pRAS1, found in both typical and atypical *A. salmonicida*, was also reported to harbours a Tn*1721* and an integron of the In4 family (Sandaa & Enger 1994; Sorum *et al*. 2003). Unfortunately, this plasmid is not sequenced and consequently it

would be perilous to postulate any evolutionary links between pRAS1 and pAsa8. As recently reviewed elsewhere, Tn*1721* is present in several strains of the genus *Aeromonas* (Piotrowska & Popowska 2015).

The pAsa8 backbone contains several genes encoding for hypothetical proteins and also has a region containing genes promoting conjugative transfers. However, many of these ORFs are likely pseudogenes or are highly derived relative to sequences in the GenBank nr/nt database (based on BLASTp). No type 2 toxin-antitoxin system was found by TAfinder. A BLASTn query of the nr/nt database with the pAsa8 sequence only gave partial alignments (the multidrug region). However, a BLASTn analysis against the Whole Genome Shotgun (WGS) database (Gammaproteobacteria, taxid:1236) resulted in significant homology to the draft genome of *Aeromonas rivuli* strain DSM 22539 (GenBank: CDBJ00000000.1). This sequence was a result of a recent large-scale study investigating Aeromonad taxonomy and was not extensively analyzed (Colston *et al*. 2014). Interestingly, it was possible to map five contigs from the draft genome of *A. rivuli* on the sequence of pAsa8 using locally CONTIguator version 2.7.4 (Galardini *et al*. 2011). The five contigs covered almost all of the pAsa8 sequence, with the exception of the region containing the Tn*1721* and the In104-like regions in pAsa8, thus indicating that a similar plasmid, without the integration of these mobile elements, could be present in the strain DSM 22539 of *A. rivuli*. A search for Tn*1721* and In104-like in the complete draft genome of *A. rivuli* was negative.

Based on the discovery of pAsa8 and the presence of the *tet*(G) genes in this plasmid which was detected for the first time in *A. salmonicida* subsp *salmonicida*, primers targeting this gene were included in the fourth multiplex PCR assay (the one detecting *tet*(C) and *tet*(H)) (see the Methods section). An illustration of the results obtained with positive controls with all the four multiplex PCR assays designed in this study is shown in Figure 17.

In addition, other primers were designed to target the various parts of pAsa8 (Supplementary Table S1). Three targets were for the pAsa8 backbone and one for the Tn*1721*. The primers targeting *tet*(G) were used to detect the presence of the In104-like since this gene was actually only found to be In104-encoded in *A. salmonicida* subsp. *salmonicida*. Only two isolates, M15448-11 and M16474-11, gave PCR-positive results

with all the primer pairs tested. No amplification occurred with any of the primer pairs in any other isolates.

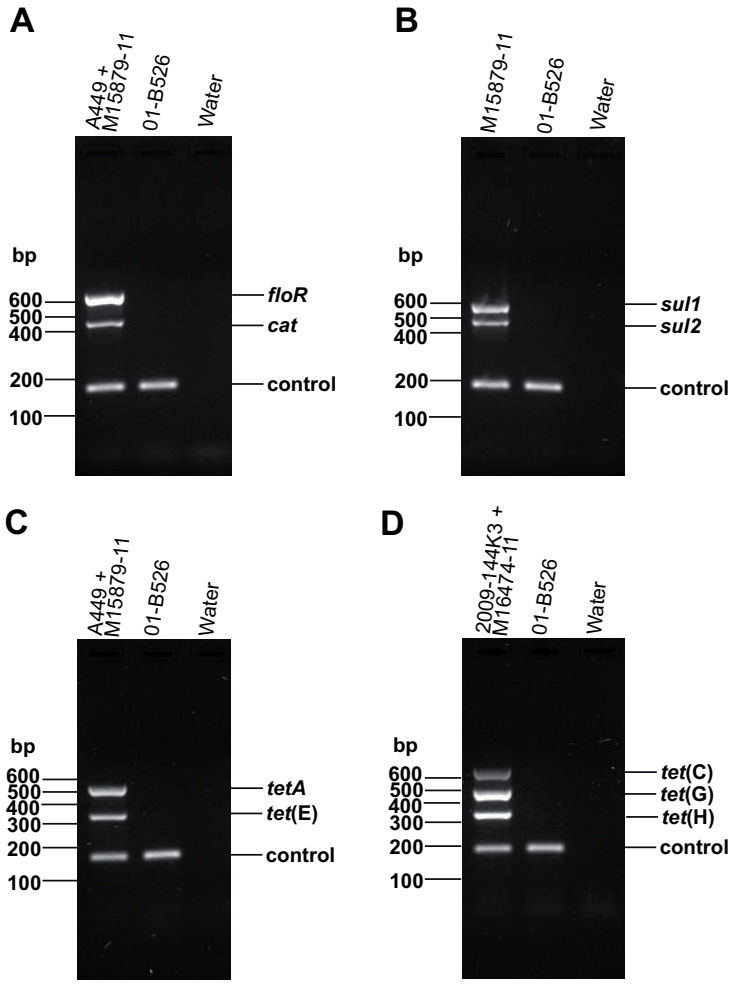

Figure 17. Multiplex PCR targeting genes coding for resistance to chloramphenicol/florfenicol (A), sulfonamides (B), and tetracyclines (C and D).

Electrophoresis gels with amplicons generated using DNA isolated from positive controls (A449 + M15879-11 in A and C, M15879-11 in B, 2009-144K3 + M16474-11 in D) and from a negative control (01-B526). A specific control for *A. salmonicida* subsp. *salmonicida* using a primer pair that amplified an element of the prophage 1 was included in each multiplex PCR reaction. The amplicon of this product can be seen at the bottom of each gel (control). A. The target *cat* (448 bp) and *floR* genes (632 bp) and B. the *sul2* (449 bp) and *sul1* (550 bp) genes were detected when present. C. Amplicons for *tet*(E) (351 bp) and *tetA* (526 pb). D. Amplicons for *tet*(H) (326 bp), *tet*(G) (460 bp), and *tet*(C) (629 bp). Water was used as a negative control.

Geographic distribution of isolates in Quebec harboring antibiotic resistance plasmids

Since a large number of isolates from Quebec were included in the present study, it was relevant to analyze the geographical distribution of the antibiotic-resistant isolates in Quebec. The province was divided into four regions: southeast (SE), southwest (SW), northeast (NE), and northwest (NW) (Figure 18 and Table 6).

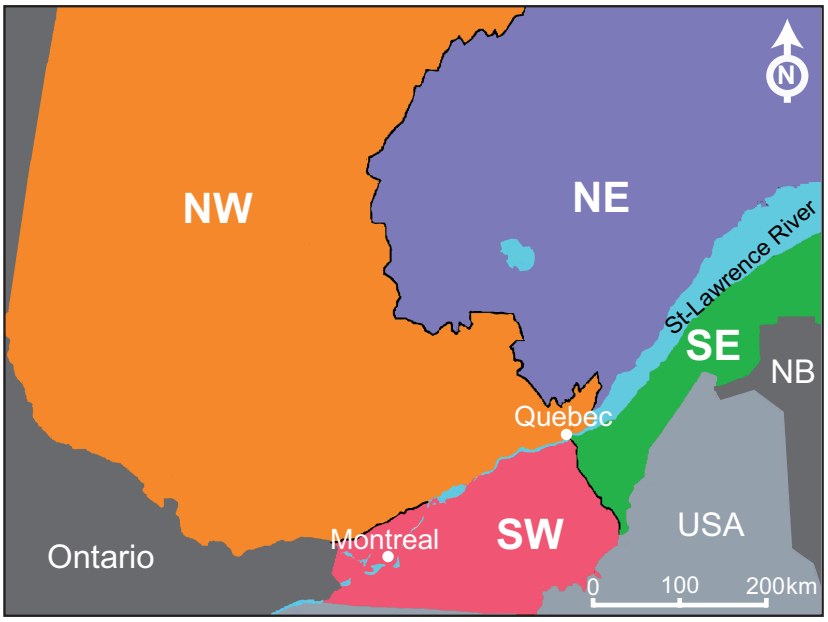

Figure 18. Map of the Quebec showing the regions to which the various isolates were assigned. Northwest (NW, orange), northeast (NE, purple), southwest (SW, pink), and southeast (SE, green). Most of the fish farms analyzed were less than 200 km from the St. Lawrence River, which crosses the province from west to east. The map has been drawn using Adobe Photoshop CS4 version 11.0.2 (www.adobe.com).

The multidrug-resistant plasmid pSN254b was identified in many isolates from the SE, SW, and NW regions, while pAsa4 variants were only found in three isolates from the NW region. The two isolates containing pAsa8 were also from the NW region. The isolates from the SE, SW and NW regions displayed a high prevalence of antibiotic resistance while those from the NE region displayed no antibiotic resistance at all.

Tableau 6. Geographical distribution of the antibiotic resistant isolates in Quebec.

Region[a]		**NE**	**SE**	**SW**	**NW**	**Quebec as a whole**
Number of isolates		16	11	14	45	86
Plasmid found in the isolates	pSN254b	0	5	6	12	23
	pAsa4-like	0	0	0	3	3
	pAsa4	0	0	0	0	0
	pAB5S9	0	0	0	0	0
	pRAS3	0	0	0	0	0
	pAsa8	0	0	0	2	2
Number of antibiotic-resistant isolates		0	5	6	17	28
% of resistant isolates[b]		0 [0.00,0.11]	45 [0.20,0.73]	43 [0.20,0.68]	38 [0.25,0.52]	33 [0.23,0.43]

a: see Figure 18
b: The binomial 95% confidence interval (Jeffreys method) is indicated between brackets.

Discussion

One of the goals of this study was to evaluate the occurrence of genes in the fish pathogen *A. salmonicida* subsp. *salmonicida* that code for resistance to chloramphenicol/florfenicol, tetracycline, and sulfamethoxazole. Based on the multiplex PCR results, sulfonamide resistance was the most common antibiotic resistance detected in the Canadian isolates. The prevalence of florfenicol resistance in Quebec is most likely due to the intensive use of this antibiotic since 1999 to treat furunculosis (Morin 2010). The selective pressure caused by exposure to this antibiotic has promoted the spread of florfenicol-resistant isolates (Dixon 2001). The multiplex PCR results showed that many isolates from Quebec contain resistance genes to the four antibiotics authorized for aquaculture use in the province. This is alarming since the Veterinary Drugs Directorate (VDD) of Health Canada has not authorized the use of any other antibiotics for treating fish infected by these multi-resistant isolates. However, given that the present study mainly investigated *A. salmonicida* subsp. *salmonicida* isolates from Quebec, more isolates from other Canadian provinces will have to be assessed to determine whether they also display the same trends.

The multiplex PCR-based method that we developed provide an accurate diagnostic tool allowing to identify genes on known plasmids in *A. salmonicida* subsp. *salmonicida* that code for resistance against the three major classes of antibiotics legally used in fish farming in Canada. While the present study focused only on *A. salmonicida* subsp. *salmonicida* isolates from Canada, this new diagnostic tool allowed us to uncover important information about the antibiotic resistance of this bacterium. This diagnostic method could also be used to study the diversity of antibiotic resistance plasmids in *A. salmonicida* subsp. *salmonicida* isolates from around the world and for conducting surveys to study temporal resistance evolution in general.

Since the discovery of transferable R-factors in *A. salmonicida* published in 1971 by Aoki (Aoki *et al*. 1971a), many R-plasmids have been found in this bacterium (Table 5). However, actually only pSN254b (25/100), pRAS3 (4/100), pAsa4-like (4/100), pAB5S9b (2/100) and pAsa8 (2/100) were found in Canadian isolates. More specifically, the pSN254b multiple antibiotic resistance-encoding plasmid has been observed in many isolates in Quebec and is a major problem when it comes to the treatment of infected fish

with antibiotics. The high prevalence of pSN254b can be mostly explained by (1) its capacity to be transferred by conjugation (McIntosh *et al*. 2008) and (2) the presence of genes conferring resistance to various antibiotics, thus enhancing the selection pressure for isolates having this plasmid. In fact, up to 40% of the *A. salmonicida* subsp. *salmonicida* bacteria isolated from diseased fish in Quebec, except in the NE region, harbor antibiotic resistance genes (Table 6). pAsa4 and pAsa8 were found in the NW region while the pSN254b plasmid was present in the SW, SE, and NW regions.

The veterinarians who collected the *A. salmonicida* subsp. *salmonicida* isolates from diseased fish proposed three reasons explaining the geographic distribution of the antibiotic resistant isolates (Uhland 2005). First, there is more infection outbreak in regions with higher aquaculture activity (mainly NW, SW and SE). Second, it is known that fish farmers exchange fish between regions which may contribute in part to the spread of the antibiotic resistant isolates. Finally, there is an increased likelihood of diagnosis for the fish farms in the surrounding regions of the veterinary service (e.g. NW and SW).

As mentioned in (Vincent *et al*. 2014), plasmids conferring resistance to antibiotics may be transferable between *A. salmonicida* subsp. *salmonicida* and human pathogens such as *Salmonella enterica* (Vincent *et al*. 2014b). This is exemplified by the IncA/C pSN254b plasmid, a variant of pSN254 found in *S. enterica* (Vincent *et al*. 2014b). The plasmids of the IncA/C group are known to be conjugative and found, in addition to *A. salmonicida* and *S. enterica*, in a broad range of bacterium (Johnson & Lang 2012) such as *Yersinia pestis* (Welch *et al*. 2007), *Klebsiella pneumonia* (Doublet *et al*. 2012), *Aeromonas hydrophila* (Aoki *et al*. 1971b; Fricke *et al*. 2009), *Photobacterium damselae* subsp. *piscicida* (Kim *et al*. 2008) and *Escherichia coli* (Call *et al*. 2010; Fernandez-Alarcon *et al*. 2011). The IncA/C plasmids are also known to regulate the excision of SGI1 (Kiss *et al*. 2015) and to serve as helpers for its mobilization in *trans* (Douard *et al*. 2010). Interestingly, pAsa8 (a new R-plasmid described here) of the M16474-11 and M15448-11 *A. salmonicida* subsp. *salmonicida* isolates appeared to have a Tn*1721* and a complex class 1 integron similar to the one found in SGI1. Even if we have a putative mechanism explaining the multiple integration events in the ancestral pAsa8 plasmid (Figure 15), it is unclear if they occurred in *A. salmonicida*, in other bacteria such as *S. enterica*, or in both.

In summary, we analyzed 100 *A. salmonicida* subsp. *salmonicida* Canadian isolates for their antibiotic resistance genes and R-plasmids repertoire to shed light on their occurrence and distribution. We found that the conjugative R-plasmid pSN254b is dominant with 25% of the isolates having it. In fact, 37% of the isolates have at least one R-plasmid. This situation is worrying considering that no government-approved antibiotics can be used against these isolates to prevent furunculosis. We suggest that *A. salmonicida* subsp. *salmonicida* should be monitored worldwide to verify the trend found in Canada revealed in this study. We also discovered and characterized a new R-plasmid named here pAsa8. This plasmid hosts multiple antibiotic resistance genes from mobile elements such as Tn*1721* and a complex class 1 integron In104-like. Since In104 is usually found within the genomic island SGI1 of the human pathogen *S. enterica*, this study reinforces the idea that *A. salmonicida* subsp. *salmonicida* could be an important reservoir of mobile DNA conferring drug resistance and raises the possibility of exchange of this genetic material with other waterborne bacteria. If indeed this is the case, it is even more worrying from a One Health perspective (i.e. the idea that human, animal and environmental health are all interconnected) (Robinson *et al*. 2016). Considering antimicrobial resistance in a One Health context is a part of the fourth intervention plan of the last report produced by the *Review on Antimicrobial Resistance* (O'Neill 2016). It is now well known that environmental bacteria, such as *Aeromonas* (Gordon *et al*. 2008), may act as a reservoir of antibiotic resistance genes (Biyela *et al*. 2004; Hatosy & Martiny 2015; Wellington *et al*. 2013). Therefore, our results add substantial evidence that there is an urgent need to develop alternative strategies to efficiently mitigate furunculosis prevalence without selecting for other resistance genes. Pre and probiotic strategies, phage therapy and vegetable extracts such as essential oils might be promising research avenues against *A. salmonicida* subsp. *salmonicida* as it is the case for other bacteria (Haba *et al*. 2014; Krylov 2014; Llewellyn *et al*. 2014).

Methods

Bacterial isolates and growth conditions

All 100 *A. salmonicida* subsp. *salmonicida* isolates used in this study were from Canada (Supplementary Table S2 online). The A449 reference strain from France (Reith *et al*.

2008), for which the annotated chromosome and plasmid sequences are publicly available, was also included. All the isolates were grown on furunculosis agar for two or three days at 18°C as previously described (Daher *et al*. 2011).

PCR analyses

The DNA templates, PCR mixtures, and program cycles used in this study have been previously described (Trudel *et al*. 2013). The PCR assays were performed at least twice. Suitable positive and negative controls were included in each assay.

The PCR primers used to identify the isolates harboring antibiotic-resistance plasmids (pAsa4, pAB5S9, pSN254, pRAS3) are listed in Supplementary Table S1 online, as are the primers used to genotype pAsa8 of the M16474-11 isolate.

Multiplex PCR

The sequences of orthologous genes causing resistance to chloramphenicol/florfenicol (*cat* and *floR*), tetracycline (*tetA*, *tet*(C), *tet*(E), *tet*(H), *tet*(G) and sulfamethoxazole (*sul1* and *sul2*) in *A. salmonicida* subsp. *salmonicida* were aligned to identify the conserved regions. The chloramphenicol resistance gene *catA2* found on the pAr-32 plasmid identified in a Japanese isolate (Aoki *et al*. 1986) was not included in the analysis due to the lack of an appropriate positive control. The alignment for each resistance gene sequence was performed using MUSCLE (Edgar 2004) and BLASTn (Altschul *et al*. 1997) through Geneious version 6.1.8 (Kearse *et al*. 2012). Primers were designed based on the conserved sequences using Primer3 (Untergasser *et al*. 2012) also through Geneious version 6.1.8.

The DNA templates were prepared by resuspending an inoculum of bacterial culture in 1 mL of SWL buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, and 0.45% Tween 20). The lysates were heated for 15 min at 95°C, and the DNA concentrations in the solutions were adjusted to 100 ng/µL. The PCR mixture contained 1X Go-Taq buffer (Promega, USA), 2 mM dNTP, 0.2 µM forward and reverse primers, 1 U of GoTaq (5 U; Promega), and 100 ng of DNA templates. Depending on the number of primers used for the multiplex PCR, various volumes of filtered water were added to adjust the final reaction volume to 20 µL. The PCR program was as follows: 2 min 30 s at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min 30 s at 68°C, followed by a final 5-min

extension step at 68°C. The PCR products were separated on 1.3% agarose gels and were stained with 0.5 µg/mL of ethidium bromide. For all the multiplex PCR, water and 01-B526, an antibiotic-sensitive isolate (Charette *et al*. 2012), were used as negative controls. The primers used for each multiplex PCR are given in Table 7.

In the first multiplex PCR, targeting *floR* and *cat*, a mix of the sequenced isolates M15879-11 and A449 (Reith *et al*. 2008; Vincent *et al*. 2014a) was used as a positive control. The *floR* gene gives resistance to both chloramphenicol and florfenicol while the *cat* gene provides resistance only to chloramphenicol (Mascaretti 2003). Bioinformatics analyses confirmed that the M15879-11 isolate bears a pSN254b plasmid similar to that of the 2004-05MF26 isolate and had the same set of antibiotic resistance genes (Vincent *et al*. 2014b, 2015). In the second multiplex PCR, developed to detect the *sul1* and *sul2* genes, the M15879-11 isolate was used as positive control. A subset of tetracycline resistance genes (*tetA* and *tet*(E)) was targeted by a third multiplex PCR. A mix of M15879-11 and A449 was used as a positive control for these genes. The last multiplex PCR assay aimed at detecting *tet*(C), *tet*(G) and *tet*(H). Two isolates were used as positive controls: 2009-144K3 (pAB5S9b and pRAS3.3) and M16474-11 (pAsa8).

Tableau 7. Primers used for the multiplex PCR.

Primer	Sequence 5'-3'	Tm (°C)	Amplicon size (bp)	Target
Multiplex PCR targeting genes coding for chloramphenicol/florfenicol resistance				
MT-ctrl-chloram-F1	GCTTACCTCAGATAATGAGTCGTC	54,8	172	Prophage 1 (Control)
MT-ctrl-chloram-R1	GCCAATAAGAGCCCTACTCTTC	55		
MT-cat-F1	CTATTTTGACAATACGCCCTGC	54,3	448	*cat*
MT-cat-R1	CTTCCCAAACGTAAATATCGGC	54		
MT-floR-F1	TTGAGCCTCTATATGGTGATGC	54,4	632	*floR*
MT-floR-R1	GTTGTCACGATCATTACAAGCG	54,3		
Multiplex PCR targeting genes coding for sulfonamide resistance				
MT-ctrl_sul-F1	TTCATTTCGTCTTGGGTCTAGC	54,8	175	Prophage 1 (Control)
MT-ctrl_sul-R1	GGACTACAGATCTACCATAATCCG	54		
MT-sul1-F1	GGGCTACCTGAACGATATCC	54,7	550	*sul1*
MT-sul1-R1	CTAGGCATGATCTAACCCTCG	54,4		
MT-sul2-F1	ATCATCTGCCAAACTCGTCG	55,2	449	*sul2*
MT-sul2-R1	TTCTTGCGGTTTCTTTCAGC	53,9		
First multiplex PCR targeting genes coding for tetracycline resistance				
MT-ctrltet1-F1	CCAGAATGACGAATTGAATGTCG	54,3	175	Prophage 1 (Control)
MT-ctrltet1-R1	GGACCTCTTTACTCCAGTCG	54,4		

MT-tetA(E)pAsa4-F1	GATGTCACACCTGAGGAATCC	55,1	351	*tetA*(E)
MT-tetA(E)pAsa4-R1	TCCGAATAAAACCCATAATGTTGC	53,9		
MT-tetApSN54b-F1	CAAGCAGGATGTAGCCTGTG	55,9	526	*tetA*
MT-tetApSN54b-R1	ATTGCCGATATCACTGATGG	52,4		
Second multiplex PCR targeting genes coding for tetracycline resistance				
MT-ctrltet2-F1	ATTCATTTCGTCTTGGGTCTAGC	55	176	Prophage 1 (Control)
MT-ctrltet2-R1	GGACTACAGATCTACCATAATCCG	54		
MT-tetHpAB5S9b-F1	ACGACTGTCTGATAAATACGGC	54,6	326	*tetH*
MT-tetHpAB5S9b-R1	ATATCGAGTGTGAAATAGCGGC	54,9		
MT-tetA(C)pRAS3-F1	CTGTAGGCATAGGCTTGGTTAT	54,4	629	*tetA*(C)
MT-tetA(C)pRAS3-R1	CTGTCCTACGAGTTGCATGATA	54,1		
MT-tetGs62-F1	GGTTCGCATCAAACCATTCG	54,8	460	tetA(G)
MT-tetGs62-R1	GCTTAGATTGGTGAGGCTCG	55,6		

DNA extraction and genomic sequencing

The total DNA of the M15879-11 and M16474-11 *A. salmonicida* subsp. *salmonicida* isolates was extracted using DNeasy Blood and Tissue kits (Qiagen, Canada) and was sequenced by next-generation sequencing (NGS) at the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval). The DNA of the M15879-11 isolate was fragmented at a 5-kb size and sequenced by pyrosequencing using a GS-FLX+ apparatus as previously described (Vincent *et al*. 2014a). The resulting sequencing reads were *de novo* assembled with Newbler version 2.5.3 (Margulies *et al*. 2005). The total draft assembly of M15879-11 was annotated by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and deposited in GenBank under accession number LAIS00000000.

The M16474-11 library was prepared using a KAPA Hyper Prep kit and was sequenced by a MiSeq (Illumina) sequencing system. The resulting reads were filtered with Trimmomatic version 0.32 (Bolger *et al*. 2014) using the manual-recommended parameters for paired-end reads. (Coil *et al*. 2015). The DNA of M16474-11 was also extracted by phenol/chloroform by following the protocol *Extracting DNA Using Phenol-Chloroform* provided by Pacific Biosciences (http://www.pacb.com). The DNA was sheared at 8-kb using a standard Covaris g-TUBE protocol and was used to prepare an Oxford nanopore technologies

sequencing library using the protocol GDE_1002_v1_revF_17Nov2015. The library was sequenced with a MinION sequencer. The basecalling was done by the Oxford nanopore technologies's Metrichor cloud service with the *2D Basecalling for SQK-MAP006* version 1.69 application. All the reads (1D and 2D) ≥ 750 bp were converted from the native HDF5/FAST5 format to FASTQ by poretools version 0.5.1 (Loman & Quinlan 2014). Finally, a hybrid assembly of both Illumina and Oxford nanopore technologies reads was performed using SPAdes version 3.7.1 (Bankevich *et al*. 2012) with kmer lengths of 21, 33, 55, 77, 99 and 127. The pAsa8 plasmid was recovered in a single contig. To assess the quality of the sequence, the Illumina reads were mapped on the pAsa8 sequence using BWA-MEM (Li & Durbin 2009) and evaluated with Pilon version 1.17 (Walker *et al*. 2014). No corrections occurred during step, reinforcing our confidence in the quality of the pAsa8 sequence. This sequence was annotated with the webserver RAST (Overbeek *et al*. 2014) and manually curated. The sequence was analyzed using TAfinder (TADB) to find putative type 2 toxin-antitoxin systems (Shao *et al*. 2011). The complete annotated sequence of pAsa8 was deposited in GenBank under the accession number KX364409.

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Accessions

The complete annotated sequences of M15879-11 and pAsa8 were deposited in GenBank under the accession numbers LAIS00000000 and KX364409, respectively.

Author's contributions

Conceived and designed the experiments: MVT, ATV, ND, SJC. Performed the experiments: MVT, ATV, SAA, ML. Analyzed the data: MVT, ATV, SAA, SJC. Contributed reagents/materials/analysis tools: ND, AIC, SJC. Wrote the paper: MVT, ATV, ND, AIC, SJC. All authors reviewed the manuscript.

Competing interests

The authors declare no competing financial interests.

References

Adams CA, Austin B, Meaden PG *et al.* Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl Environ Microbiol* 1998;**64**:4194-201.

Allmeier H, Cresnar B, Greck M *et al.* Complete nucleotide sequence of Tn*1721*: gene organization and a novel gene product with features of a chemotaxis protein. *Gene* 1992;**111**:11-20.

Altschul SF, Madden TL, Schaffer AA *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;**25**:3389-402.

Aoki T, Egusa S, Kimura T *et al.* Detection of R factors in naturally occurring *Aeromonas salmonicida* strains. *Appl Microbiol* 1971a;**22**:716-7.

Aoki T, Egusa S, Ogata Y *et al.* Detection of resistance factors in fish pathogen *Aeromonas liquefaciens*. *J Gen Microbiol* 1971b;**65**:343-9.

Aoki T, Mitoma Y, Crosa JH. The characterization of a conjugative R-plasmid isolated from *Aeromonas salmonicida*. *Plasmid* 1986;**16**:213-8.

Bankevich A, Nurk S, Antipov D *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012;**19**:455-77.

Bi S, Yan H, Chen M *et al.* New variant *Salmonella* genomic island 1-U in *Proteus mirabilis clinical* and food isolates from South China. *J Antimicrob Chemother* 2011;**66**:1178-9.

Biyela PT, Lin J, Bezuidenhout CC. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci Technol* 2004;**50**:45-50.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;**30**:2114-20.

Boyd DA, Peters GA, Ng L *et al.* Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica Typhymurium* DT104. *FEMS Microbiol Lett* 2000;**189**:285-91.

Call DR, Singer RS, Meng D *et al.* blaCMY-2-positive IncA/C plasmids from *Escherichia coli* and *Salmonella enterica* are a distinct component of a larger lineage of plasmids. *Antimicrob Agents Chemother* 2010;**54**:590-6.

Charette SJ, Brochu F, Boyle B *et al.* Draft genome sequence of the virulent strain 01-B526 of the fish pathogen *Aeromonas salmonicida*. *J Bacteriol* 2012;**194**:722-3.

Coil D, Jospin G, Darling AE. A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics* 2015;**31**:587-9.

Colston SM, Fullmer MS, Beka L *et al.* Bioinformatic genome comparisons for taxonomic and phylogenetic assignments using *Aeromonas* as a test case. *MBio* 2014;**5**:e02136.

Daher RK, Filion G, Tan SG *et al.* Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions. *Vet Microbiol* 2011;**152**:353-60.

Dallaire-Dufresne S, Tanaka KH, Trudel MV *et al.* Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* 2014;**169**:1-7.

Dixon BA. The biology of antibiotic resistance. *World Aquac* 2001;**32**:63-5.

Douard G, Praud K, Cloeckaert A *et al.* The *Salmonella* genomic island 1 is specifically mobilized in trans by the IncA/C multidrug resistance plasmid family. *PLoS One* 2010;**5**:e15302.

Doublet B, Boyd D, Douard G *et al.* Complete nucleotide sequence of the multidrug resistance IncA/C plasmid pR55 from *Klebsiella pneumoniae* isolated in 1969. *J Antimicrob Chemother* 2012;**67**:2354-60.

Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;**32**:1792-7.

Fernandez-Alarcon C, Singer RS, Johnson TJ. Comparative genomics of multidrug resistance-encoding IncA/C plasmids from commensal and pathogenic *Escherichia coli* from multiple animal sources. *PLoS One* 2011;**6**:e23415.

Fricke WF, Welch TJ, McDermott PF *et al.* Comparative genomics of the IncA/C multidrug resistance plasmid family. *J Bacteriol* 2009;**191**:4750-7.

Galardini M, Biondi EG, Bazzicalupo M *et al.* CONTIGuator: a bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol Med* 2011;**6**:11.

Gordon L, Cloeckaert A, Doublet B *et al.* Complete sequence of the *floR*-carrying multiresistance plasmid pAB5S9 from freshwater *Aeromonas bestiarum*. *J Antimicrob Chemother* 2008;**62**:65-71.

Haba E, Bouhdid S, Torrego-Solana N *et al.* Rhamnolipids as emulsifying agents for essential oil formulations: antimicrobial effect against *Candida albicans* and methicillin-resistant *Staphylococcus aureus*. *Int J Pharm* 2014;**476**:134-41.

Hatosy SM, Martiny AC. The ocean as a global reservoir of antibiotic resistance genes. *Appl Environ Microbiol* 2015;**81**:7593-9.

Hiney M, Olivier G. Furunculosis (*Aeromonas salmonicida*). In: Woo P, Bruno D (eds.) *Fish diseases and disorders, vol III: viral, bacterial and fungal infections*: CAB publishing Oxford, 1999,341–425.

Johnson TJ, Lang KS. IncA/C plasmids: An emerging threat to human and animal health? *Mob Genet Elements* 2012;**2**:55-8.

Kearse M, Moir R, Wilson A *et al.* Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012;**28**:1647-9.

Kim MJ, Hirono I, Kurokawa K *et al.* Complete DNA sequence and analysis of the transferable multiple-drug resistance plasmids (R plasmids) from *Photobacterium damselae* subsp. *piscicida* isolates collected in Japan and the United States. *Antimicrob Agents Chemother* 2008;**52**:606-11.

Kiss J, Nagy B, Olasz F. Stability, entrapment and variant formation of *Salmonella* genomic island 1. *PLoS One* 2012;**7**:e32497.

Kiss J, Papp PP, Szabo M *et al.* The master regulator of IncA/C plasmids is recognized by the *Salmonella* Genomic island *SGI1* as a signal for excision and conjugal transfer. *Nucleic Acids Res* 2015;**43**:8735-45.

Krylov VN. Bacteriophages of *Pseudomonas aeruginosa*: long-term prospects for use in phage therapy. *Adv Virus Res* 2014;**88**:227-78.

L'Abee-Lund TM, Sorum H. Functional Tn*5393*-like transposon in the R plasmid pRAS2 from the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida* isolated in Norway. *Appl Environ Microbiol* 2000;**66**:5533-5.

L'Abee-Lund TM, Sorum H. Class 1 integrons mediate antibiotic resistance in the fish pathogen *Aeromonas salmonicida* worldwide. *Microb Drug Resist* 2001;**7**:263-72.

L'Abee-Lund TM, Sorum H. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. *Plasmid* 2002;**47**:172-81.

Lei CW, Zhang AY, Liu BH *et al.* Molecular characteristics of *Salmonella* genomic island 1 in *Proteus mirabilis* isolates from poultry farms in China. *Antimicrob Agents Chemother* 2014;**58**:7570-2.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;**25**:1754-60.

Llewellyn MS, Boutin S, Hoseinifar SH *et al.* Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Front Microbiol* 2014;**5**:207.

Loman NJ, Quinlan AR. Poretools: a toolkit for analyzing nanopore sequence data. *Bioinformatics* 2014;**30**:3399-401.

Margulies M, Egholm M, Altman WE *et al.* Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005;**437**:376-80.

Mascaretti OA. *Bacteria versus antibacterial agents: an integrated approach.* Washington D.C.: ASM Press, 2003.

McIntosh D, Cunningham M, Ji B *et al.* Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *J Antimicrob Chemother* 2008;**61**:1221-8.

Michael GB, Hall R, Fanning S *et al.* Antimicrobial Resistance in *Salmonella*. In: Barrow PA, Methner U (eds.) *Salmonella in domestic animals*: 2n edition Edition, 2013, 120-35.

Morin R. L'utilisation des antibiotiques pour combattre la furonculose chez l'omble de fontaine génère de l'antibiorésistance chez *Aeromonas salmonicida*. *L'aquicole* 2010;**15**:2-6.

Mulvey MR, Boyd DA, Olson AB *et al.* The genetics of *Salmonella* genomic island 1. *Microbes Infect* 2006;**8**:1915-22.

O'Neill J. Tackling drug-resistant infections globally: final report and recommendations. *The review on antimicrobial resistance*, 2016.

Overbeek R, Olson R, Pusch GD *et al.* The SEED and the Rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res* 2014;**42**:D206-14.

Piotrowska M, Popowska M. Insight into the mobilome of *Aeromonas* strains. *Front Microbiol* 2015;**6**:494.

Qin S, Qi H, Zhang Q *et al.* Emergence of extensively drug-resistant *Proteus mirabilis* harboring a conjugative NDM-1 plasmid and a novel *Salmonella* genomic island 1 variant, SGI1-Z. *Antimicrob Agents Chemother* 2015;**59**:6601-4.

Reith ME, Singh RK, Curtis B *et al.* The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* 2008;**9**:427.

Robinson TP, Bu DP, Carrique-Mas J *et al.* Antibiotic resistance is the quintessential One Health issue. *Trans R Soc Trop Med Hyg* 2016;**110**:377-80.

Sandaa RA, Enger O. Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. *Appl Environ Microbiol* 1994;**60**:4234-8.

Shao Y, Harrison EM, Bi D *et al.* TADB: a web-based resource for type 2 toxin-antitoxin loci in bacteria and archaea. *Nucleic Acids Res* 2011;**39**:D606-11.

Siebor E, Neuwirth C. Emergence of *Salmonella* genomic island 1 (*SGI1*) among *Proteus mirabilis* clinical isolates in Dijon, France. *J Antimicrob Chemother* 2013;**68**:1750-6.

Sorum H, L'Abee-Lund TM, Solberg A *et al.* Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 2003;**47**:1285-90.

Takaya A, Watanabe M, Yamamoto T. Organization of Tn*2610* containing two transposition modules. *Antimicrob Agents Chemother* 2006;**50**:1143-7.

Trudel MV, Tanaka KH, Filion G *et al.* Insertion sequence *AS5* (IS*AS5*) is involved in the genomic plasticity of *Aeromonas salmonicida*. *Mob Genet Elements* 2013;**3**:e25640.

Uhland CF. Rapport des activités du Laboratoire d'Ichtyopathologie (1 avril 2004 – 31 mars 2005). In: ichtyopathologie Sdde (ed.). Saint-Hyacinthe (Québec), 2005, 16 pages.

Untergasser A, Cutcutache I, Koressaar T *et al.* Primer3--new capabilities and interfaces. *Nucleic Acids Res* 2012;**40**:e115.

Vincent AT, Boyle B, Derome N *et al.* Improvement in the DNA sequencing of genomes bearing long repeated elements. *J Microbiol Methods* 2014a;**107**:186-8.

Vincent AT, Emond-Rheault JG, Barbeau X *et al.* Antibiotic resistance due to an unusual ColE1-type replicon plasmid in *Aeromonas salmonicida*. *Microbiology* 2016;**162**:942-953

Vincent AT, Tanaka KH, Trudel MV *et al.* Draft genome sequences of two *Aeromonas salmonicida* subsp. *salmonicida* isolates harboring plasmids conferring antibiotic resistance. *FEMS Microbiol Lett* 2015;**362**:fnv002.

Vincent AT, Trudel MV, Paquet VE *et al.* Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug resistance, interspecies exchanges, and plasmid reshaping. *Antimicrob Agents Chemother* 2014b;**58**:7367-74.

Walker BJ, Abeel T, Shea T *et al.* Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 2014;**9**:e112963.

Welch TJ, Fricke WF, McDermott PF *et al.* Multiple antimicrobial resistance in plague: an emerging public health risk. *PLoS One* 2007;**2**:e309.

Wellington EM, Boxall AB, Cross P *et al.* The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect Dis* 2013;**13**:155-65.

Chapitre 4 – Article 3

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

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Résumé

Il est maintenant bien connu que la bactérie *A. salmonicida* subsp. *salmonicida* possède plusieurs plasmides avec des gènes de résistance aux antibiotiques. Dans cette étude, nous avons découvert et caractérisé un petit plasmide de 5,2 kb qui possède un gène *cat*, encodant pour une chloramphénicol acétyltransférase impliquée dans la résistance au chloramphénicol. Ce plasmide a la particularité d'être très similaire au petit plasmide cryptique pAsa2, qui est trouvé dans toutes les souches d'*A. salmonicida* subsp. *salmonicida*, mais qui ne possède aucune fonction connue. En fait, il est possible que pAsa7 soit dérivé de pAsa2 et soulève donc l'hypothèse que les petits plasmides cryptiques, comme pAsa2, puissent être des vecteurs modelables afin que la bactérie puisse acquérir de nouveaux gènes. De plus, nous avons démontré que pAsa7 est en haut nombre de copies, et par conséquent le gène *cat* également, ce qui se traduit par une haute résistance au chloramphénicol.

Abstract

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 nucleotides 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 to 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 plasmids.

Introduction

Mankind is now facing a worldwide concern: the rise of bacterial strains resistant to antibiotics (Reardon 2014). Human infections are not the only aspect of this problem; farming is also concerned. The aquaculture industry is negatively touched by superbug infections (Cabello *et al.* 2013; Labella *et al.* 2013). The bacterial fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* is the aetiological agent of furunculosis, a disease responsible for important economic losses (Dallaire-Dufresne *et al.* 2014a).

This bacterium has a notable plasmidome involved in antibiotic resistance in several isolates (Adams *et al.* 1998; L'Abée-Lund and Sørum 2002; Piotrowska and 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 kbp), pSN254b (152.2 kbp), pAB5S9b (25.5 kbp) and pAr-32 (~47 kb) are plasmids conferring 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 pAB5S9b, 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), pAsal1 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 ColE2-type replicons while pAsa2 is a ColE1-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 ColE1-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 at infecting fish (Beaz-Hidalgo & Figueras 2012; Dacanay *et al.* 2006; Dautremepuits *et al.* 2006).

Restriction fragment profiles

A plasmid miniprep kit (Feldan) was used, as recommended by the manufacturer, to extract <50 kbp 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 *Eco*RI (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* into 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 JYFG00000000. 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 ParaAT.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 over the one of *gyrB*. The same procedure was performed for the large plasmids pAsa4, pSN254b and pAB5S9b 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/indraniel/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) at 18 °C. This web server is known to perform accurate predictions of the ColE1-type RNA I and II (Kim *et al.* 2005). Moreover, the predicted structures were compared with the published one of the original ColE1 found experimentally (Tomizawa 1986). The structures were designed using varna version 3.9 (Darty *et al.* 2009). The pairing between RNA I and II (hug complex) for both

pAsa2 and pAsa7 was predicted using free2bind (http://sourceforge.net/projects/free2bind/) at 18 °C.

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+Γ 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 8.1.17 (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 ggtree 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 MOE (Chemical Computing Group) for homology modelling with default parameters generating 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 TEXShade (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 furunculosis agar at 18 °C for 48 to 72 h. Several colonies of each isolate were suspended in fresh LB medium (EMD 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 (10^6 to 10^1 copy number). 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 OD_{595}. 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 rRNA 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 for RT-qPCR (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 pAsal1) (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 plasmidic-DNA isolation, digestion by the *Eco*RI 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 *Eco*RI restriction site and produces fragments of different sizes, except for pAsa1 and pAsa2, to be resolved by a standard electrophoresis (pAsal1, 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 (Figure 19).

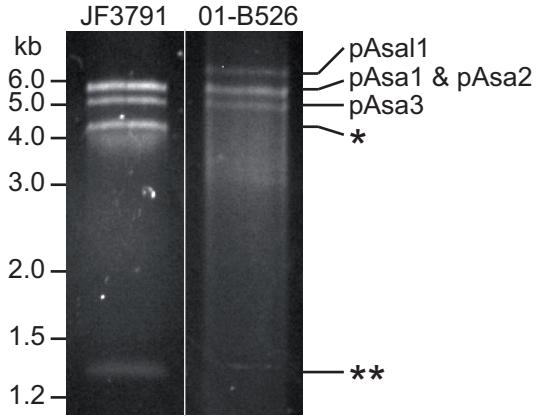

Figure 19. Plasmid profiles of isolates 01-B526 and JF3791.
Isolate 01-B526 exhibits the standard plasmid profile: pAsa1, pAsa2, pAsa3 and pAsal1, while JF3791 does not bear the pAsal1 plasmid and shows two unusual bands (* and **).

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, pAB5S9b, pSN254b or pAr-32 (Reith *et al.* 2008; Sørum *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 (Figure 20).

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 *Eco*RI sites, compared with one for pAsa2 (Figure 20), thus explaining the bands at ~1.3 and ~4.0 kbp in the plasmidic profile (Figure 19).

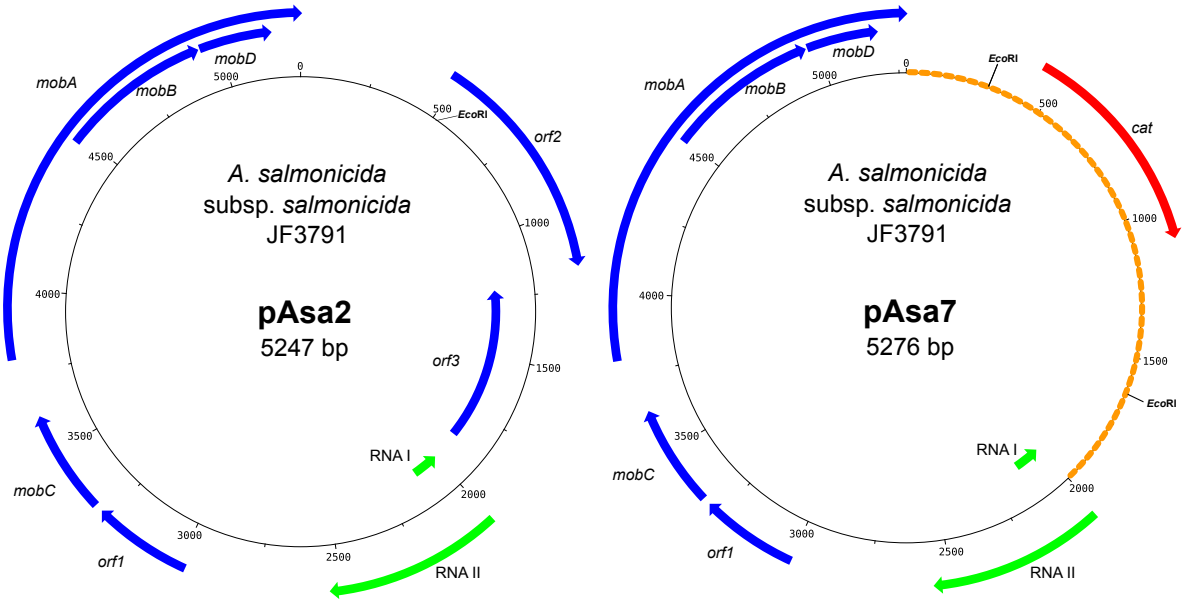

Figure 20. Maps of pAsa2 and pAsa7 from the Swiss strain JF3791.
The arrows in blue, green and red represent, respectively, the genes encoding proteins, regulator RNAs and a protein causing CHL resistance. The orange dotted line on the pAsa7 map represents the section of this plasmid that is divergent compared with pAsa2. The *Eco*RI sites are indicated on each map. Each tick mark represents a 250 bp step.

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 (Figure 20). 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 8). The analyses showed that only the *mobB* gene might have a bias to gain non-synonymous substitutions.

Tableau 8. Mutational comparison between gene sequences of pAsa2 and pAsa7

Gene	S*	NS†	NS/S	(S+NS)/length‡
orf1	13.00	7.00	0.54	0.07
mobC	16.00	8.00	0.50	0.08
mobA	35.00	39.00	1.11	0.05
mobB	8.00	19.00	2.37	0.06
mobD	6.00	6.00	1.00	0.05

*: Synonymous substitution
†: Nonsynonymous substitution
‡: The value used corresponds to the alignment length.

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 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 (Figure 21). 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 9). 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 pAB5S9b known to bear a *floR* gene (Vincent *et al.* 2015), were also used to have a more complete panel of CHL resistance genes.

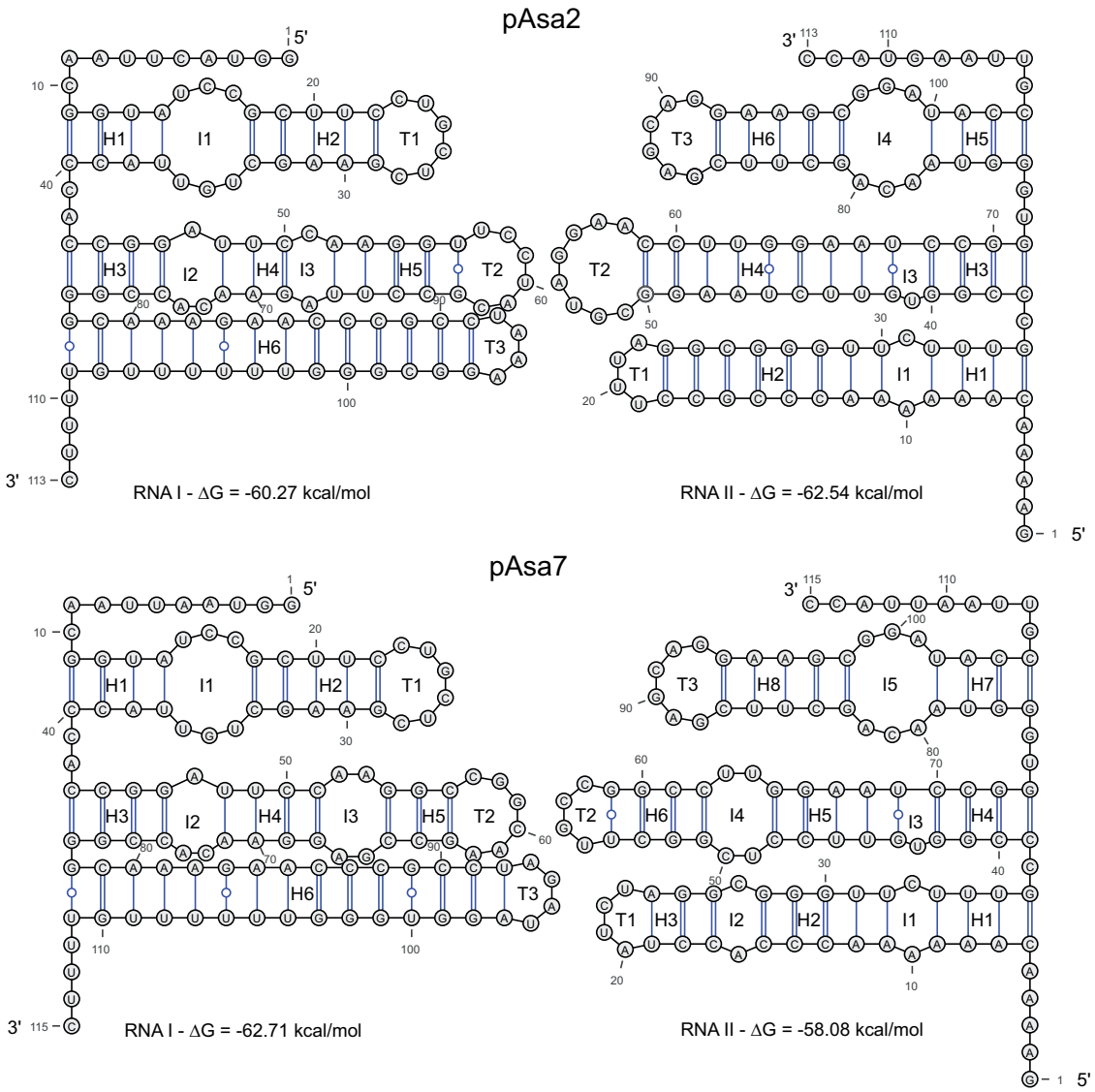

Figure 21. Predicted RNA I and RNA II secondary structures for pAsa2 and pAsa7.
The nomenclature used is the one of Leontis & Westhof (2001). The 'H', 'I' and 'T' mean, respectively, 'Helix', 'Internal loop' and 'Terminal loop'. The change in free energy (ΔG) required for the two RNA sequences to hybridize in kilocalories per mole (kcal mol −1, where 1 kcal=4184 J) is given for each RNA. For clarity purposes, only the sequence corresponding to the RNA I is shown for the RNA II of pAsa2 and pAsa7.

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 9). 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 9). 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).

Tableau 9. MIC of chloramphenicol for some known *A. salmonicida* subsp. *salmonicida* isolates

Strain	MIC (µg/ml)	Plasmid	Resistance gene
01-B526	2	N/A*	N/A
JF3224	2	N/A	N/A
2004-05MF26	64	pSN254b	*floR*
2009-144K3	96	pAB5S9b	*floR*
01-B526+pAsa7	128	pAsa7	*cat*-pAsa7
JF3224+pAsa7	128	pAsa7	*cat*-pAsa7
JF3791	192	pAsa7	*cat*-pAsa7
RS 534	> 384	pAsa4	*cat*-pAsa4
A449	> 384	pAsa4	*cat*-pAsa4

*: N/A means none-applicable

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 pAB5S9b 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 pAB5S9b and pSN254b, meaning that the promoter regions are also the same. An investigation using the amount of sequencing reads showed that pSN254b and pAB5S9b 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 pAB5S9b 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 (Figure 22) ($P<0.01$ 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*.

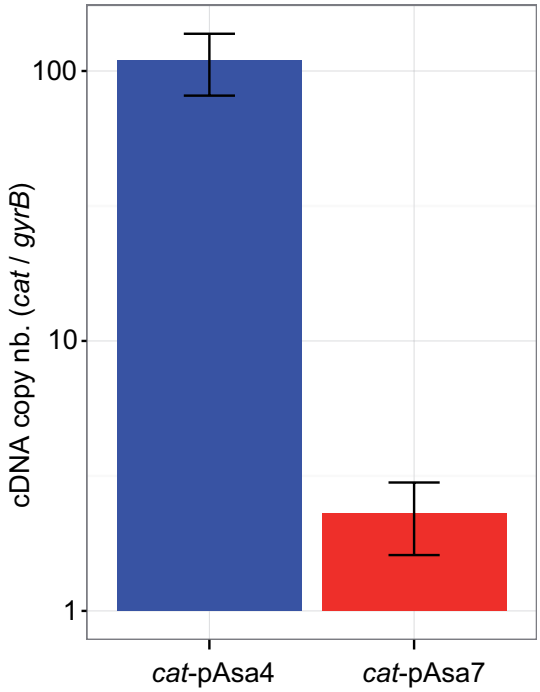

Figure 22. Fold-difference in the expression level of *cat*-pAsa4 and *cat*-pAsa7 over that of *gyrB*. The error bars correspond to the standard deviation.

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 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 (Figure 23). 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 (Figure 23). 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.

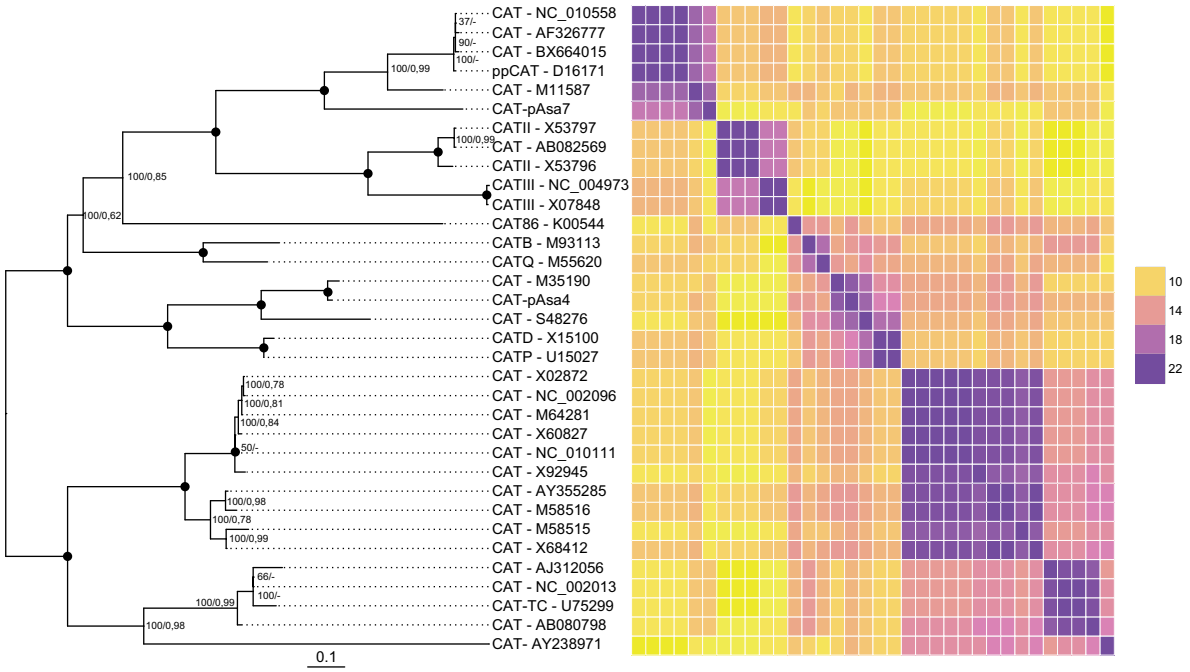

Figure 23. Molecular phylogeny of 34 CAT sequences coupled with an identity matrix of 22 residues of the catalytic site.

On the left, the molecular phylogeny of 34 CAT sequences is presented. The statistical values (bootstrap/posterior probability) are indicated at each node. Dotted nodes indicate that the statistical values were optimal (100/1.00). The accession numbers corresponding to the CARD database (McArthur *et al*. 2013) are indicated for each sequence. On the right, a pairwise identity matrix comparing 22 residues in the catalytic site for the 34 cat sequences is shown. The matrix is coloured according to the level of identity for the pairwise alignment of the 22 residues in the catalytic site. Identical and diverging catalytic site residue compositions are coloured from purple to yellow respectively.

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 9), 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 Figure 24. The CHL substrate is bound mostly through similar hydrophobic contacts and by two polar interactions. However, as shown in Figure 24, 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.

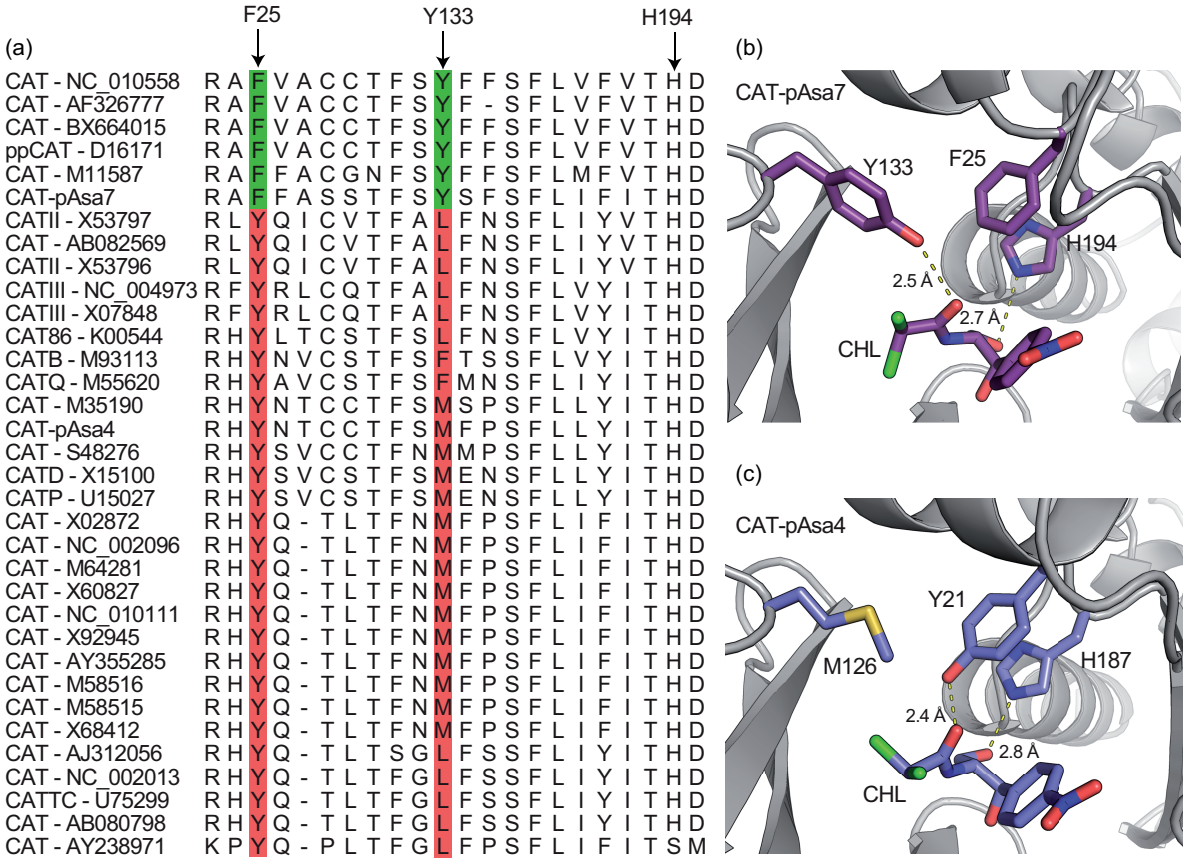

Figure 24. Divergent substrate binding between CAT-pAsa7 and CAT-pAsa4.
Multiple sequence alignment for 22 important residues in the active site (a) (Biswas *et al*. 2012). Structural representations of CAT-pAsa7 (b) and CAT-pAsa4 (c) are in grey cartoon. Residues involved in the structural reorganization 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).

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 (Attéré *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 (Attéré *et al.* 2015). The same study also reported that pAsa2 plasmids are very stable with especially few mutations between their sequences (Attéré *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 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 (Figure 21). 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 9). This unsuspected 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 pAsal1 and pAsal1 to pAsal1-B-C-D, 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 *Aeromonas hydrophila* and *Aeromonas sobria* (Han *et al.*, 2012a,b; Piotrowska and 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).

Acknowledgments

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Abbreviations

A. salmonicida, *Aeromonas salmonicida*; CHL, chloramphenicol; CAT, chloramphenicol acetyltransferase; MIC, minimum inhibitory concentration; NGS, next-generation sequencing; OD, optical density; TTSS, type three secretion system

References

Adams C, Austin B, Meaden P *et al.* Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl Environ Microbiol* 1998;**64**:4194–201.

Aoki T, Mitoma Y, Crosa JH. The characterization of a conjugative R-plasmid isolated from *Aeromonas salmonicida*. *Plasmid* 1986;**16**:213–8.

Attéré SA, Vincent AT, Trudel MV *et al.* Diversity and homogeneity among small plasmids of *Aeromonas salmonicida* subsp. *salmonicida* linked with geographical origin. *Front Microbiol* 2015;**6**:1274.

Beaz-Hidalgo R, Figueras MJ. Molecular detection and characterization of furunculosis and other *Aeromonas* fish infections. In: Carvalho E (ed.). *Health and Environment in Aquaculture*. Intech, 2012, 97–132.

Beitz E. TEXshade: shading and labeling of multiple sequence alignments using LATEX2 epsilon. *Bioinformatics* 2000;**16**:135–9.

Belland RJ, Trust TJ. Cloning of the gene for the surface array protein of *Aeromonas salmonicida* and evidence linking loss of expression with genetic deletion. *J Bacteriol* 1987;**169**:4086–91.

Belland RJ, Trust TJ. *Aeromonas salmonicida* plasmids: Plasmid-directed synthesis of proteins in vitro and in *Escherichia coli* minicells. *Microbiology* 1989;**135**:513–24.

Biswas T, Houghton JL, Garneau-Tsodikova S *et al.* The structural basis for substrate versatility of chloramphenicol acetyltransferase CATI. *Protein Sci* 2012;**21**:520–30.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;**30**:2114–20.

Boyd J, Williams J, Curtis B *et al.* Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. *Plasmid* 2003;**50**:131–44.

Burr SE, Frey J. Analysis of type III effector genes in typical and atypical *Aeromonas salmonicida*. *J Fish Dis* 2007;**30**:711–4.

Burr SE, Pugovkin D, Wahli T *et al.* Attenuated virulence of an *Aeromonas salmonicida* subsp. *salmonicida* type III secretion mutant in a rainbow trout model. *Microbiology* 2005;**151**:2111–8.

Cabello FC, Godfrey HP, Tomova A *et al.* Antimicrobial use in aquaculture re-examined: Its relevance to antimicrobial resistance and to animal and human health. *Environ Microbiol* 2013;**15**:1917–42.

Camps M. Modulation of ColE1-like plasmid replication for recombinant gene expression. *Recent Pat DNA Gene Seq* 2010;**4**:58–73.

Charette SJ, Brochu F, Boyle B *et al.* Draft genome sequence of the virulent strain 01-B526 of the fish pathogen *Aeromonas salmonicida* . *J Bacteriol* 2012;**194**:722–3.

Chen CY, Lindsey RL, Strobaugh TP *et al.* Prevalence of ColE1-like plasmids and kanamycin resistance genes in *Salmonella enterica* serovars. *Appl Environ Microbiol* 2010;**76**:6707–14.

Coil D, Jospin G, Darling AE. A5-miseq : an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics* 2015;**31**:587-9.

Dacanay A, Knickle L, Solanky KS *et al.* Contribution of the type III secretion system (TTSS) to virulence of *Aeromonas salmonicida* subsp. *salmonicida*. *Microbiology* 2006;**152**:1847–56.

Dallaire-Dufresne S, Emond-Rheault J-G, Attéré SA *et al.* Optimization of a plasmid

electroporation protocol for *Aeromonas salmonicida* subsp. *salmonicida*. *J Microbiol Methods* 2014a;**98**:44–9.

Dallaire-Dufresne S, Tanaka KH, Trudel MV *et al.* Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* 2014a;**169**:1–7.

Darriba D, Taboada GL, Doallo R *et al.* ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics* 2011;**27**:1164–1165.

Darty K, Denise A, Ponty Y. VARNA: Interactive drawing and editing of the RNA secondary structure. *Bioinformatics* 2009;**25**:1974–5.

Dautremepuits C, Fortier M, Croisetiere S *et al.* Modulation of juvenile brook trout (*Salvelinus fontinalis*) cellular immune system after *Aeromonas salmonicida* challenge. *Vet Immunol Immunopathol* 2006;**110**:27–36.

Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;**32**:1792–7.

Fehr D, Casanova C, Liverman A *et al.* AopP, a type III effector protein of *Aeromonas salmonicida*, inhibits the NF-kappaB signalling pathway. *Microbiology* 2006;**152**:2809–18.

García-Alcalde F, Okonechnikov K, Carbonell J *et al.* Qualimap: Evaluating next-generation sequencing alignment data. *Bioinformatics* 2012;**28**:2678–9.

Han JE, Kim JH, Choresca CH *et al.* Prevalence of *tet* gene and complete genome sequencing of *tet* gene-encoded plasmid (pAHH01) isolated from *Aeromonas species* in South Korea. *J Appl Microbiol* 2012a;**112**:631–8.

Han JE, Kim JH, Choresca JH *et al.* First description of ColE-type plasmid in *Aeromonas* spp. carrying quinolone resistance (*qnrS2*) gene. *Lett Appl Microbiol* 2012b;**55**:290–4.

Hänninen ML, Hirvelä-Koski V. Molecular and phenotypic methods for the

characterization of atypical *Aeromonas salmonicida*. *Vet Microbiol* 1997;**56**:147–58.

Kim D, Rhee Y, Rhodes D *et al.* Directed evolution and identification of control regions of ColE1 plasmid replication origins using only nucleotide deletions. *J Mol Biol* 2005;**351**:763–75.

L'Abée-Lund TM, Sørum H. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. *Plasmid* 2002;**47**:172–81.

Labella A, Gennari M, Ghidini V *et al.* High incidence of antibiotic multi-resistant bacteria in coastal areas dedicated to fish farming. *Mar Pollut Bull* 2013;**70**:197–203.

Lartillot N, Lepage T, Blanquart S. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* 2009;**25**:2286–8.

Leontis NB, Stombaugh J, Westhof E. The non-Watson-Crick base pairs and their associated isostericity matrices. *Nucleic Acids Res* 2002;**30**:3497–531.

Leontis NB, Westhof E. Geometric nomenclature and classification of RNA base pairs. *RNA* 2001;**7**:499–512.

Leslie AG. Refined crystal structure of type III chloramphenicol acetyltransferase at 1.75 A resolution. *J Mol Biol* 1990;**213**:167–86.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;**25**:1754–60.

Li H, Handsaker B, Wysoker A *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* 2009;**25**:2078–9.

Lin-Chao S, Chen WT, Wong TT. High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. *Mol Microbiol* 1992;**6**:3385–93.

McArthur AG, Waglechner N, Nizam F *et al.* The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother* 2013;**57**:3348–57.

Meyer R. Functional organization of MobB, a small protein required for efficient conjugal transfer of plasmid R1162. *J Bacteriol* 2011;**193**:3904–11.

Murray IA, Lewendon A, Williams JA *et al.* Alternative binding modes for chloramphenicol and 1-substituted chloramphenicol analogues revealed by site-directed mutagenesis and X-ray crystallography of chloramphenicol acetyltransferase. *Biochemistry* 1991;**30**:3763–70.

Piotrowska M, Popowska M. Insight into the mobilome of *Aeromonas* strains. *Front Microbiol* 2015;**6**:494.

Reardon S. Antibiotic resistance sweeping developing world. *Nat News* 2014;**509**:141–2.

Reith ME, Singh RK, Curtis B *et al.* The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* 2008;**9**:427.

Rutherford K, Parkhill J, Crook J *et al.* Artemis: sequence visualization and annotation. *Bioinformatics* 2000;**16**:944–5.

Schmieder R, Lim YW, Rohwer F *et al.* TagCleaner: Identification and removal of tag sequences from genomic and metagenomic datasets. *BMC Bioinformatics* 2010;**11**:341.

Schrödinger L. The PyMOL Molecular Graphics System. 2017.

Sørum H, L'Abée-Lund TM, Solberg A *et al.* Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 2003;**47**:1285–90.

Stamatakis A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;**30**:1312–3.

Team RC. R: A language and environment for statistical computing. *R Found Stat Comput Vienna, Austria* 2013.

Tomizawa J-I. Control of ColE1 plasmid replication: Binding of RNA I to RNA II and inhibition of primer formation. *Cell* 1986;**47**:89–97.

Tomizawa J. Control of ColE1 plasmid replication: The process of binding of RNA I to the primer transcript. *Cell* 1984;**38**:861–70.

Chemical Computing Group. Molecular Operating Environment (MOE). 2017. 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7

Vendeix FAP, Munoz AM, Agris PF. Free energy calculation of modified base-pair formation in explicit solvent: A predictive model. *RNA* 2009;**15**:2278–87.

Vincent AT, Tanaka KH, Trudel M V *et al.* Draft genome sequences of two *Aeromonas salmonicida* subsp. *salmonicida* isolates harboring plasmids conferring antibiotic resistance. *FEMS Microbiol Lett* 2015;**362**:1–4.

Vincent AT, Trudel MV, Freschi L *et al.* Increasing genomic diversity and evidence of constrained lifestyle evolution due to insertion sequences in *Aeromonas salmonicida*. *BMC Genomics* 2016;**17**:1–12.

Vincent AT, Trudel M V, Paquet VE *et al.* Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug-resistance, interspecies exchanges, and plasmid reshaping. *Antimicrob Agents Chemother* 2014;**58**:7367–74.

Wang Z, Yuan Z, Xiang L *et al.* tRNA-dependent cleavage of the ColE1 plasmid-encoded RNA I. *Microbiology* 2006;**152**:3467–76.

Wróbel B, Wegrzyn G. Replication regulation of ColE1-like plasmids in amino acid-starved *Escherichia coli*. *Plasmid* 1998;**39**:48–62.

Wu YC, Liu ST. A sequence that affects the copy number and stability of pSW200 and ColE1. *J Bacteriol* 2010;**192**:3654–60.

Xia X. DAMBE5: A comprehensive software package for data analysis in molecular

biology and evolution. *Mol Biol Evol* 2013;**30**:1720–8.

Zhang Z, Xiao J, Wu J *et al.* ParaAT: A parallel tool for constructing multiple protein-coding DNA alignments. *Biochem Biophys Res Commun* 2012;**419**:779–81.

Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;**31**:3406–15.

Chapitre 5 – Article 4

Increasing genomic diversity and evidence of constrained lifestyle evolution due to insertion sequences in *Aeromonas salmonicida*

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Running title: Lifestyle dichotomy of *A. salmonicida*

Résumé

La bactérie *A. salmonicida* possède cinq sous-espèces, dont quatre connues comme étant psychrophiles et une seule, *pectinolytica*, comme étant mésophile, et donc avec la capacité de croître à 37°C. Le fait que des sous-espèces d'une même bactérie aient des modes de vie aussi différents était une opportunité d'étudier les déterminants pouvant expliquer cette divergence évolutive. Une investigation génomique de toutes les sous-espèces d'*A. salmonicida* connues ainsi que de trois nouvelles souches mésophiles a permis de mettre en évidence que les séquences d'insertion (IS) pourraient aider à expliquer pourquoi certaines souches poussent à 37°C alors que d'autres en sont incapables. Ces éléments d'ADN mobiles sont connus comme étant impliqués dans des réarrangements génomiques majeurs chez les souches d'*A. salmonicida* subsp. *salmonicida* lorsque celles-ci sont cultivées à des températures avoisinant 25°C. Nous avons trouvé que les souches psychrophiles possèdent plus d'IS et surtout qu'elles possèdent celles causant des réarrangements.

Abstract

Background: Aeromonads make up a group of Gram-negative bacteria that includes human and fish pathogens. The *Aeromonas salmonicida* species has the peculiarity of including five known subspecies. However, few studies of the genomes of *A. salmonicida* subspecies have been reported to date.

Results: We sequenced the genomes of additional *A. salmonicida* isolates, including three from India, using next-generation sequencing in order to gain a better understanding of the genomic and phylogenetic links between *A. salmonicida* subspecies. Their relative phylogenetic positions were confirmed by a core genome phylogeny based on 1645 gene sequences. The Indian isolates, which formed a sub-group together with *A. salmonicida* subsp. *pectinolytica*, were able to grow at either at 18°C and 37°C, unlike the *A. salmonicida* psychrophilic isolates that did not grow at 37°C. Amino acid frequencies, GC content, tRNA composition, loss and gain of genes during evolution, pseudogenes as well as genes under positive selection and the mobilome were studied to explain this intraspecies dichotomy.

Conclusion: Insertion sequences appeared to be an important driving force that locked the psychrophilic strains into their particular lifestyle in order to conserve their genomic integrity. This observation, based on comparative genomics, is in agreement with previous results showing that insertion sequence mobility induced by heat in *A. salmonicida* subspecies causes genomic plasticity, resulting in a deleterious effect on the virulence of the bacterium. We provide a proof-of-concept that selfish DNAs play a major role in the evolution of bacterial species by modeling genomes.

Keywords: *Aeromonas salmonicida*; phylogeny; mesophilic; psychrophilic; insertion sequence

Background

The *Aeromonas* genus (also known as aeromonads) has a complex taxonomy, with fourteen species officially recognized in the latest edition of the *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan & Joseph 2005). However, recent works like the one of Colston *et al*. in 2014 showed, based on molecular phylogenies (house-keeping genes and core genomes), average nucleotide identities (ANI), and *in silico* DNA-DNA hybridization (*is*DDH), that aeromonads have a greater taxonomic richness (Colston *et al.* 2014).

Aeromonads can be also divided into two groups based on their ability or inability to grow at a mesophilic temperature (37°C) (Palumbo *et al.* 1985; Park & Ha 2014; Percival & Williams 2014). The best-known representatives of the mesophilic group are *A. hydrophila*, *A. caviae*, and *A. veronii*, all of which are human pathogens (Palumbo *et al.* 1985; Percival & Williams 2014). The psychrophilic group (constituted by species that cannot grow at 37°C) includes *A. salmonicida*, which can be further divided into five subspecies: *salmonicida*, *smithia*, *achromogenes*, and *masoucida* (Percival & Williams 2014) as well as *pectinolytica*, which is an exception to the rule since it grows well at 37°C and is considered to be mesophilic (Pavan *et al.* 2000).

A. salmonicida is of particular interest since this aeromonad species is known to include fish pathogens causing important economical loss worldwide (Janda & Abbott 2010; Dallaire-Dufresne *et al.* 2014). The genomes of all the subspecies have been sequenced and have been deposited in public databases such as GenBank or Sequence Read Archive (SRA). The sequenced isolates come from geographical origins as diverse as Argentina, Chile, Canada, the United Kingdom, France, Switzerland, Korea, and Japan. However, among these isolates only the genome of *A. salmonicida* subsp. *salmonicida* A449 was completely sequenced, assembled and annotated (Reith *et al.* 2008). A striking result of this study was the discovery of 10 types of insertion sequences (ISs), which were present in 102 copies (88 complete and 14 partial), compared to the genome of *A. hydrophila*, which contained no ISs (Seshadri *et al.* 2006).

Recently, *A. salmonicida* isolates have also been obtained in India from food samples at a local market (Nagar *et al.* 2013). The origin of these *A. salmonicida* isolates (i.e., not

directly from water or diseased fish) in a tropical climate is very intriguing in terms of the spread of *A. salmonicida* species. With the exception of the *pectinolytica* subsp., which was isolated from a polluted river in Argentina (Pavan *et al.* 2000), all the other *A. salmonicida* subsp. were isolated from infected hosts living in cold water (Reith *et al.* 2008; Charette *et al.* 2012; Roger *et al.* 2012; Han *et al.* 2013; Emond-Rheault *et al.* 2015a; Valdes *et al.* 2015; Vincent *et al.* 2015).

We sequenced the genomes and examined the genomic elements of the Indian isolates in order to gain a better understanding of the genome architecture and the wide diversity of *A. salmonicida* species. Consequently, we performed an optimized core-genome phylogeny of the aeromonads including previously sequenced *A. salmonicida* genomes as well as genomes sequenced specifically for the present study. A comparison of these genomes revealed that among aeromonads, *A. salmonicida* subspecies are unusually diverse. It also uncovered at least one new mesophilic subspecies and showed that *A. salmonicida* was evolving from a mesophilic to a psychrophilic lifestyle due to the loss of its ability to proliferate in mesophilic environments. We propose that insertion sequences are an important driving force, which may have locked some *A. salmonicida* subspecies into a psychrophilic lifestyle in order to conserve their genomic integrity.

Results and Discussion

Sequencing new aeromonad genomes

To shed light on the evolution and diversity of *A. salmonicida* species, we first used next-generation sequencing (NGS) to sequence the complete genomes of five additional isolates, including one member of the subspecies *smithia* (JF4097), one of the subspecies *salmonicida* (RS 534), and three isolates from India with an unclear taxonomy (Y47, Y567, and Y577) (Nagar *et al.* 2013). The results of the *de novo* assembly for the five isolates are presented in Additional file 1.

Phylogenetic analysis of *A. salmonicida*

A robust optimized molecular phylogeny of 43 aeromonads was inferred from 1645 gene sequences (see Additional file 1 and 2) to study the taxonomy of this genus and more specifically the *salmonicida* species. Since our study mainly focused on *A. salmonicida*, we

were surprised to see that according to our phylogeny, *A. salmonicida* CBA100, a recently deposited Chilean isolate (Valdes *et al.* 2015), was phylogenetically closer to *A. bestiarum* than to *A. salmonicida* (see Additional file 1). To verify the relatedness of the CBA100 isolate and *A. bestiarum*, the average nucleotide identity (ANI) values were computed for some key taxa (see Additional file 1). The ANI values were in agreement with the molecular phylogeny since the ANI value of the CBA100 isolate and *A. bestiarum* was above 96%, meaning that the CBA100 isolate likely belong to the *A. bestiarum* species and not *A. salmonicida* as initially proposed (Valdes *et al.* 2015). A recent study based on *in silico* DNA-DNA hybridization (*is*DDH) also suggested that CBA100 isolate is a member of the *A. bestiarum* group (Pavan *et al.* 2015).

The Indian isolates (Y47, Y567, and Y577) had basal positions among the *salmonicida* species (Figure 25) and the ANI analysis confirmed that these taxa are *salmonicida* species (see Additional file 1). Isolate Y577 shared a clade with *A. salmonicida* subsp. *pectinolytica* while isolates Y47 and Y567 formed a basal clade to the *masoucida* subspecies. The subspecies *smithia* formed a clade with the subspecies *achromogenes* while the subspecies *salmonicida* RS 534 strain clustered among the other isolates of this subspecies (Figure 25).

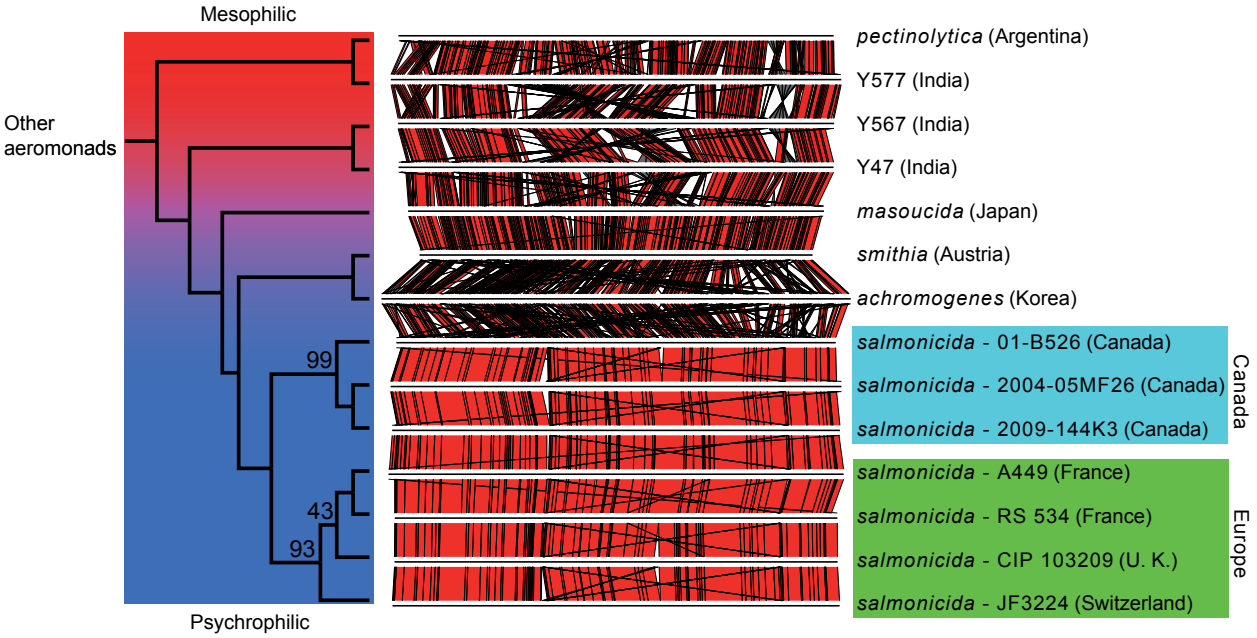

Figure 25. View of the *salmonicida* species section of the phylogenetic tree with proportional branch lengths.

See Additional file 1 for the complete tree for all the aeromonads. Putative chromosome sequences were constructed for all the taxa and were compared based on their phylogenetic positions. The red alignments show identity between direct sequences while the gray ones show between inverted sequences. Only the subspecies of each *A. salmonicida* strain are indicated on the right. The geographic provenance of each taxon is indicated in brackets. Only bootstrap values below 100 are shown. The gradient from red to blue represents the ability of each taxon to grow at a mesophilic temperature (red) or at a strict psychrophilic one (blue).

The complete taxon sampling of the *salmonicida* subspecies coupled with the high level of accuracy engendered by the high number of markers (696,249 positions and 420,006 alignment patterns) of our molecular phylogenetic analysis clustered the European and the Canadian isolates independently (Figure 25). It is interesting to note that the bootstrap value at the node corresponding to the split between the European and the Canadian isolates is 100, which suggests an important statistical robustness. Previous studies using low resolution approaches such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) have indicated that *salmonicida* subspecies strains are genetically homogeneous (Belland & Trust 1988; Hänninen *et al.* 1995; García *et al.* 2000; O'hIci *et al.* 2000) and with a clonal population structure (McCormick *et al.* 1990; Umelo & Trust 1998). Another study, based on the sequence of the gene *tapA*, proposed that there was no difference between the European isolates and those found in North America (Nilsson *et al.* 2006). However, recent studies have shown that isolates from different geographical regions may bear specific variants of the *AsaGEI* genomic island and that *AsaGEI* can be used to track the geographical provenance of *salmonicida* isolates (Emond-Rheault *et al.* 2015a, 2015b). The present study confirms that there are differences between European and Canadian isolates at the molecular level. On the other hand, the branch lengths were small (see Additional file 1), meaning that the dichotomy between the European and Canadian isolates is a relatively recent event and/or that the European and Canadian isolates have a similar mutation rate. Additional *salmonicida* isolates from other regions need to be analyzed to determine whether they also cluster differently.

Mesophilic/psychrophilic dichotomy

Since *A. salmonicida* subsp. *pectinolytica* is a mesophilic strain and is closely related to the Indian isolates (Figure 25), we grew the Indian isolates (Y47, Y567 and Y577) at 18°C and 37°C to clarify whether they were mesophilic as well. They grew very well at 37°C and could thus be considered as mesophilic (Figure 26A). *A. salmonicida* subsp. *masoucida*, which is considered a psychrophilic subspecies (Percival & Williams 2014), tolerated and

even grew at 37°C albeit slowly and to a lower density before declining. This capacity of *A. salmonicida* subsp. *masoucida* to grow moderately at 37°C was also reported in a previous large-scale phenotypic study without, however, being clearly mentioned (Austin *et al.* 1989). This is a key observation given that *A. salmonicida* subsp. *masoucida* is positioned in the molecular phylogeny directly after the mesophilic clades and shares the same basal node as the psychrophilic clades (Figure 25).

The 18°C temperature has been chosen for additional growth tests since it is at this temperature that psychrophilic *A. salmonicida* subsp. *salmonicida* strains are usually the more efficient to infect fish (Dacanay *et al.* 2006; Dautremepuits *et al.* 2006; Beaz-Hidalgo & Figueras 2012). All the tested isolates, including the mesophiles, grew well at 18°C (Figure 26B). Even if 18°C is the most efficient temperature for *A. salmonicida* to infect fish, we have also tested the growth of these isolates at 7°C. The growth curves showed the same patterns than at 18°C, with the mesophilic strains growing more efficiently than the psychrophilic ones (see Additional file 1). Interestingly, the psychrophilic isolates had a lower growth rate capacity than the mesophilic ones at psychrophilic temperatures. This suggests that the psychrophilic isolates did not gain the ability to be psychrophilic but, in fact, experienced an alteration of their overall physiology leading to the loss of their capacity to grow in a mesophilic environment. In addition, the growth profiles of the *pectinolytica* subspecies and Y577 were different, which indicates, in agreement with the overall genomic organization (Figure 25), that they may belong to different subspecies with a near common ancestor. This is also possible for Y47 and Y567 since they do not present the same growth profile.

Interestingly, the isolate representing the *smithia* subspecies grew minimally at 18°C and not at all at 37°C (Figure 26). According to the *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan & Joseph 2005), the requirements of this subspecies are not different than those of the other *salmonicida* subspecies. However, we cannot rule out the possibility that the *smithia* subspecies has specific unknown growth requirements.

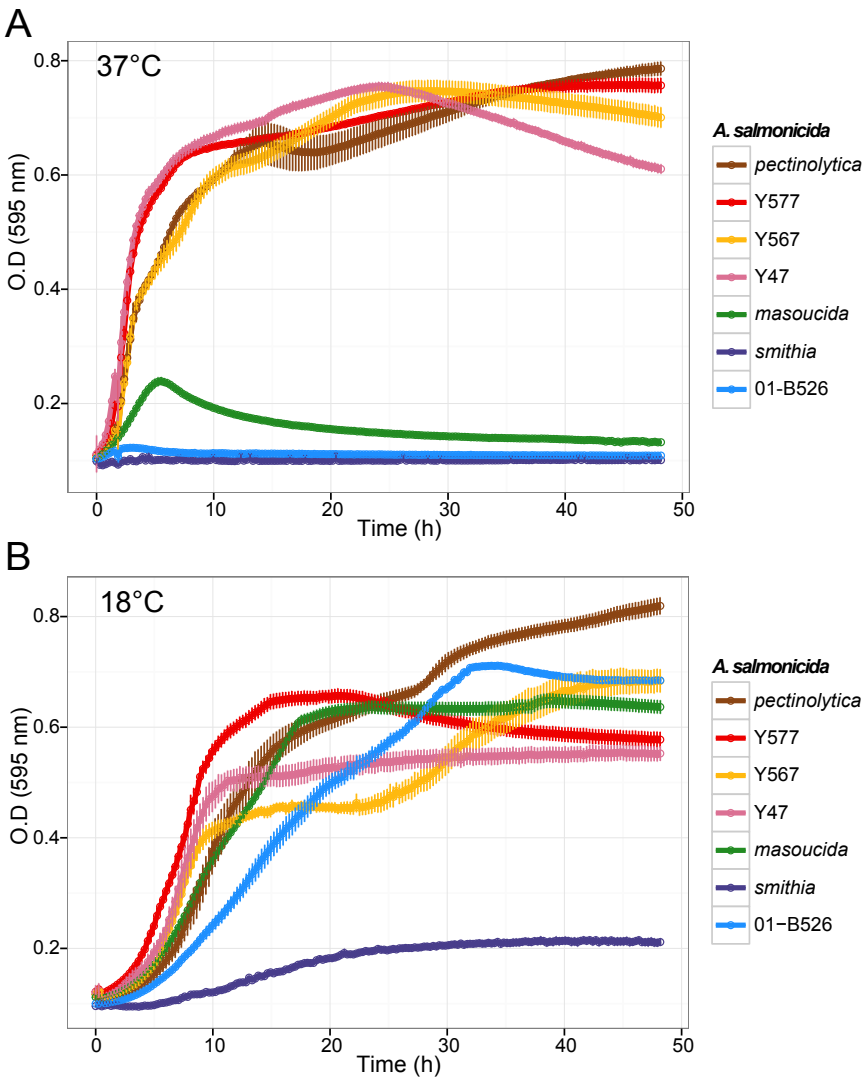

Figure 26. Growth curves at 37°C (A) and 18°C (B) for selected *A. salmonicida* subspecies.
The growth curves were determined three times in independent experiments. The means of three replicates with standard error of the means are shown for each subspecies.

Genomic features responsible for the dichotomy

Based on our dataset, it is tempting to suggest that *A. salmonicida* subsp. *masoucida* lies at the interface of a mesophilic/psychrophilic lifestyle. *A. salmonicida* could be an interesting model to study how bacteria gradually evolve from a mesophilic to a psychrophilic lifestyle. We postulate here that, from an evolutionary point of view, *A. salmonicida* is as an example of a recent evolution in lifestyle.

Previous interesting studies have investigated bacteria from different genera to uncover the genomic elements responsible for the mesophilic and psychrophilic lifestyles (Methé *et al.* 2005; Metpally & Reddy 2009). However, comparing bacteria from different genera to

infer how this type of adaptation has occurred is biased by the noise of unrelated genotypic variations. As reviewed elsewhere (Barria *et al.* 2013), bacterial adaptation to different temperatures implies many major physiological changes. Since mesophilic and psychrophilic isolates of *A. salmonicida* are phylogenetically close to each other, genomic variations among these isolates should mostly be related to differences in lifestyle (i.e., mesophilic versus psychrophilic).

Many studies have reported differences in the amino acid compositions of proteins from psychrophilic and mesophilic strains (Saunders *et al.* 2003; Methé *et al.* 2005; Riley *et al.* 2008; Metpally & Reddy 2009). The ratios of the amino acids of *A. salmonicida* isolates that can grown at 37°C and those that cannot were computed, and the results were analyzed using an unpaired t-test (see Additional file 3). Unlike other studies, we found few significant ($p<0.05$) differences in amino acid composition (only for Gly, His, and Val) between the two groups. This result was expected given that there was no significant difference between the %G+C values of the two groups (see Additional file 3). However, it is important to note that we were limited by the small number of *salmonicida* subspecies, which resulted in a low statistical power (four mesophilic isolates and three psychrophilic isolates). In addition, it has been already reported that such analyses may give inconsistent results (D'Amico *et al.* 2006).

The total number of tRNA genes (ttRNA) and tRNA diversity (dtRNA) are also correlated with the optimal growth temperature (Satapathy *et al.* 2010), with the psychrophilic bacteria harboring more tRNA genes than the mesophilic ones in order to compensate for a lower diffusion rate. However, despite the low statistical power no significant difference was found between the *A. salmonicida* mesophilic and psychrophilic isolates (see Additional file 3). The fact that there was no significant difference between mesophilic and psychrophilic *A. salmonicida* subspecies in terms of genomic elements that can normally be used to discriminate between lifestyles tends to reinforce the hypothesis that the dichotomy in *A. salmonicida* lifestyles is a recent event and that one lifestyle could be derived from the other (i.e., some strains have lost the ability to grow at 37°C).

We used an in-house Perl script (see "Methods" section) to find the pan-genome of *A. salmonicida* in order to shed light on the genomic elements responsible for this

dichotomy. The resulting binary matrix (i.e., presence/absence) was used to map the characters (i.e., the genes) on a phylogenetic tree based on the core genome (Figure 27). This analysis made it possible to determine which genes were acquired and which were lost during evolution and, consequently, may have played a role in the adaption of a given isolate. Given the mesophilic-to-psychrophilic gradient, we investigated the gene repertoires for the branch separating *A. salmonicida* subsp. *masoucida* from the mesophilic isolates (branch 1) and the branch separating *A. salmonicida* subsp. *masoucida* from the psychrophilic isolates (branch 2) (Figure 27).

The functional categories of the genes gained and lost at branches 1 and 2 inferred their potential roles (see Additional file 1). The genes related to branch 1 were present in many functional categories, which made sense given that the mesophilic-to-psychrophilic transition is a complex process and cannot be reduced to a few genes or functional categories. However, it is interesting to note that some categories had acquired or lost many genes. The three most affected categories are transcription (K), cell motility (N), and mobilome (X) (Figure 27). In the case of branch 2, the three functional categories exhibiting most important changes are energy production and conversion (C) (only losses for this category), carbohydrate transport and metabolism (G), replication, recombination, and repair (L) (Figure 27). Interestingly only gains have been detected for the category related to the mobilome (X) (see Additional file 1).

The X category (mobilome) appeared to be interesting at both branches. However, since mobile elements are the main cause of contig breaks during the genome assembly process (Vincent *et al.* 2014, 2015), the genes in these categories were likely under-estimated by our analysis. As reviewed elsewhere (Tanaka *et al.* 2013), *A. salmonicida* subsp. *salmonicida* insertion sequences (ISs) are involved in large-scale mutations such as the loss of the type three secretion system (TTSS) (Daher *et al.* 2011; Emond-Rheault *et al.* 2015a) (see also Additional file 1), loss of the pAsal1 plasmid (Tanaka *et al.* 2012), the formation of the pAsa6 plasmid from pAsa5 (Najimi *et al.* 2009), and the disruption of *vapA* (Gustafson *et al.* 1994), a gene encoding the A-layer (Chu *et al.* 1991). More importantly, IS*AS11*, which is involved in the loss of the TTSS locus and the pAsal1 plasmid, is more active when the temperature reaches 25°C and above (Tanaka *et al.* 2012; Emond-Rheault

et al. 2015a). Lastly, the distribution of IS*AS4* (also known as IS*630*) in aeromonads is even enough to construct a clustering of the genus (Studer *et al.* 2013), which showed without doubt that *salmonicida* subspecies isolates harbor large numbers of IS*AS4*. Given all of the above, especially knowing that the IS*AS11* is temperature-sensitive, it is tempting to speculate that ISs are involved in the mesophilic-to-psychrophilic transition.

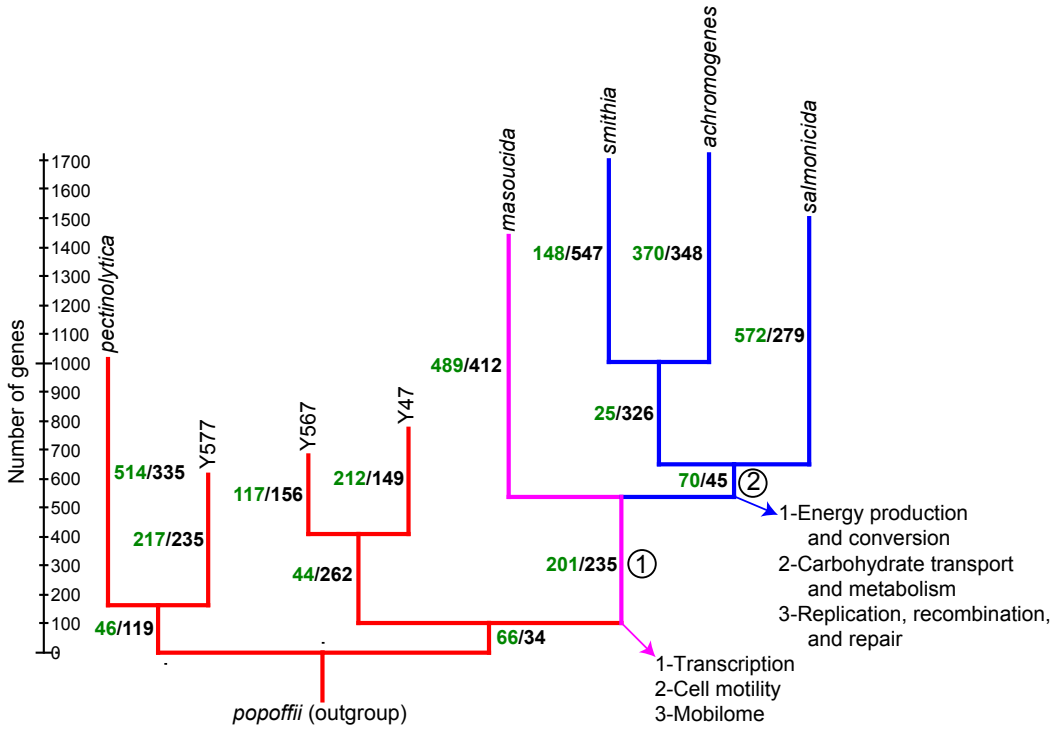

Figure 27. Distribution of the pan-genome on a phylogenetic tree for some key taxa.
The phylogenetic tree was based on the tree found using the core genome. The green and black values indicate the number of genes acquired and lost, respectively, for the specific branch using the parsimonious Dollo model. The branch lengths represent the total number of genes acquired or lost. The three functional categories for which genes are the most affected for branches 1 and 2 are indicated. For more details on the analysis of the functional categories see Additional file 1. For *A. salmonicida* subsp. *salmonicida* the strain used was 01-B526.

The involvement of ISs in lifestyle evolution

All the genomes in the present study were in a draft state, with the exception of the *A. salmonicida* subsp. *salmonicida* A449 reference strain (Reith *et al.* 2008). Rigorous study of IS diversity in these genomes is complicated since these mobile elements are one of the main factors behind contig breaks during *de novo* assembly (Beatson & Walker 2014; Vincent *et al.* 2014, 2015). However, since the drawback is mainly an algorithmic one during the *de novo* assembly process, we decided to address this issue by working directly with sequencing reads. The high sequencing depth provided by Illumina

technology allowed us to study IS diversity directly from the raw sequencing data. As indicated in the "Methods" section, the relative abundance of 70 ISs known to be present in *Aeromonas* was computed for the studied members of the *salmonicida* species.

The results of our study of IS diversity showed (1) a dichotomy between mesophilic and the psychrophilic isolates for their IS repertoire and (2) globally a higher number of ISs in the psychrophilic isolates (Figure 28). In fact, among the 70 ISs studied, 8 were significantly present in the mesophilic isolates and it was possible to see a gradient following the phylogenetic position (Figure 28A). In the case of the psychrophilic isolates, a striking observation was the high amount of the ISs for the subspecies *masoucida* and *smithia* (Figure 28B). The ISAs4 was the most abundant IS for both *masoucida* and *smithia*. This IS was previously postulated to be in high copy number in *A. salmonicida* subspecies, at exception of the *salmonicida* subspecies (Nilsson *et al.* 2006). As indicated previously, IS*AS11*, which causes major rearrangements at high temperatures, was one of the most common ISs in the psychrophilic isolates. For example, the total assembly of the *smithia* subspecies was shown to harbor a very high abundance of IS*AS11*. This observation suggested that the probability of composite transposons is extremely high and, as such, the probability of large-scale genomic rearrangements is also extremely high.

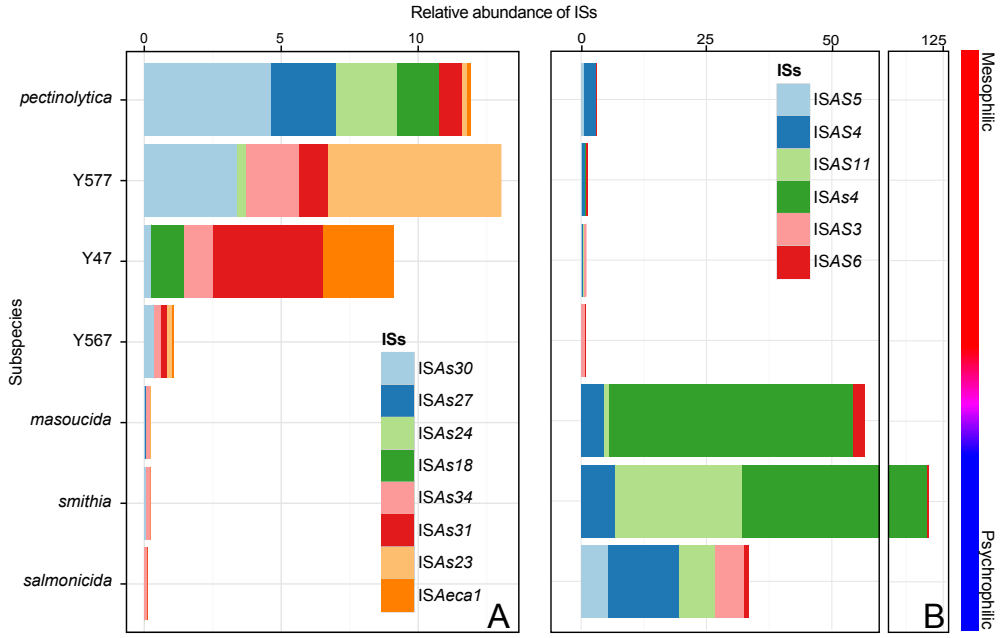

Figure 28. Relative abundance of ISs in *A. salmonicida* subspecies.
The distribution of 8 ISs significantly found in the mesophilic isolates (A) and 6 in the psychrophilic isolates (B) have been determined in a representative set of sequenced *A. salmonicida* isolates.

The high copy number of IS*AS11* in the *smithia* subspecies can be partially explained by the presence of the small-high-copy plasmid pJF4097 (see Additional file 1). This plasmid, which is in approximately 40 copies and bears an IS*AS11*, significantly increased the absolute number of IS*AS11* copies. This is a similar situation to that of the *salmonicida* subspecies like 01-B526 in which the pAsal1 plasmid makes an important contribution to the IS*AS11* pool (Fehr *et al.* 2006). Of note, both plasmids have different replicons, pJF4097 is a ColE1-type whereas pAsal1 is a ColE2-type replicon. The *smithia* subspecies grew much more slowly at 18°C than the other psychrophilic isolates (Figure 26B). It is possible that the metabolic burden caused by the high-copy plasmid and the ISs are responsible for the slow growth of this isolate at 18°C. In fact, we found that around 14% of the subsp. *smithia* genome was devoted to ISs. As reviewed recently, ISs have a major impact on genome architecture and evolution and an IS expansion might eventually results in a significant reduction of the genome (Siguier *et al.* 2014). This genome reduction might significantly change the bacterial lifestyle, for example by enhancing the transition of a free-living to host-dependent bacteria (Siguier *et al.* 2014). It is not impossible that the lifestyle of *A. salmonicida* subsp. *smithia* is currently experiencing dependence to its host, thus explaining the low growth of the bacterium under laboratory conditions.

We believe that it is important to take the absolute number of ISs into consideration since even plasmidic ISs can undergo rearrangements with genomic ISs. For example, a manual investigation of the draft genome of the *A. salmonicida* subsp. *achromogenes* AS03 strain (Han *et al.* 2013) revealed the presence of an IS*AS11* adjacent to the *aopP* gene in a chromosomal contig, just like the pAsal1 plasmid (Fehr *et al.* 2006).

As recently reviewed (Siguier *et al.* 2014), ISs not only play significant role in bacterial evolution by shaping the genomic architecture, they can also (1) insert into genes and thus generate pseudogenes and (2) influence expression of neighbor genes. These two genetic impacts are subtler than large-scale deletions or rearrangements, but significantly alter the bacterial behavior, regulation, and lifestyle. Consequently we investigated the pseudogenes annotated in the reference strain A449 (Reith *et al.* 2008). On all 155 known pseudogenes, 21 (13.5%) were caused by the insertion of an IS. From these 21 pseudogenes, only one

could make sense in a context of psychrophilic/mesophilic lifestyle dichotomy: ASA_1469, which was disrupted by an IS*AS4*. This gene encodes a dihydrolipoamide acetyltransferase, a protein member of the pyruvate dehydrogenase complex. This complex has been associated to bacterial lifestyle adaptation (de Kok *et al.* 1998; Qiu *et al.* 2006; Dziewit & Bartosik 2014). An investigation of the sequences of every *A. salmonicida* isolate included in this study showed that all the mesophilic subspecies (*pectinolytica*, Y577, Y567 and Y47) harbor a complete and likely functional gene (i.e. ASA_1469) whereas the psychrophilic subspecies (*smithia*, *achromogenes* and *salmonicida*) and the intermediate one (*masoucida*) have a truncated gene caused by an insertion of IS*AS4* at the same site. This suggests that IS*AS4* was inserted in the gene in the common ancestor of all the psychrophilic (including *masoucida*) subspecies.

Taking altogether, we postulate that ISs play a significant role in the genomic evolution of the *salmonicida* species and that the psychrophilic subspecies may be "locked" into their lifestyle to conserve their genomic integrity. As shown in other studies and confirmed by the present study (see Additional file 1), IS*AS11*s induce major genomic instabilities and prevent bacteria harboring them from growing without inducing genomic rearrangements at 25°C and above. Moreover, the ISs IS*AS1* and IS*AS2* are also known to be more active around 30°C (Gustafson *et al.* 1994). Given all of this, it is reasonable to postulate that other ISs may also induce deletions and rearrangements at higher temperature. In fact, although temperature-sensitive ISs were not frequently documented, there was at least one other case in *Burkholderia multivorans* which was reported (Ohtsubo *et al.* 2005). Given the present study, these ISs (i.e., the temperature-sensitive) should be investigated in other psychrophilic bacteria to verify if they can be implied in lifestyle related to growth temperature.

Genes under positive selection for specific lineages

A. salmonicida can be seen as an ideal model for studying relationships between genomic features and bacterial lifestyles given the spectrum of lifestyles of the various subspecies. It would be interesting to determine whether some genes have undergone a positive selection in specific lineages. The core genome of *A. salmonicida*, composed of a balanced dataset of mesophilic and psychrophilic isolates, contained 2758 genes (see Additional file 4), 322 of

which were under positive selection in at least one lineage (see the "Methods" section for the details on how the analyses were performed). The categorization of the genes based on lifestyle revealed that those in mesophilic lineages had undergone a more extensive positive selection process than in the psychrophilic lineages (Figure 29). This leads to at least two hypotheses, which are not mutually exclusive. The first hypothesis is that the mesophilic isolates are able to grow more efficiently than the psychrophilic isolates (Figure 26) and consequently replicate their genomes more often, making them more prone to accumulating mutations that allow them to respond more quickly to changes in their environment. The second hypothesis is that, since the mesophilic isolates grow well over a wide range of temperatures, many of their genes are subjected to evolutionary pressure to be able to efficiently colonize and adapt to different environments and hosts (which are actually unknown), unlike the psychrophilic isolates, which are subject to less positive selection. However, this does not explain why the psychrophilic isolates evolved from mesophilic isolates.

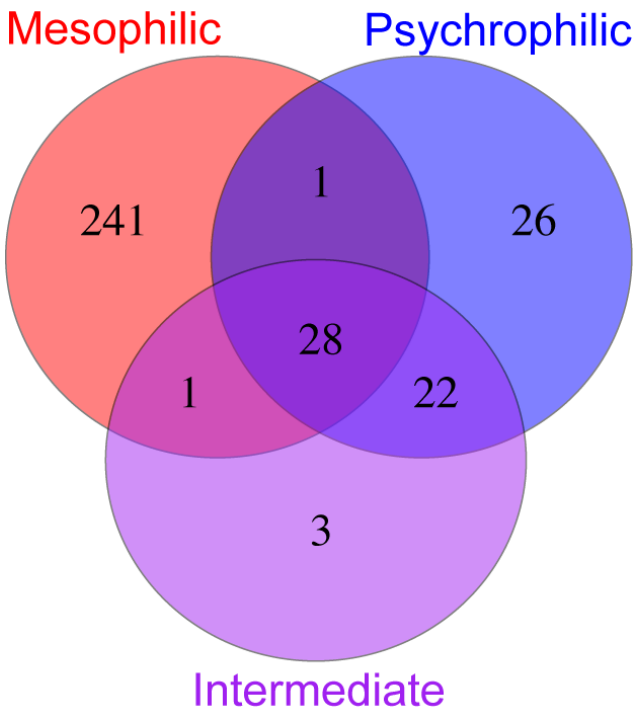

Figure 29. Venn diagram representing the genes under positive selection in the mesophilic, psychrophilic, and intermediate lineages.

Conclusion

Our dataset, which is based on a robust core genome molecular phylogeny, revealed that *A. salmonicida* isolates are much more diverse than previously thought and that they run the gamut of mesophilic, intermediate, and psychrophilic lifestyles. Since mesophilic isolates grow better at 18°C than psychrophilic isolates, the psychrophilic lifestyle of *A. salmonicida* isolates may be the result of genetic drift rather than adaptation. Our dataset revealed that the ISs may be an important driving and genomic modeling force that pushed and locked the psychrophilic isolates into this lifestyle, much like the transposable elements that have been shown to drive the evolution of *Drosophila melanogaster* (González *et al.* 2008; González & Petrov 2009) and ants (Schrader *et al.* 2014). On a more fundamental level, our study provided another important proof-of-concept that selfish DNA can produce evolutionary innovations. However, while it is still unclear why psychrophilic isolates have been conserved throughout the evolution of *A. salmonicida*, there are some possible explanations, including (1) an adaptive selection for psychrophilic hosts like salmonids, (2) a neutral selection, (3) a bottleneck that reduced the effective size of a sub-population of *A. salmonicida*, allowing an enhanced genetic drift and the fixation of the psychrophilic effect in the population, which seems caused by the invasion of ISs, or (4) a relaxation of the selective pressure to grow in mesophilic environments.

Methods

Bacterial isolates and growth conditions

The *A. salmonicida* subsp. *smithia* JF4097 isolate was isolated from a diseased Arctic char (*Salvelinus alpinus*) during an ulcerative and hemorrhagic outbreak in Austria (Goldschmidt-Clermont *et al.* 2009). The *A. salmonicida* subsp. *salmonicida* RS 534 isolate, which is also known as A450 (Belland & Trust 1987), was isolated in France. The Indian Y47, Y567, and Y577 isolates were isolated from a chicken and two species of fish (*Ompok bimaculatus* and *Aristichthys nobilis*), respectively, sold as food at a local market in Mumbai (Nagar *et al.* 2011). The isolates used in our study were grown on a furunculosis agar (Hänninen & Hirvelä-Koski 1997) at 18°C for 24 to 72 h. No ethical approval was necessary for any aspect of this study.

DNA extraction, sequencing, and assembling

The total genomic DNAs of the five isolates were extracted using DNeasy Blood and Tissue kits (Qiagen, Canada). The sequencing libraries were prepared using KAPA Hyper Prep kits and were sequenced by NGS on a MiSeq instrument (Illumina technology) by the Plateforme d'Analyse Génomique of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval). The resulting sequencing reads were assembled *de novo* into contigs using the A5-miseq pipeline version 20140401 (Coil *et al.* 2014). The assemblies were annotated through the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and deposited under accession numbers [GenBank:JZTF00000000, GenBank:JZTG00000000, GenBank:JZTH00000000, GenBank:JZTI00000000, GenBank:JYFF00000000] for Y47, Y567, Y577, JF4097, and RS 534, respectively. The total genomic DNAs of *A. salmonicida* subsp. *pectinolytica* 34mel^T and *A. salmonicida* subsp. *masoucida* NBRC 13784^T were also extracted and sequenced as described above. However, the DNA of these isolates were only sequenced to compare ISs, as shown in Figure 28 (see Results and Discussion), and were not *de novo* assembled.

Phylogenetic analyses

To perform a robust core genome phylogeny, we wrote an in-house Perl script called CoreFinder.pl that relies on BioPerl modules (Stajich *et al.* 2002) to find the genes involved in the core genome. The script uses coding sequences extracted from a GenBank file and sequentially performs tblastn (Altschul *et al.* 1997) searches in fasta or multi-fasta (for draft genomes) files. We used *A. hydrophila* ATCC 7966^T (Seshadri *et al.* 2006), which is the *A. hydrophila* type strain, as a reference. The genome of this strain has been well studied and has a high-quality annotation. The others aeromonads used in the present study are listed in the Additional file 1.

The coding sequences (CDSs) found by CoreFinder.pl that were involved in the core genomes of the 43 isolates studied (see Additional file 2) were extracted using another in-house Perl script, which uses tblastn in loop. All the gene sequences were aligned using MUSCLE version 3.5 (Edgar 2004). The resulting alignments were filtered with BMGE version 1.12 (Criscuolo & Gribaldo 2010) to remove constant characters. Lastly, all

individual filtered alignments were concatenated into a matrix. This matrix and all the in-house scripts are available upon request to the corresponding author.

The phylogenetic analysis was performed by maximum-likelihood using RAxML version 8.1.17 (Stamatakis 2014) with the GTR+Γ model and 1000 rapid bootstraps. The best-fit model GTR+Γ was previously chosen by computing the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) using jModelTest version 2.1.7 (Darriba *et al.* 2012) (see Additional file 1). The resulting tree was visualized using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). To represent the tree, we used a midpoint rooting, an effective method in which the root is defined by the midpoint between the two most divergent operational taxonomic units (OTUs) (Hess & De Moraes Russo 2007). The complete optimization steps are available in Additional file 1.

Assignment of functional categories

An in-house Perl script was written to perform blastp searches to find correspondences between our protein sequences and the Clusters of Orthologous Groups (COGs) database (2014 update) maintained by NCBI (Tatusov *et al.* 2003) in order to assign a functional category to each of them.

Alignment of the pseudo-chromosomes

To compare the general structure of the chromosome for all isolates of the *salmonicida* species used in this study, we generated pseudo-chromosomes by mapping contigs onto the reference strain A449 of the subspecies *salmonicida* (Reith *et al.* 2008), the only chromosome actually fully assembled for the *salmonicida* species, using CONTIGuator version 2.7.4 (Galardini *et al.* 2011). The pseudo-chromosome sequences generated were aligned using Easyfig version 2.1 (Sullivan *et al.* 2011) with default parameters.

Bacterial growth

A. salmonicida subsp. *pectinolytica* (34mel^T), Y577, Y567, Y47, *A. salmonicida* subsp. *smithia* (JF4097), *A. salmonicida* subsp. *masoucida* (NBRC 13784^T), and *A. salmonicida* subsp. *salmonicida* (01-B526) were inoculated on furunculosis agar (Hänninen & Hirvelä-Koski 1997) or on tryptic soy agar (TSA) from frozen stocks and were grown at 18°C for 24 to 48 h. The isolates were then inoculated in 2 ml of lysogeny broth (LB) and were

incubated at 18°C overnight. The turbidity was adjusted to an optical density of 0.1 at 595 nm (OD_{595}), and the cultures were incubated at 18°C or 37°C with shaking at 200 rpm in a Tecan Infinite F200 PRO microplate reader (Tecan, USA). The ODs were read automatically every 15 min for 48 h. The experiments were performed in triplicate.

Frequency of occurrence of amino acids, %GC, and tRNA composition

The frequency of occurrence of amino acids and the %GC were found for each isolate presented in Additional file 3 using respectively pepstats and geecee included in EMBOSS version 6.6.0.0 (Rice *et al.* 2000). Finally, the tRNA composition (dtRNA and ttRNA) for each isolate was found using tRNA-scan-SE version 1.3.1 (Lowe & Eddy 1997) by specifying the bacterial search mode. The unpaired t-tests were performed using the statistical framework R (Team 2013).

Pan-genome analyses

An in-house Perl script was used to find the pan-genome of the key taxa (available upon request to the corresponding author). Briefly, the translated coding sequences were extracted from the corresponding GenBank files of each taxon. Since all the genomes had been annotated using PGAP at approximately the same time, there was no bias caused by different annotation processes. The *A. popoffii* genome served as an outgroup. Reciprocal best blast analyses were then performed using blastp. Genes were considered orthologous if their translated sequences shared at least 60% similarity over at least 85% of their length. The script encoded the pan-genome as a binary matrix (presence/absence). The genes were considered as Dollo characters in MacClade version 4.08 (Maddison & Maddison 2000) and were mapped on a phylogenetic tree based on the tree generated using the core genome. This approach has been used in other studies (Brouard *et al.* 2010; Turmel *et al.* 2015).

Relative IS abundance

The relative abundances of 70 known ISs for the aeromonads listed in ISfinder (Siguier *et al.* 2006) were determined using the sequencing reads. Briefly, the sequencing reads for each taxon were filtered using Trimmomatic version 0.32 (Bolger *et al.* 2014) with the parameters suggested in the manual. The resulting filtered sequencing reads were then mapped using BWA (BWA-MEM algorithm) version 0.7.9a-r786 (Li & Durbin 2009) on

contigs sequences resulting from a *de novo* assembly (see above for the assembly process) to verify the number of high-quality reads. The reads were then aligned on the 70 IS sequences still using BWA (BWA-MEM algorithm) version 0.7.9a-r786 (Li & Durbin 2009). The total abundance of each IS was determined by comparing the mapped reads over the total number of high-quality reads and standardized for the IS length using the results of tools included in SAMtools version 0.1.19-44428cd (Li *et al.* 2009).

Genes under selection

Since we knew the lifestyles and molecular phylogeny of the isolates, we determined whether some genes were under positive selection (dN/dS> 1) for specific lineages. The core genome was found using the same dataset of eight *A. salmonicida* isolates used to find the pan-genome and using the same method used to construct the molecular phylogeny. The well-annotated *A. salmonicida* subsp. *salmonicida* A449 chromosome (Reith *et al.* 2008) was used as a reference in this case. All the sequences were codon-aligned using PRANK version 140603 (Löytynoja & Goldman 2005). All the aligned gene sequences were assessed with HyPhy version 2.2.4 (Kosakovsky Pond *et al.* 2005) using the adaptive branch-site random effects likelihood (aBSREL) method (Smith *et al.* 2015). A gene was considered under positive selection for the tested lineage if the p-value (using the Holm-Bonferroni method in HyPhy) was below 0.05.

Availability of supporting data

The sequence datasets obtained during this project have been deposited in the NCBI GenBank database under the accession numbers [GenBank: JZTF00000000, GenBank: JZTG00000000, GenBank: JZTH00000000, GenBank: JZTI00000000, GenBank: JYFF00000000] for Y47, Y567, Y577, JF4097, and RS 534, respectively. The phylogenetic matrix and the in-house Perl scripts are available upon request to the corresponding author.

Abbreviations

aBSREL: adaptive branch-site random effects likelihood; ANI: average nucleotide identities; CDS: coding sequence; COG: clusters of orthologous group; IS: insertion sequence; *is*DDH: *in silico* DNA-DNA hybridization; LB: lysogeny broth; NGS: next-generation sequencing; OD: optical density; OTU: operational taxonomic unit; PGAP:

prokaryotic genome annotation pipeline; RAPD: random amplified polymorphic DNA; RFLP: restriction fragment length polymorphism; TSA: tryptic soy agar; TTSS: type three secretion system.

Competing Interests

The authors declare that they have no competing interests

Authors's contributions

A.T.V. and S.J.C. designed the research protocol; A.T.V., M.V.T., C.G.T and V.N. performed the research; A.T.V., L.F., C.G.T and S.J.C. analyzed the data; and A.T.V., R.C.L., and S.J.C. wrote the paper. All authors read and approved the final manuscript.

Additional files

Additional file 1: Contains additional experimental procedures and results (Table S1. Aeromonads used in the study; Table S2. The five best models and their –InL, AIC, and BIC values; Table S3. Assembly results; Table S4. Phylogenetic features; Table S5. Biochemical tests used for the mesophilic *A. salmonicida* strains; Figure S1. Conceptual schematization of the in-house CoreFinder.pl Perl script; Figure S2. Number of genes involved in the core genome based on the similarity percent used with the CoreFinder.pl script; Figure S3. Relative abundance of 26 functional categories for genes used to construct the phylogenetic matrixes at 40 and 80% similarity; Figure S4. Molecular core genome phylogeny of 43 aeromonads (80% similarity); Figure S5. Molecular phylogeny of 43 aeromonads (40% similarity); Figure S6. Average nucleotide identity (ANI) analyses for some *A. salmonicida* subspecies included in this study. Figure S7. Growth curves at 7°C for selected *A. salmonicida* subspecies; Figure S8. The three high-copy plasmids found in the Indian strain Y47; Figure S9. The high-copy plasmid pJF4097 found in *A. salmonicida* subsp. *smithia*; Figure S10. Result of the PCR assay confirming that the RS 534 strain lost its TTSS by the recombination of two IS*AS11*s; Figure S11. Pan-genome analysis of selected *A. salmonicida* subspecies; Figure S12. Functional categories of the genes under positive selection in the *A. salmonicida* mesophilic lineages).

Additional file 2: List of the coding sequences (CDSs) being in the core genomes of the 43 aeromonad strains studied.

Additional file 3: The frequency of occurrence of amino acids and the %GC found for each *A. salmonicida* isolate.

Additional file 4: List of the coding sequences (CDSs) being in the core genomes of the 8 *A. salmonicida* strains studied.

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References

Altschul SF, Madden TL, Schäffer AA *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;**25**:3389–402.

Austin DA, McIntosh D, Austin B. Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. *Syst Appl Microbiol* 1989;**11**:277–90.

Barria C, Malecki M, Arraiano CM. Bacterial adaptation to cold. *Microbiol (United Kingdom)* 2013;**159**:2437–43.

Beatson SA, Walker MJ. Tracking antibiotic resistance. *Sci* 2014;**345**:1454–5.

Beaz-Hidalgo R, Figueras MJ. Molecular detection and characterization of furunculosis and other *Aeromonas* fish infections. In: Carvalho E (ed.). *Health and Environment in Aquaculture*. Intech, 2012, 97–132.

Belland RJ, Trust TJ. Cloning of the gene for the surface array protein of *Aeromonas salmonicida* and evidence linking loss of expression with genetic deletion. *J Bacteriol* 1987;**169**:4086–91.

Belland RJ, Trust TJ. DNA:DNA reassociation analysis of *Aeromonas salmonicida*. *J Gen Microbiol* 1988;**134**:307–15.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;**30**:2114–20.

Brouard JS, Otis C, Lemieux C *et al.* The exceptionally large chloroplast genome of the green alga *Floydiella terrestris* illuminates the evolutionary history of the Chlorophyceae. *Genome Biol Evol* 2010;**2**:240–56.

Charette SJ, Brochu F, Boyle B *et al.* Draft genome sequence of the virulent strain 01-B526 of the fish pathogen *Aeromonas salmonicida*. *J Bacteriol* 2012;**194**:722–3.

Chu S, Cavaignac S, Feutrier J *et al.* Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. *J Biol Chem* 1991;**266**:15258–65.

Coil D, Jospin G, Darling AE. A5-miseq : an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics* 2015;**31**: 587-9.

Colston SM, Fullmer MS, Beka L *et al.* Bioinformatic genome comparisons for taxonomic and phylogenetic assignments using *Aeromonas* as a test case. *MBio* 2014;**5**:1–13.

Criscuolo A, Gribaldo S. BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evol Biol* 2010;**10**:210.

D'Amico S, Collins T, Marx J-C *et al.* Psychrophilic microorganisms: challenges for life.

EMBO Rep 2006;**7**:385–9.

Dacanay A, Knickle L, Solanky KS *et al.* Contribution of the type III secretion system (TTSS) to virulence of *Aeromonas salmonicida* subsp. *salmonicida*. *Microbiology* 2006;**152**:1847–56.

Daher RK, Filion G, Tan SGE *et al.* Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions. *Vet Microbiol* 2011;**152**:353–60.

Dallaire-Dufresne S, Tanaka KH, Trudel MV *et al.* Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* 2014;**169**:1–7.

Darriba D, Taboada GL, Doallo R *et al.* jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 2012;**9**:772.

Dautremepuits C, Fortier M, Croisetiere S *et al.* Modulation of juvenile brook trout (*Salvelinus fontinalis*) cellular immune system after *Aeromonas salmonicida* challenge. *Vet Immunol Immunopathol* 2006;**110**:27–36.

Dziewit L, Bartosik D. Plasmids of psychrophilic and psychrotolerant bacteria and their role in adaptation to cold environments. *Front Microbiol* 2014;**5**:596.

Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;**32**:1792–7.

Emond-Rheault J-G, Vincent AT, Trudel MV *et al.* AsaGEI2b: a new variant of a genomic island identified in the *Aeromonas salmonicida* subsp. *salmonicida* JF3224 strain isolated from a wild fish in Switzerland. *FEMS Microbiol Lett* 2015a;**362**:fnv093.

Emond-Rheault J-G, Vincent AT, Trudel MV *et al.* Variants of a genomic island in *Aeromonas salmonicida* subsp. *salmonicida* link isolates with their geographical origins. *Vet Microbiol* 2015b;**175**:68–76.

Fehr D, Casanova C, Liverman A *et al.* AopP, a type III effector protein of *Aeromonas salmonicida*, inhibits the NF-kappaB signalling pathway. *Microbiology* 2006;**152**:2809–18.

Galardini M, Biondi EG, Bazzicalupo M *et al.* CONTIGuator: a bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol Med* 2011;**6**:11.

García JA, Larsen JL, Dalsgaard I *et al.* Pulsed-field gel electrophoresis analysis of *Aeromonas salmonicida* ssp. *salmonicida*. *FEMS Microbiol Lett* 2000;**190**:163–6.

Goldschmidt-Clermont E, Hochwartner O, Demarta A *et al.* Outbreaks of an ulcerative and haemorrhagic disease in Arctic char *Salvelinus alpinus* caused by *Aeromonas salmonicida* subsp. *smithia*. *Dis Aquat Organ* 2009;**86**:81–6.

González J, Lenkov K, Lipatov M *et al.* High rate of recent transposable element-induced adaptation in *Drosophila melanogaster*. *PLoS Biol* 2008;**6**:2109–29.

González J, Petrov DA. The adaptive role of transposable elements in the *Drosophila* genome. *Gene* 2009;**448**:124–33.

Gustafson CE, Chu S, Trust TJ. Mutagenesis of the paracrystalline surface protein array of *Aeromonas salmonicida* by endogenous insertion elements. *J Mol Biol* 1994;**237**:452–63.

Han JE, Kim JH, Shin SP *et al.* Draft genome sequence of *Aeromonas salmonicida* subsp. *achromogenes* AS03, an atypical strain isolated from crucian carp (*Carassius carassius*) in the Republic of Korea. *Genome Announc* 2013;**1**:e00791–13.

Hänninen ML, Hirvelä-Koski V. Molecular and phenotypic methods for the characterization of atypical *Aeromonas salmonicida*. *Vet Microbiol* 1997;**56**:147–58.

Hänninen ML, Ridell J, Hirvelä-Koski V. Phenotypic and molecular characteristics of *Aeromonas salmonicida* subsp. *salmonicida* isolated in southern and northern Finland. *J Appl Bacteriol* 1995;**79**:12–21.

Hess PN, De Moraes Russo CA. An empirical test of the midpoint rooting method. *Biol J Linn Soc* 2007;**92**:669–74.

Janda JM, Abbott SL. The genus *Aeromonas*: Taxonomy, pathogenicity, and infection. *Clin Microbiol Rev* 2010;**23**:35–73.

de Kok A, Hengeveld AF, Martin A *et al.* The pyruvate dehydrogenase multi-enzyme complex from Gram-negative bacteria. *Biochim Biophys Acta* 1998;**1385**:353–66.

Kosakovsky Pond SL, Frost SDW, Muse S V. HyPhy: Hypothesis testing using phylogenies. *Bioinformatics* 2005;**21**:676–9.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;**25**:1754–60.

Li H, Handsaker B, Wysoker A *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* 2009;**25**:2078–9.

Lowe TM, Eddy SR. tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* 1997;**25**:955–64.

Löytynoja A, Goldman N. An algorithm for progressive multiple alignment of sequences with insertions. *Proc Natl Acad Sci U S A* 2005;**102**:10557–62.

Maddison WP, Maddison DR. *MacClade 4: Analysis of Phylogeny and Character Evolution. Version 4.0*, 2000.

Martin-Carnahan A, Joseph S. *Aeromonadales* ord. nov. In: Brenner D, Krieg N, Staley J, et al. (eds.). *Bergey's Manual® of Systematic Bacteriology SE - 12*. Springer US, 2005, 556–87.

McCormick WA, Stevenson RM, MacInnes JI. Restriction endonuclease fingerprinting analysis of Canadian isolates of *Aeromonas salmonicida*. *Can J Microbiol* 1990;**36**:24–32.

Methé BA, Nelson KE, Deming JW *et al.* The psychrophilic lifestyle as revealed by the

genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. *Proc Natl Acad Sci USA* 2005;**102**:10913–8.

Metpally RPR, Reddy BVB. Comparative proteome analysis of psychrophilic versus mesophilic bacterial species: Insights into the molecular basis of cold adaptation of proteins. *BMC Genomics* 2009;**10**:11.

Nagar V, Shashidhar R, Bandekar JR. Prevalence, characterization, and antimicrobial resistance of *Aeromonas* strains from various retail food products in Mumbai, India. *J Food Sci* 2011;**76**:M486–92.

Nagar V, Shashidhar R, Bandekar JR. Characterization of *Aeromonas* strains isolated from Indian foods using *rpoD* gene sequencing and whole cell protein analysis. *World J Microbiol Biotechnol* 2013;**29**:745–52.

Najimi M, Balado M, Lemos ML *et al.* Genetic characterization of pAsa6, a new plasmid from *Aeromonas salmonicida* subsp. *salmonicida* that encodes a type III effector protein AopH homolog. *Plasmid* 2009;**61**:176–81.

Nilsson WB, Gudkovs N, Strom MS. Atypical strains of *Aeromonas salmonicida* contain multiple copies of insertion element IS*Asa4* useful as a genetic marker and a target for PCR assay. *Dis Aquat Organ* 2006;**70**:209–17.

O'hIci B, Olivier G, Powell R. Genetic diversity of the fish pathogen *Aeromonas salmonicida* demonstrated by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses. *Dis Aquat Organ* 2000;**39**:109–19.

Ohtsubo Y, Genka H, Komatsu H *et al.* High-temperature-induced transposition of insertion elements in *Burkholderia multivorans* ATCC 17616. *Appl Environ Microbiol* 2005;**71**:1822–8.

Palumbo SA, Morgan DR, Buchanan RL. Influence of temperature, NaCl, and pH on the growth of *Aeromonas hydrophila*. *J Food Sci* 1985;**50**:1417–21.

Park S, Ha S-D. Effect of temperature on the growth kinetics and predictive growth model

of *Aeromonas hydrophila* on squid (*Sepioteuthis sepioidea*). *Food Sci Biotechnol* 2014;**23**:307–12.

Pavan ME, Abbott SL, Zorzópulos J *et al. Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase- positive subspecies isolated from a heavily polluted river. *Int J Syst Evol Microbiol* 2000;**50**:1119–24.

Pavan ME, Pavan EE, López NI *et al.* Living in an extremely polluted environment: clues from the genome of melanin-producing *Aeromonas salmonicida* subsp. *pectinolytica* 34mel^{T}. *Appl Environ Microbiol* 2015;**81**:5235–48.

Percival SL, Williams DW. Chapter Three - *Aeromonas*. In: Gray of WD (Second E (ed.). London: Academic Press, 2014, 49–64.

Qiu Y, Kathariou S, Lubman DM. Proteomic analysis of cold adaptation in a Siberian permafrost bacterium--*Exiguobacterium sibiricum* 255-15 by two-dimensional liquid separation coupled with mass spectrometry. *Proteomics* 2006;**6**:5221–33.

Reith ME, Singh RK, Curtis B *et al.* The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* 2008;**9**:427.

Rice P, Longden I, Bleasby A. EMBOSS: The european molecular biology open software suite. *Trends Genet* 2000;**16**:276–7.

Riley M, Staley JT, Danchin A *et al.* Genomics of an extreme psychrophile, *Psychromonas ingrahamii*. *BMC Genomics* 2008;**9**:210.

Roger F, Marchandin H, Jumas-Bilak E *et al.* Multilocus genetics to reconstruct aeromonad evolution. *BMC Microbiol* 2012;**12**:62.

Satapathy SS, Dutta M, Ray SK. Higher tRNA diversity in thermophilic bacteria: A possible adaptation to growth at high temperature. *Microbiol Res* 2010;**165**:609–16.

Saunders NFW, Thomas T, Curmi PMG *et al.* Mechanisms of thermal adaptation revealed

from the genomes of the Antarctic archaea *Methanogenium frigidum* and *Methanococcoides burtonii*. *Genome Res* 2003;**13**:1580–8.

Schrader L, Kim JW, Ence D *et al.* Transposable element islands facilitate adaptation to novel environments in an invasive species. *Nat Commun* 2014;**5**:5495.

Seshadri R, Joseph SW, Chopra AK *et al.* Genome sequence of *Aeromonas hydrophila* ATCC 7966T: Jack of all trades. *J Bacteriol* 2006;**188**:8272–82.

Siguier P, Gourbeyre E, Chandler M. Bacterial insertion sequences: Their genomic impact and diversity. *FEMS Microbiol Rev* 2014;**38**:865–91.

Siguier P, Perochon J, Lestrade L *et al.* ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 2006;**34**:D32–6.

Smith MD, Wertheim JO, Weaver S *et al.* Less is more: An adaptive branch-site random effects model for efficient detection of episodic diversifying selection. *Mol Biol Evol* 2015;**32**:1342–53.

Stajich JE, Block D, Boulez K *et al.* The bioperl toolkit: Perl modules for the life sciences. *Genome Res* 2002;**12**:1611–8.

Stamatakis A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;**30**:1312–3.

Studer N, Frey J, Vanden Bergh P. Clustering subspecies of *Aeromonas salmonicida* using IS*630* typing. *BMC Microbiol* 2013;**13**:13–36.

Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualiser. *Bioinformatics* 2011;**27**:1009–10.

Tanaka KH, Dallaire-Dufresne S, Daher RK *et al.* An insertion sequence-dependent plasmid rearrangement in *Aeromonas salmonicida* causes the loss of the type three secretion system. *PLoS One* 2012;**7**:e33725.

Tanaka KH, Frenette M, Charette SJ. IS-mediated loss of virulence by *Aeromonas*

salmonicida: A tangible piece of an evolutionary puzzle. *Mob Genet Elements* 2013;**3**:e23498.

Tatusov RL, Fedorova ND, Jackson JD *et al.* The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* 2003;**4**:41.

Team RC. R: A language and environment for statistical computing. *R Found Stat Comput Vienna, Austria* 2013.

Turmel M, Otis C, Lemieux C. Dynamic evolution of the chloroplast genome in the green algal classes pedinophyceae and trebouxiophyceae. *Genome Biol Evol* 2015;**7**:2062–82.

Umelo E, Trust TJ. Physical map of the chromosome of *Aeromonas salmonicida* and genomic comparisons between *Aeromonas* strains. *Microbiology* 1998;**144**:2141–9.

Valdes N, Espinoza C, Sanhueza L *et al.* Draft genome sequence of the Chilean isolate *Aeromonas salmonicida* strain CBA100. *FEMS Microbiol Lett* 2015;**362**:fnu062.

Vincent AT, Boyle B, Derome N *et al.* Improvement in the DNA sequencing of genomes bearing long repeated elements. *J Microbiol Methods* 2014;**107**:186–8.

Vincent AT, Tanaka KH, Trudel MV *et al.* Draft genome sequences of two *Aeromonas salmonicida* subsp. *salmonicida* isolates harboring plasmids conferring antibiotic resistance. *FEMS Microbiol Lett* 2015;**362**:1–4.

Chapitre 6 – Article 5

Study of mesophilic *Aeromonas salmonicida* A527 strain sheds light on the species lifestyles and taxonomic dilemma

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Résumé

La bactérie *A. salmonicida* comprend cinq sous-espèces : *salmonicida*, *smithia*, *achromogenes*, *masoucida* et *pectinolytica*. Cette dernière est une sous-espèce mésophile capable de croître à une large gamme de températures, y compris 37°C, tandis que les autres sous-espèces sont psychrophiles, se limitant donc à des températures plus basses. L'étude des différences entre les sous-espèces mésophiles et psychrophiles est entravée par l'absence de génomes fermés provenant des souches mésophiles. Une étude antérieure a rapporté que les séquences d'insertion (IS), qui peuvent induire des réarrangements génomiques à des températures autour de 25°C, pourraient être l'un des déterminants expliquant cette dichotomie (mésophilie versus psychrophilie). Le génome de la souche mésophile A527 d'*A. salmonicida* a été séquencé, fermé et analysé. Ce génome soutient l'hypothèse que les IS sont des éléments majeurs pour expliquer la dichotomie d'*A. salmonicida*. De plus, l'analyse phylogénétique réalisée pour positionner la souche A527 soulève un problème concernant la taxonomie d'*A. salmonicida*.

Abstract

The Gram-negative bacterium *Aeromonas salmonicida* contains five subspecies: *salmonicida*, *smithia*, *achromogenes*, *masoucida* and *pectinolytica*. *Pectinolytica* is a mesophilic subspecies with the ability to thrive at a wide range of temperatures, including 37°C, while the four other subspecies are psychrophilic, thus restricted to lower temperatures. The psychrophilic subspecies are known to infect a wide range of fishes. However, there is no evidence of pathogenicity for the mesophilic subspecies *pectinolytica*. Study of the differences between the mesophilic and psychrophilic subspecies is hampered by the lack of completely sequenced and closed genomes from the mesophilic subspecies. A previous study reported that insertion sequences, which can induce genomic rearrangements at temperatures around 25°C, could be one of the determinants explaining the differences in lifestyle (mesophilic or psychrophilic) between the subspecies. In this study, the genome of mesophilic strain A527 of *A. salmonicida* was sequenced, closed and analyzed to investigate the mesophilic/psychrophilic discrepancy. This reference genome supports the hypothesis that insertion sequences are major determinants of the lifestyle differences between the *A. salmonicida* subspecies. Moreover, the phylogenetic analysis done to position strain A527 within the taxonomy raises an issue regarding the intraspecies structure of *A. salmonicida*.

Introduction

The first mention of the Gram-negative bacterium *Aeromonas salmonicida* was in 1894 (Emmerich & Weibel 1894). It was reported to be the subspecies *salmonicida* (named *Bacillus der Forellenseuche* at that time), causing the fish disease furunculosis. As detailed elsewhere (Austin & Austin 2016), the taxonomic positioning and the name of the bacterium became confusing over time, due to some articles that were written using different names to describe the bacterium, and in other research, the creation of novel bacterial families, including *Aeromonadaceae* (Colwell *et al.* 1986).

Currently, there are five officially recognized *A. salmonicida* subspecies: *salmonicida*, *smithia*, *achromogenes*, *masoucida,* and *pectinolytica* (Martin-Carnahan & Joseph 2005). The subspecies *salmonicida* is considered to be the usual etiologic agent of furunculosis in salmonids, while the four other subspecies are designated as "atypical" according to their phenotypic and biochemical differences and their ability to infect a wide range of fishes (Martin-Carnahan & Joseph 2005; Dallaire-Dufresne *et al.* 2014; Austin & Austin 2016). Strains of the subspecies *salmonicida*, *smithia*, *achromogenes* and *masoucida* are psychrophilic and thus, their growth is restricted to temperatures below 25°C (Austin & Austin 2016).

In 2000, the *pectinolytica* subspecies was isolated from a polluted river (Matanza River, Argentina) but it does not have any known (fish) host (Pavan *et al.* 2000) and there is no record of its pathogenicity. This subspecies, by having the ability to grow well at 37°C, is considered mesophilic and challenged the current knowledge on *A. salmonicida*, thereby suggesting a greater diversity.

More recently, a study reported the characterization of three Indian mesophilic *A. salmonicida* strains (Y47, Y567 and Y577) from undetermined subspecies and suggested that insertion sequences could be major determinants in the temperature-related lifestyle dichotomy between species of *A. salmonicida* (Vincent *et al.* 2016b). This hypothesis is mainly due to the fact that genomes from mesophilic strains seem to have fewer insertion sequences and that their repertoire is different from the one of psychrophilic strains. Several studies reported experimental evidence that insertion sequences in *A. salmonicida* can be

the cause of major genomic disturbance events when the strains are grown at temperatures above their optimal growth temperature. For example, growing *A. salmonicida* subsp. *salmonicida* at temperatures around 25°C resulted in the disruption of genes (Gustafson *et al.* 1994), the loss of the type three secretion system (TTSS) locus (Daher *et al.* 2011), the loss of the small pAsal1 plasmid (Tanaka *et al.* 2012), and reshaping of the pAsa4 plasmids (Tanaka *et al.* 2016). However, complete and closed genomic sequences are only available for reference strain A449, of the *salmonicida* subspecies, (Reith *et al.* 2008) and for three likely psychrophilic strains [S44 (BioSample: SAMN07276874), S68 (BioSample: SAMN07276873) and S121 (BioSample: SAMN07276469)]. The taxonomy for these three strains is unclear, which limits genomic interpretation and analysis.

In this study, the complete genome of the mesophilic strain *A. salmonicida* A527 was sequenced, closed and analyzed to shed light on the unusual lifestyle dichotomy of the species. The genomic information gained herein also raised a taxonomic issue about the subspecies delineation of *A. salmonicida*.

Methods

A. salmonicida strain A527 was isolated in a market in Mumbai (India) from a dead giant river prawn (*Macrobrachium rosenbergii*) (Nagar *et al.* 2011). As indicated previously (Nagar *et al.* 2011), 25 g of the prawn was added to 225 mL of tryptic soya broth, homogenized, and incubated at 30°C for 24 h. The resulting culture was then plated on starch ampicillin agar and incubated again at 30°C for 24 h. A single colony that showed typical *Aeromonas* characteristics was picked at random and re-streaked as above. A first taxonomic assignment was made by sequencing the 16S rRNA gene and then by searching homologous sequences in GenBank database. Finally, a pulsed field gel electrophoresis was performed to obtain a DNA fingerprint of the strain and to compare it with reference strains of various *Aeromonas* species (Nagar *et al.* 2011).

Bacterial samples were recovered from frozen stocks at –80°C, plated on TSA, and incubated at 18°C for 48 h before being used in subsequent analyses. The growth kinetics were realized at 18°C and 37°C in a Tecan Infinite F200 PRO microplate reader (Tecan, USA) as already described elsewhere (Vincent *et al.* 2016b). Phenotypic tests were

performed using API 20E strips (bioMérieux) as described by the manufacturer. The strips were incubated for 72 h at 18°C and analyzed. The tests for motility, production of pigments, catalase, oxidase, utilization of gluconate, haemolytic capacity, degradation of casein and tributyrin, growth on MacConkey agar and finally Gram/KOH were also performed as described elsewhere (Gerhardt 1994; Cowan *et al.* 2003).

The DNA was extracted from strain A527 with phenol and chloroform, following a protocol called *Extracting DNA Using Phenol-Chloroform*, provided by Pacific Biosciences (http://www.pacb.com). The SMRT long-reads technology from PacBio was chosen to generate a high quality assembly able to bypass the large, repeated elements, such as insertion sequences and ribosomal operons (Vincent *et al.* 2014a). PacBio reads were processed and *de novo* assembled with the RS_HGAP_Assembly.3 pipeline as implemented in SMRT Analysis 2.3.0 [https://github.com/PacificBiosciences/SMRT-Analysis/wiki/SMRT-Analysis-Software-Installation-v2.3.0]. The tool Circlator version 1.5.1 (Hunt *et al.* 2015) was used to circularize the chromosome sequence. The resulting sequence was annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) of the NCBI and was deposited in GenBank under the accession number CP022550. The insertion sequences contained in the *A. salmonicida* A527 genome were annotated using ISsaga (Varani *et al.* 2011) and manually curated.

The pan-genome of all available *A. salmonicida* genomes (a total of 32, including the genome of strain A527 and at least one representative of each of the five official subspecies), in addition to representatives of all other *Aeromonas* species (a total of 28), was evaluated using GET_HOMOLOGUES version 20170609 (Contreras-Moreira & Vinuesa 2013) (see Supp. Fig. S1 and Table S1). The sequences of the soft core genes (genes that are present in more than 95% of taxa) were aligned by codons with TranslatorX version 1.1 (Abascal *et al.* 2010), and filtered using BMGE version 1.12 (Criscuolo & Gribaldo 2010). Finally, the resulting 2,044 sequences (after removing the paralogous genes) were concatenated into a partitioned supermatrix with 882,290 positions. The phylogenetic analysis itself was performed with IQ-TREE version 1.6.beta2, where the best-fit model was found for each partition by performing 10,000 ultrafast bootstraps (UFBoot) (Nguyen *et al.* 2015). The resulting tree was visualized and midpoint-rooted by

FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). Average Nucleotide Identity (ANI) values were obtained with pyani (https://github.com/widdowquinn/pyani).

The tools TXSScan (Abby *et al.* 2016) and PHASTER (Arndt *et al.* 2016) were used to predict secretion systems and prophage sequences, respectively. The antibiotic resistance genes were predicted by the Resistance Gene Identifier (RGI) available online through the Comprehensive Antibiotic Resistance Database (CARD) (Jia *et al.* 2016). Finally, the presence of CRISPR clusters was assessed using CRISPRFinder online (Grissa *et al.* 2007).

Result and Discussion

General features

A. salmonicida strain A527 was isolated from a dead giant river prawn (*Macrobrachium rosenbergii*) that showed no sign of disease during a sampling campaign for a study that evaluated the presence of *Aeromonas* bacteria at various food retailers in Mumbai (India) (Nagar *et al.* 2011). It is unclear if *M. rosenbergii* is A527's host, or if it was contaminated with the *A. salmonicida* strain. Therefore, the host is considered as unknown. Given the reported mesophilic/psychrophilic lifestyle dichotomy present in the *A. salmonicida* species (Vincent *et al.* 2016b), A527's capacity to grow at 18°C and 37°C was tested. Predictably, as the strain was initially recovered at 30°C (Nagar *et al.* 2011), A527 grew efficiently at both temperatures (Figure 30).

A. salmonicida strain A527 shares some signature metabolic activities with other mesophilic *A. salmonicida* strains (see Supp. Fig. S2), such as the production of acid from sorbitol and arabinose, and the presence of active L-lysine decarboxylase and β-galactosidase. Both A527 and strain 34mel^T of the subspecies *pectinolytica* were cytochrome *c* oxidase positive, as are the psychrophilic *A. salmonicida* strains [NBRC 13784^T (subspecies *masoucida*) and 01-B526 (subspecies *salmonicida*)], meaning that they can reduce molecules of oxygen to water in the aerobic respiratory chain (Iwata 1998). Other mesophilic Indian strains Y577, Y567 and Y47 are cytochrome *c* oxidase negative. Notwithstanding, a clustering based on phenotypes permitted the generation of two distinct groups (see Supp. Fig. S2): the first containing the psychrophilic subspecies *masoucida* and *salmonicida*, and a second group with the mesophilic Indian *A. salmonicida* strains (A527,

Y47, Y567 and Y577) and the mesophilic subspecies *pectinolytica*. However, it is actually impossible to assign a known subspecies to the strain A527 based on the 35 tests performed in the present study.

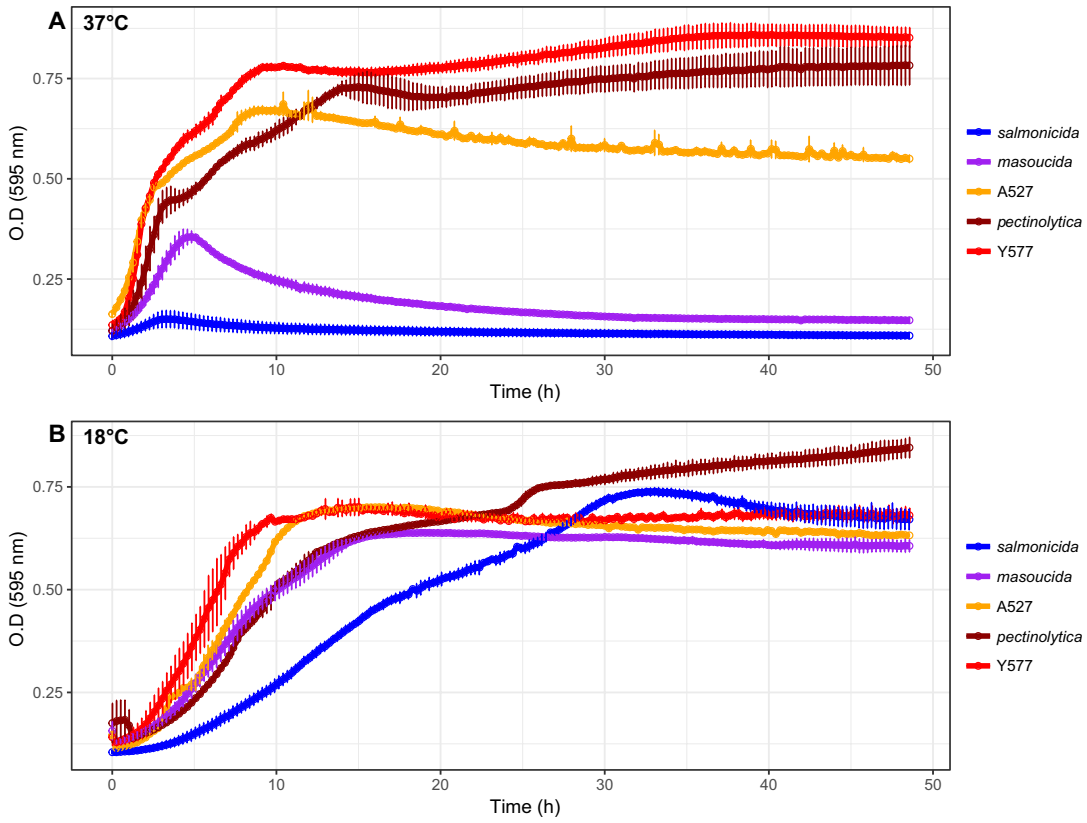

Figure 30. Growth profiles of selected *A. salmonicida* strains.
Growth profiles of A527 and selected mesophilic (*pectinolytica* 34mel^T and Y577) and psychrophilic (*salmonicida* 01-B526) *A. salmonicida* strains at 37°C (A) and 18°C (B). The strain NBRC 13784^T of the *masoucida* subspecies was also added given its intermediate ability to grow at 37°C (Vincent *et al.* 2016b). All the curves were done in triplicate and the standard error bars are shown.

Since no closed genome from a mesophilic strain of *A. salmonicida* was available, the A527 genome was sequenced by PacBio, which led to the assembly of a single contig. This 4,806,250 bp-long genome has a guanine-cytosine (GC) content of 58.66%. These values are close to the ones reported for the four other *A. salmonicida* closed genomes (see Supplementary Table S2). However, a comparison between these genomes showed major differences within their architecture (Figure 31). An investigation of the A527 genome using TXSScan (Abby *et al.* 2016) predicted genes coding for proteins involved in putative complete Type I and II secretion systems, and Type IV pilus. The same tool also predicted the mandatory genes to produce flagella.

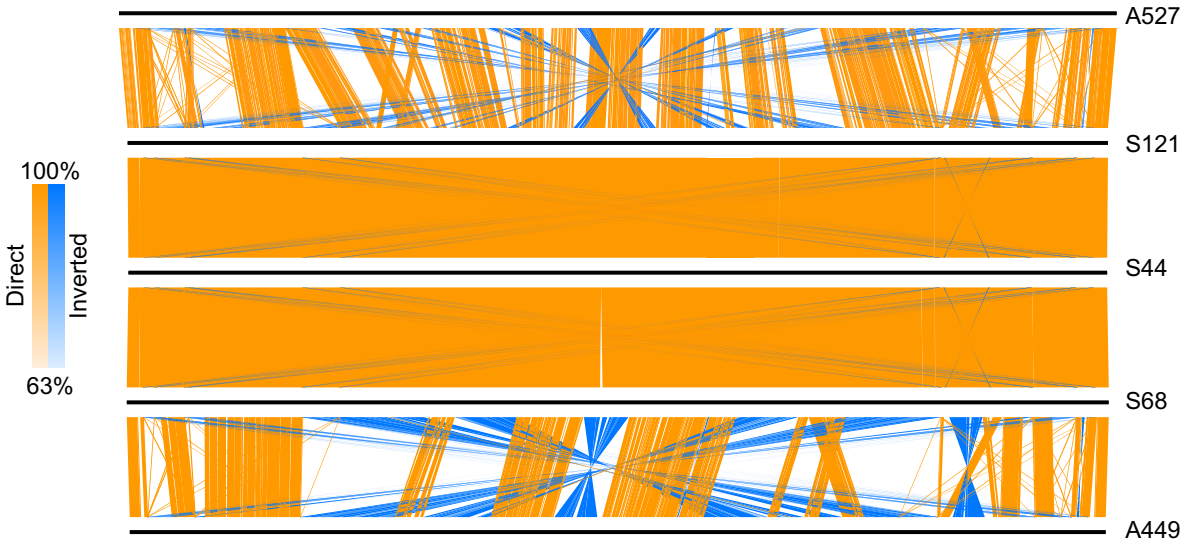

Figure 31. Comparison between the complete closed genomes of *A. salmonicida* strains. Only the genomes of strains A527 (CP022550), S121 (NZ_CP022175.1), S44 (NZ_CP022181.1), S68 (NZ_CP022186.1) and A449 (NC_009348.1) are compared since they are the only closed genomes available for *A. salmonicida*. The direct and inverted matches are in orange and blue, respectively. The strain A449 is the only strain in this figure with an official subspecies assignment (*salmonicida*).

Recent studies reported antibiotic resistance as a major issue for treating *A. salmonicida* subsp. *salmonicida* infections (Vincent *et al.* 2014b, 2016a; Piotrowska & Popowska 2015; Trudel *et al.* 2016). However, little is known about the antibiotic resistance of atypical *A. salmonicida* strains (L'Abée-Lund & Sørum 2001; Casas *et al.* 2005). The genome of *A. salmonicida* strain A527 possesses several genes that code for efflux pumps putatively involved in antibiotic resistance (see Supp. Fig. S3). PHASTER (Arndt *et al.* 2016) predicted a complete prophage in the genome of A527 (including *attL* and *attR* sequences). Three distinct prophages were already reported in genomes of strains of *A. salmonicida* subsp. *salmonicida*. One of them, named Prophage 3 (Emond-Rheault *et al.* 2015), exhibits sequelog sequences (Varshavsky 2004) with the prophage found in A527. No CRISPR was found in the genome of A527.

Phylogenetic analysis

The phylogenetic position of this new strain was assessed by molecular phylogeny. Strain A527 clustered along with other mesophilic strains (*pectinolytica* 34mel^T, Y577, Y567 and Y47) (Figure 32). The positions of strains of the subspecies *salmonicida*, *smithia*, *achromogenes* and *masoucida* respect the previously proposed topology (Vincent *et al.* 2016b; Vincent & Charette 2017). The two newly published *A. salmonicida* subsp.

salmonicida Chinese strains BG and YK (Long *et al.* 2016) were basal to those from Canada and Europe. Strains S44, S68 and S121, for which the genomes are closed, form a clade with the subspecies *masoucida*. However, there is currently no additional information on these latter strains, preventing conclusions about their phylogenetic position.

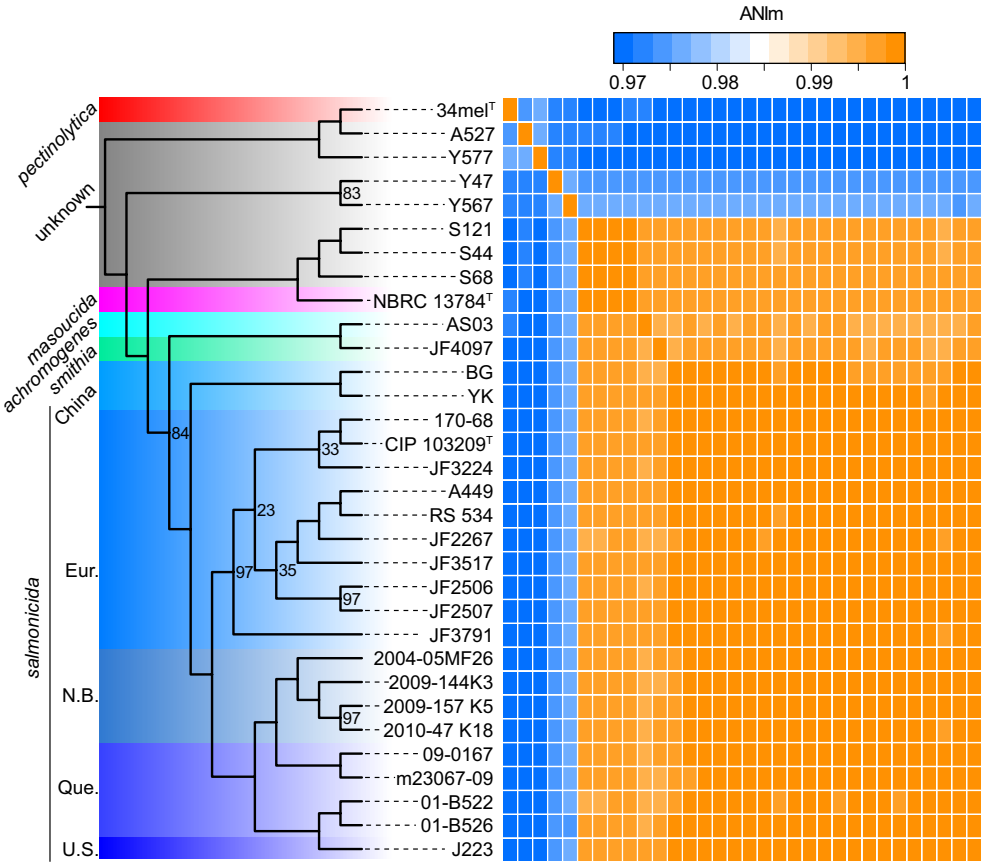

Figure 32. Cladogram showing the phylogenetic relations between all available *A. salmonicida* strains with a sequenced genome (draft or closed).

Only bootstrap values inferior to 100 are shown at the corresponding nodes. The heatmap represents the ANI values. The complete tree with all 60 taxa is shown in supplementary material (Figure S4). The strains of the *salmonicida* subspecies cluster according to the geographical regions where they have been isolated: China, Europe (Eur.), United States (U.S.) and the Canadian provinces of New-Brunswick (N.B.) and Quebec (Que.).

We correlated the phylogenetic positions with the average nucleotide identity (ANI), a measure that helps to define species boundaries (Richter & Rosselló-Móra 2009). It was previously estimated that an ANI cutoff value of 96% is appropriate for *Aeromonas* species delineations (Colston *et al.* 2014). The present analysis showed, as expected, that all *A. salmonicida* strains belong to the same species and that the psychrophilic and mesophilic strains form two distinct groups, as suggested by the clustering based on the biochemical tests (see Supp. Fig. S2). In addition, all psychrophilic strains share high ANI values, while

mesophilic strains are much more distant, even between each other (Figure 32). For example, strain A527 shares ANI values of 0.974 and 0.975 with strain 34mel^T of *pectinolytica* and Y577, respectively, and 0.970 with A449, of the subspecies *salmonicida*.

The fact that the ANIs between the genomes of A527 and its closest relatives are almost as distant as with a strain of a psychrophilic subspecies such as *salmonicida* suggest that strain A527 should possibly be considered to be a member of a new subspecies. However, this reasoning could also be applied to Y577, Y567 and Y47, which are also distant. In correlation with the ANI values, all the mesophilic strains harbour long branch lengths compared to the psychrophilic strains (see Supp. Fig. S4). The fact that the psychrophilic strains have short branch lengths is of interest, because the genome architecture of reference strain A449 of subspecies *salmonicida* was shown to be divergent from those of strains S44, S68 and S121 (Figure 31).

Taken altogether, the above data presents a taxonomic dilemma, specifically about the intraspecies structure of *A. salmonicida* (Austin 2011; Austin & Austin 2016). Since the psychrophilic subspecies *salmonicida*, *smithia*, *achromogenes* and *masoucida* are considered taxonomically different despite their high ANI values (~99%), each mesophilic strain should perhaps also be considered to be a different subspecies, as they are even more divergent. Although this could be due to a sampling bias, only one member of the many putative new subspecies has been isolated, which seems unusual.

As shown in Figure 32, there is a clear sequencing bias towards *A. salmonicida* subsp. *salmonicida*, mainly given its recurrent presence in fish farms (Dallaire-Dufresne *et al.* 2014). More genomes from strains belonging to other subspecies should be sequenced to learn about their genomic signatures. We now know that some strains of the species *salmonicida* are mesophilic. This opens the door to sample environments that may previously not have been considered likely locations for *A. salmonicida*.

The level of diversity and heterogeneity in the biochemical characteristics of the *A. salmonicida* strains is not an exact reflection of the genomic diversity and heterogeneity. As indicated above, only a few features can distinctively separate psychrophilic and mesophilic *A. salmonicida* isolates. However, analysis of other mesophilic *A. salmonicida*

strains is required to robustly assign subspecies and biochemical signatures to them, as was done for the psychrophilic *A. salmonicida* subspecies (Austin *et al.* 1989). For all of these reasons, strain A527 has not yet been associated with a subspecies. The classification scheme for *A. salmonicida* must be clarified before considering defining new subspecies.

Investigation of the insertion sequences

Obtaining the complete closed genome of strain A527 was an opportunity to substantiate the reported trend that genomes from mesophilic *A. salmonicida* strains harbour fewer insertion sequences than those from psychrophilic subspecies, and that their insertion sequence repertoire is divergent (Vincent *et al.* 2016b). The genome of A527 was predicted to harbour 74 complete and 21 fragments of insertion sequences distributed in 19 types and 10 families (Table 10). These high numbers are close to those of well-annotated strain A449 of the subspecies *salmonicida*, which has 88 complete and 14 partial insertion sequences (Reith *et al.* 2008). According to the ISfinder database (Siguier *et al.* 2006), several predicted insertion sequences in A527 were previously found in *A. salmonicida* (Table 10). However, there is no mention regarding the strains or even the subspecies in the database. Although A527's genome includes a high number of insertion sequences, as in the genome of the A449 strain, its repertoire is different. Of the 19 types in A527, only IS*Ahy2* (annotated as IS*AS4* in A449) and IS*As19* (not annotated) are found in the genome of A449. It is interesting to note that IS*As18*, IS*As23*, IS*As24*, IS*As30* and IS*As31* were listed to be specific to mesophilic *A. salmonicida* (Vincent *et al.* 2016b).

Although the insertion sequences are not directly annotated in the genomes of S44, S68 and S121 as they are for *A. salmonicida* subsp. *salmonicida* A449, we investigated their presence and those shared with A527's genome. Again, only a few shared insertion sequences were found. In strain S44, insertion sequences IS*5*, IS*Kpn3* and IS*Ec35* were found to be present on large plasmid pS44-1 (NZ_CP022176.1). Interestingly, three IS*Kpn3*s are predicted to be present in A527's genome. This insertion sequence was originally found in *Klebsiella pneumoniae* plasmid pRDDHA (Verdet *et al.* 2006) and more recently in the large plasmid pAsa4c of *A. salmonicida* subsp. *salmonicida* strain JF2267 (Tanaka *et al.* 2016). Notably, several freestanding transposase genes, not clearly associated with known insertion sequences, were found in A527's genome. This highlights

that putative new insertion sequences could eventually be found in the A527 genome and that the ISfinder database should continue to be updated (Siguier *et al.* 2006).

Tableau 10. Complete and partial ISs found in the genome of *A. salmonicida* A527

IS	Family	Complete	Partial	Host[a]	Presence in other closed *A. salmonicida* genomes			
					A449	S44	S68	S121
IS*1396*	IS*L3*	0	1	*Serratia marcescens*	No	No	No	No
IS*Aeca4*	IS*3*	7	2	*Aeromonas caviae*	No	No	No	No
IS*Aeme5*	IS*66*	0	1	*Aeromonas media*	No	No	No	Yes
IS*Aeme17*	IS*21*	1	0	*Aeromonas media*	No	No	No	No
IS*Aeme21*	IS*481*	0	2	*Aeromonas media*	No	No	No	No
IS*Ahy2*	IS*630*	8	2	*Aeromonas hydrophila*	Yes[b]	Yes	Yes	Yes
IS*As13*	IS*5*	3	0	*Aeromonas salmonicida*	No	No	No	No
IS*As15*	IS*5*	1	1	*Aeromonas salmonicida*	No	No	No	No
IS*As18*	IS*4*	5	1	*Aeromonas salmonicida*	No	No	No	No
IS*As19*	IS*481*	2	2	*Aeromonas salmonicida*	Yes[c]	No	No	No
IS*As23*	IS*1595*	3	2	*Aeromonas salmonicida*	No	No	No	No
IS*As24*	IS*110*	4	4	*Aeromonas salmonicida*	No	No	No	No
IS*As30*	IS*4*	4	1	*Aeromonas salmonicida*	No	No	No	No
IS*As31*	IS*3*	10	0	*Aeromonas salmonicida*	No	No	No	No
IS*Ave3*	IS*3*	1	0	*Aeromonas veronii*	No	No	No	No
IS*Ec35*	IS*5*	0	1	*Escherichia coli*	No	Yes	No	No
IS*Kpn3*	IS*1595*	2	1	*Klebsiella pneumoniae*	No	Yes	No	No
IS*UnCu16*	IS*66*	20	0	*uncultured bacterium*	No	No	No	No
IS*5*	IS*5*	3	0	*Escherichia coli*	No	Yes	No	Yes
Total		**74**	**21**					

a: Host in which the insertion sequence is listed in ISfinder.
b: IS*Ahy2* showed a high identity with IS*AS4* of the *A. salmonicida* subsp. *salmonicida* A449 genome.
c: This insertion sequence is currently not annotated in the *A. salmonicida* subsp. *salmonicida* A449 genome.

Insertion sequences can drive novelty in terms of adaptation and genome plasticity (Vandecraen *et al.* 2017) and were already known to cause major genomic alterations in *A. salmonicida*, such as plasmid reshaping (Tanaka *et al.* 2016, 2017) and the disruption of *vapA* (Gustafson *et al.* 1994), a gene coding for a protein involved in the A-layer virulence factor (Chu *et al.* 1991). *A. salmonicida* genomes display high rearrangement capabilities while having a slow mutation rate for coding sequences (Figure 31 and Supp. Fig. S4). Insertion sequences could be one of the determinants causing this asymmetric evolution by driving recombination. Also, it is known that insertion sequences can modify

regulation/transcription of genes (Vandecraen *et al.* 2017) and further studies should assess if these elements are involved in similar alterations in *A. salmonicida*.

Conclusion

This study describes the first complete sequenced and closed genome of a mesophilic *A. salmonicida* strain, as a reference to investigate the dichotomy between psychrophilic and mesophilic *A. salmonicida* subspecies. As already suggested by another study (Vincent *et al.* 2016b), the content in insertion sequences is a major difference between the genomes of mesophilic and psychrophilic *A. salmonicida* strains, and further studies are needed to assess if these elements are involved in creating the lifestyle differences between psychrophilic and mesophilic *A. salmonicida* subspecies. To get a more complete picture of the evolution of *A. salmonicida* and to help clarify the taxonomy of this species, reference closed genomes for subspecies *achromogenes*, *smithia*, *masoucida* and *pectinolytica* are required.

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References

Abascal F, Zardoya R, Telford MJ. TranslatorX: Multiple alignment of nucleotide sequences guided by amino acid translations. *Nucleic Acids Res* 2010;**38**:W7–13.

Abby SS, Cury J, Guglielmini J *et al.* Identification of protein secretion systems in bacterial genomes. *Sci Rep* 2016;**6**:23080.

Arndt D, Grant JR, Marcu A *et al.* PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* 2016;**44**:W16–21.

Austin B, Austin DA. Aeromonadaceae Representative (*Aeromonas salmonicida*). In: Austin B, Austin DA (eds.). *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*. Cham: Springer International Publishing, 2016, 215–321.

Austin B. Taxonomy of bacterial fish pathogens. *Vet Res* 2011;**42**:20

Austin DA, McIntosh D, Austin B. Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. *Syst Appl Microbiol* 1989;**11**:277–90.

Casas C, Anderson EC, Ojo KK *et al.* Characterization of pRAS1-like plasmids from atypical North American psychrophilic *Aeromonas salmonicida*. *FEMS Microbiol Lett* 2005;**242**:59–63.

Chu S, Cavaignac S, Feutrier J *et al.* Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. *J Biol Chem* 1991;**266**:15258–65.

Colston SM, Fullmer MS, Beka L *et al.* Bioinformatic genome comparisons for taxonomic and phylogenetic assignments using *Aeromonas* as a test case. *MBio* 2014;**5**:1–13.

Colwell RR, Macdonell MT, De Ley J. Proposal to recognize the family *Aeromonadaceae* fam. nov. *Int J Microbiol* 1986;**36**:473–7.

Contreras-Moreira B, Vinuesa P. GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis. *Appl Environ Microbiol* 2013;**79**:7696–701.

Cowan ST, Steel KJ, Barrow GI *et al. Cowan and Steel's manual for the identification of medical bacteria*. 3rd ed., 1. Cambridge, UK: Cambridge University Press, 2003.

Criscuolo A, Gribaldo S. BMGE (block mapping and gathering with entropy): a new software for selection of phylogenetic informative regions from multiple sequence

alignments. *BMC Evol Biol* 2010;**10**:210.

Daher RK, Filion G, Tan SGE *et al.* Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions. *Vet Microbiol* 2011;**152**:353–60.

Dallaire-Dufresne S, Tanaka KH, Trudel MV *et al.* Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* 2014;**169**:1–7.

Emmerich R, Weibel E. Über eine durch Bakterien erzengte Seuche unter den Forellen. *Arch für Hyg und Bakteriol* 1894;**21**:1–21.

Emond-Rheault J-G, Vincent AT, Trudel MV *et al.* Variants of a genomic island in *Aeromonas salmonicida* subsp. *salmonicida* link isolates with their geographical origins. *Vet Microbiol* 2015;**175**:68–76.

Gerhardt P. *Methods for general and molecular bacteriology*. Washington: American Society for Microbiology, 1994.

Grissa I, Vergnaud G, Pourcel C. CRISPRFinder: a web tool to identify clustered regularly interspace short palindromic repeats. *Nucleic Acids Res* 2007;**35**:52–7.

Gustafson CE, Chu S, Trust TJ. Mutagenesis of the paracrystalline surface protein array of *Aeromonas salmonicida* by endogenous insertion elements. *J Mol Biol* 1994;**237**:452–63.

Hunt M, Silva N De, Otto TD *et al.* Circlator: automated circularization of genome assemblies using long sequencing reads. *Genome Biol* 2015;**16**:294.

Iwata S. Structure and function of bacterial cytochrome *c* oxidase. *J Biochem* 1998;**123**:369–75.

Jia B, Raphenya AR, Alcock B *et al.* CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res*

2016;**45(D1)**:D566-73

L'Abée-Lund TM, Sørum H. Class 1 integrons mediate antibiotic resistance in the fish pathogen *Aeromonas salmonicida* worldwide. *Microb Drug Resist* 2001;**7**:263–72.

Long M, Nielsen TK, Leisner JJ *et al. Aeromonas salmonicida* subsp. *salmonicida* strains isolated from Chinese freshwater fish contain a novel genomic island and possible regional-specific mobile genetic elements profiles. *FEMS Microbiol Lett* 2016;**363**:fnw190.

Martin-Carnahan A, Joseph S. *Aeromonadales* ord. nov. In: Brenner D, Krieg N, Staley J, et al. (eds.). *Bergey's Manual® of Systematic Bacteriology SE - 12*. Springer US, 2005, 556–87.

Nagar V, Shashidhar R, Bandekar JR. Prevalence, characterization, and antimicrobial resistance of *Aeromonas* strains from various retail food products in Mumbai, India. *J Food Sci* 2011;**76**:M486–92.

Nguyen LT, Schmidt HA, Von Haeseler A *et al.* IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 2015;**32**:268–74.

Pavan ME, Abbott SL, Zorzópulos J *et al. Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. *Int J Syst Evol Microbiol* 2000;**50**:1119–24.

Piotrowska M, Popowska M. Insight into the mobilome of *Aeromonas* strains. *Front Microbiol* 2015;**6**:494.

Reith ME, Singh RK, Curtis B *et al.* The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* 2008;**9**:427.

Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci USA* 2009;**106**:19126–31.

Siguier P, Perochon J, Lestrade L *et al.* ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 2006;**34**:D32–6.

Tanaka KH, Dallaire-Dufresne S, Daher RK *et al.* An insertion sequence-dependent plasmid rearrangement *in Aeromonas salmonicida* causes the loss of the type three secretion system. *PLoS One* 2012;**7**:e33725.

Tanaka KH, Vincent AT, Emond-Rheault J-G *et al.* Plasmid composition in *Aeromonas salmonicida* subsp. *salmonicida* 01-B526 unravels unsuspected type three secretion system loss patterns. *BMC Genomics* 2017;**18**:528.

Tanaka KH, Vincent AT, Trudel MV *et al.* The mosaic architecture of *Aeromonas salmonicida* subsp. *salmonicida* pAsa4 plasmid and its consequences on antibiotic resistance. *PeerJ* 2016;**4**:e2595.

Trudel MV., Vincent AT, Attéré SA *et al.* Diversity of antibiotic-resistance genes in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida*: dominance of pSN254b and discovery of pAsa8. *Sci Rep* 2016;**6**:35617.

Vandecraen J, Chandler M, Aertsen A *et al.* The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit Rev Microbiol* 2017;**43**:709-30.

Varani AM, Siguier P, Gourbeyre E *et al.* ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. *Genome Biol* 2011;**12**:R30.

Varshavsky A. “Spalog” and “sequelog”: neutral terms for spatial and sequence similarity. *Curr Biol* 2004;**14**:R181–3.

Verdet C, Benzerara Y, Gautier V *et al.* Emergence of DHA-1-producing *Klebsiella* spp. in the parisian region: Genetic organization of the *ampC* and *ampR* genes originating from *Morganella morganii*. *Antimicrob Agents Chemother* 2006;**50**:607–17.

Vincent AT, Boyle B, Derome N *et al.* Improvement in the DNA sequencing of genomes bearing long repeated elements. *J Microbiol Methods* 2014a;**107**:186–8.

Vincent AT, Charette SJ. Phylogenetic analysis of the fish pathogen *Aeromonas salmonicida* underlines the dichotomy between European and Canadian strains for the *salmonicida* subspecies. *J Fish Dis* 2017;**40**:1241–7.

Vincent AT, Emond-Rheault J-G, Frenette M *et al.* Antibiotic resistance due to an unusual ColE1-type replicon plasmid in *Aeromonas salmonicida*. *Microbiology* 2016a;**162**:942–53.

Vincent AT, Trudel MV, Freschi L *et al.* Increasing genomic diversity and evidence of constrained lifestyle evolution due to insertion sequences in *Aeromonas salmonicida*. *BMC Genomics* 2016b;**17**:1–12.

Vincent AT, Trudel M V, Paquet VE *et al.* Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug-resistance, interspecies exchanges, and plasmid reshaping. *Antimicrob Agents Chemother* 2014b;**58**:7367–74.

Chapitre 7 – Article 6

Characterization and diversity of phages infecting *Aeromonas salmonicida* subsp. *salmonicida*

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Résumé

L'étude des phages d'*A. salmonicida* remonte à des décennies. Malgré cela, très peu ont été caractérisés au niveau génomique. Dans la présente étude, les génomes de 12 phages infectant *A. salmonicida*, dont trois nouvellement isolés, ont été séquencés et analysés. Il a été possible de mettre en place une catégorisation génomique de ces phages ainsi que de ceux dont les génomes étaient déjà disponibles publiquement. Des gènes sous-sélection positive ont été trouvés et nous avons renforcé une hypothèse stipulant que les génomes de certains phages infectant *A. salmonicida* possèdent des gènes pour des ARNt puisque le répertoire de l'hôte est inefficace pour traduire certains codons rares. De plus, les 12 phages ont été confrontés à 65 souches d'*A. salmonicida*, dont la majorité est de la sous-espèce *salmonicida*. Cette caractérisation lytique a mis en lumière trois groupes de phages selon le nombre de souches lysées.

Abstract

Phages infecting *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of the fish disease furunculosis, have been isolated for decades but very few of them have been characterized. Here, the host range of 12 virulent phages, including three isolated in the present study, was evaluated against a panel of 65 *A. salmonicida* isolates, including representatives of the psychrophilic subspecies *salmonicida*, *smithia*, *masoucida,* and the mesophilic subspecies *pectinolytica.* This bacterial set also included three isolates from India suspected of being members of a new subspecies. Our results allowed to elucidate a lytic dichotomy based on the lifestyle of *A. salmonicida* (mesophilic or psychrophilic) and more generally, on phage types (lysotypes) for the subspecies *salmonicida*. The genomic analyses of the 12 phages from this study with those available in GenBank led us to propose an *A. salmonicida* phage pan-virome. Our comparative genomic analyses also suggest that some phage genes were under positive selection and *A. salmonicida* phage genomes having a discrepancy in GC% compared to the host genome encode tRNA genes to likely overpass the bias in codon usage. Finally, we propose a new classification scheme for *A. salmonicida* phages.

Introduction

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is a major fish pathogen that is responsible for significant economic losses in the aquaculture industry worldwide (Dallaire-Dufresne *et al.* 2014). More recently, genomic studies on *A. salmonicida* have led to a better understanding of its evolutionary history and global diversity, including its large number of mobile genetic elements (Emond-Rheault *et al.* 2015a, 2015b; Vincent *et al.* 2016) as well as its lifestyle (psychrophilic or mesophilic) (Pavan *et al.* 2000; Vincent *et al.* 2016).

As with other bacteria, *A. salmonicida* subsp. *salmonicida* can be infected by viruses, namely bacteriophages or phages. Moreover, it is generally recognized that phages play significant roles in microbial ecology through bacterial lysis, reprograming of host metabolism and horizontal gene transfer (Stern & Sorek 2011; Koskella & Brockhurst 2014; Klimenko *et al.* 2016). One of the first studies on phages infecting *A. salmonicida* was published in 1933 and reported the presence of these biological entities in English rivers (Todd 1933). Another study on *A. salmonicida* phages published in 1970 (Popoff & Vieu 1970) identified various lysotypes (i.e., groups of bacterial strains based on their sensitivity to specific phages), suggesting that *A. salmonicida* is subject to infection by dynamic and diverse groups of phages. It was later shown that the A-layer, a bacterial surface structure implicated in virulence (Kay *et al.* 1981), may also be a phage receptor in *A. salmonicida* (Ishiguro *et al.* 1983). Both virulent phages, which produce virions after lysis of the host cell, and temperate phages, which can integrate their genome into the host bacterial chromosome, have been isolated for *A. salmonicida* (Paterson *et al.* 1969; Popoff & Vieu 1970; Ishiguro *et al.* 1980; Ishiguro *et al.* 1984; Petrov *et al.* 2010; Kim *et al.* 2012a, 2012b, 2012c).

With the rise of bacterial strains resistant to antibiotics, phages are now being revisited as a potential complement or alternative to antibiotics to treat bacterial infections (Reardon 2014). For example, phages were explored to treat or prevent furunculosis, a disease caused by *A. salmonicida* subsp. *salmonicida* (Richards 2014). Although phage therapy remains a promising strategy, there are potential drawbacks associated with the use of phages as antibacterial agents. For example, lysogenic conversion, the process whereby the host

capitalizes on the genes encoded by prophage to enhance its own fitness, has conferred new capabilities in a wide range of bacterial species. It has now been established that some genes related to drug resistance (Modi *et al.* 2013), metabolism (Paul 2008) and virulence (Waldor & Mekalanos 1996) in several bacteria are a result of lysogenic conversion. Thus, before using phages as therapeutics, it is important to characterize them at the genomic and phenotypic levels.

Although viruses are the most abundant biological entities on earth and are implicated in various important ecological processes (Sime-Ngando 2014), their genomic sequences represent only a small fraction in public databases. For example, only 10 complete genome sequences of phages infecting *A. salmonicida* are currently available (phages 25, 56, 31, 65, 44RR2.8t, Aes508, AS4, AS5, AS7, and PX29). These phages are classified in the *Myoviridae* family (dsDNA genome, long contractile tail), except phage AS7, which is a member of the *Podoviridae* family (dsDNA genome, short tail). The genomes of five *Aeromonas* phages (Aeh1, Aes012, CC2, pAh6-C, ΦO18P) infecting other species (*hydrophila, media*) are also available in GenBank.

Based on comparative genomic analyses with other phages (Petrov *et al.* 2010; Comeau *et al.* 2012), *A. salmonicida* phages have been classified by the International Committee on Taxonomy of Viruses (ICTV) into several different taxa. For example, the *Secunda5virus* genus of the *Myoviridae* family includes the viral species *Aeromonas virus 25, Aeromonas virus 31, Aeromonas virus Aes508,* and *Aeromonas virus AS4.* The *Biquartavirus* genus of the *Myoviridae* family comprises the species *Aeromonas virus 44RR2.8t.* In addition, there are several species of *Aeromonas* myophages that have yet to be assigned a genus, such as *Aeromonas virus 65.*

Here, we have significantly increased the genomic information of *Aeromonas* phages. The complete genomes of 12 phages infecting *A. salmonicida* subsp. *salmonicida* were obtained and analyzed. The antimicrobial potential of these phages was also evaluated against a panel of 65 strains of *A. salmonicida*. Additionally, we propose a revised classification scheme for phages infecting *A. salmonicida* subsp. *salmonicida*.

Results and Discussion

A. salmonicida phages

One of the goals of this study was to isolate and characterize new virulent phages able to infect *A. salmonicida* subsp. *salmonicida* and to assess the diversity of phages infecting this species. Water samples were collected from three rivers, including two passing through fish farms within the Province of Quebec (Canada). All samples tested were found to contain *A. salmonicida* subsp. *salmonicida* phages. Three phages were purified and named SW69-9, L9-6 and Riv-10. As shown in Figure 33, the three phages belong to the *Myoviridae* family with an elongated capsid and a contractile tail. As determined by one-step growth curves (see Supplementary Fig. S1 online), the burst sizes were very low at 3, 2 and 2 new virions per infected cell for phages SW69-9, L9-6 and Riv-10, respectively while the latent period was long at 145, 150 and 142 minutes. Such low burst sizes were not unexpected since *Aeromonas virus 31*, which is genetically close (see Pan-genome analysis section), is known to have a burst size of 7 new virions per infected cell (Popoff 1971, 1973).

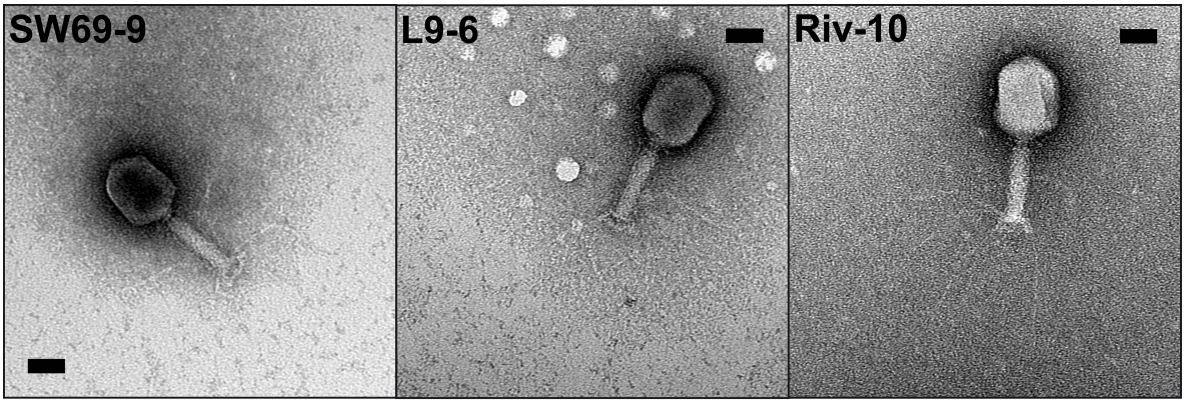

Figure 33. Micrographs of phages SW69-9, L9-6 and Riv-10.
The average head/tail length and diameter are indicated below each phage. The bars represent 50 nm.

Nine other virulent phages infecting *A. salmonicida* were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses (phage 3 (HER84), 31 (HER105), 32 (HER106), 51 (HER108), 56 (HER109), 59.1 (HER100), 65 (HER110), 44RR2.8t (HER98), and Asp37 (HER99)). The electron micrographs of these phages are available online on the Félix d'Hérelle Reference Center website (http://www.phage.ulaval.ca) and are all classified in the *Myoviridae* family.

Genomic characterization

The complete genomes of the 12 phages (9 from the Félix d'Hérelle Reference Center for Bacterial Viruses and the 3 phages isolated from the environmental samples in this study) were sequenced using Illumina technology and *de novo* assembled, resulting in a final database of 18 *A. salmonicida* subsp. *salmonicida* phage genomes, which includes the six genomes already available through GenBank, namely the podophage AS7 and the myophages 25, Aes508, AS4, AS5, and PX29 (Table 11). It should be noted that we resequenced the genomes of phages 31, 56 [deposited as vB_AsaM-56], 65, and 44RR2.8t and subsequently renamed them 31.2, 56, 65.2 and 44RR2.8t.2. Non-synonymous mutations were present between the two versions of the four genomes (see Supplementary Table S1 online), suggesting that either these phages have evolved or that these discrepancies are due to the use of a different sequencing technology and genome assembly software.

A striking feature was the pronounced divergence in GC% between phages with small (56% ± 1.29) or medium/large (42.27% ± 2.19) genome (Table 11). These results were surprising because in general the GC% of phage genomes tend to correspond with the GC content of their bacterial hosts (Xia & Yuen 2005), which in the case of *A. salmonicida* subsp. *salmonicida*, is 58.5%. A previous study on codon co-evolution showed that *A. salmonicida* phage genomes with small GC% encode tRNA genes that permit the phages to presumably bypass codons overused in the phage genes, since the repertoire of tRNAs from the host is likely inadequate to translate efficiently phage mRNAs (Prabhakaran *et al.* 2014). Our larger dataset of phages allowed us to investigate whether phages with low or high GC% can be statistically differentiated based on (1) the relative synonymous codon usage (RSCU) and (2) the amino acids composition. Principal component analysis (PCA) showed in both cases a clear separation between genomes having low or high GC% (see Supplementary Fig. S2 online). Interestingly, although the genome with the lowest GC% (phage 65.2 with 37%) generally clustered with other low-GC% genomes (see Pan-genome analysis section), it was a distinct outlier, which is concordant with its extreme GC%. Also, an analysis of our dataset showed a perfect correlation between the presence or absence of tRNA genes and the GC% of the genomes (Table 11).

Tableau 11. Phages infecting *A. salmonicida* used in this study.

Name	HER[a]	Taxonomy	Isolation	Genome size (bp)	CDSs	tRNAs	GC%	Integrase	GenBank	Reference
Phages having a small genome										
AS7	N/A[b]	*T7-like*[c]	N/A	41,572	53	0	57	No	JN651747.1	(Kim *et al.* 2012c)
51	108	*Myoviridae*	France	43,551	84	0	55	Yes[d]	KY290953	(Popoff & Vieu 1970)
56	109	*Myoviridae*	France	43,551	84	0	55	Yes[d]	KY290954	(Popoff & Vieu 1970)
59.1	100	*Myoviridae*	Canada	46,057	87	0	54	Yes	KY290950	(Paterson *et al.* 1969)
3	84	*Myoviridae*	France	46,349	83	0	57	Yes	KY290947	(Popoff & Vieu 1970)
Asp37	99	*Myoviridae*	Canada	47,977	83	0	57	Yes	KY290949	(Paterson *et al.* 1969)
32	106	*Myoviridae*	France	48,252	83	0	57	Yes	KY290952	(Popoff & Vieu 1970)
Phages having a medium genome										
Aes508	N/A	*Myoviridae* (*Secunda5virus*)	N/A	160,646	230	10	41	No	JN377894.1	N/A
25	85	*Myoviridae* (*Secunda5virus*)	France	161,475	232	11	41	No	DQ529280.1	(Popoff & Vieu 1970)
AS4	N/A	*Myoviridae* (*Secunda5virus*)	Korea	163,875	268	15	41	No	HM452125.1	(Kim *et al.* 2012a)
44RR2.8t.2	98	*Myoviridae* (*Biquartavirus*)	Canada	173,590	253	16	44	No	KY290948	(Paterson *et al.* 1969)
31.2	105	*Myoviridae* (*Secunda5virus*)	France	172,957	245	16	44	No	KY290951	(Popoff & Vieu 1970)
SW69-9	523	*Myoviridae*	Canada	173,097	249	16	44	No	KY290958	This study
L9-6	524	*Myoviridae*	Canada	173,578	251	16	44	No	KY290956	This study
Riv-10	525	*Myoviridae*	Canada	174,311	249	16	44	No	KY290957	This study
Phages having a large genome										
PX29	N/A	*Myoviridae*	N/A	222,006	322	24	42	No	GU396103.1	(Petrov *et al.* 2010)
AS5	N/A	*Myoviridae*	Korea	225,268	333	25	43	No	HM452126.1	(Kim *et al.* 2012b)
65.2	110	*Myoviridae*	France	236,567	410	18	37	No	KY290955	(Popoff & Vieu 1970)

a: Refers to the Felix d'Hérelle collection (http://www.phage.ulaval.ca) number.
b: Means none applicable.
c: Only based on bioinformatics inference.
d: Low confidence in the gene's identity .

Pan-genome analysis

The pan-genome (i.e., whole gene repertoire of a study group) of our current phage dataset was determined. An analysis that included all the ORFs from all 18 phage genomes (3599 sequences) grouped them into 1222 clusters. As expected considering phage diversity, no cluster contained ORFs from all phage genomes. Consequently, there is no core-genome when one takes all 18 genomes into consideration (see Supplementary Fig. S3 online). Although via manual curation we were able to identify a large terminase subunit that was present in all of the genomes, the percent sequence similarity was so low between some sequences (see Supplementary Fig. S4 online) that they were not considered as homologous by GET_HOMOLOGUES (see Methods section). However, even with a high degree of

sequence divergence, phylogenetic clusters based on large terminase subunit sequences are well known to correlate with DNA packaging strategies (Merrill *et al.* 2016). We assembled a database of the large terminase subunit sequences of the 18 *A. salmonicida* phages from this study and 78 other terminase sequences from phages known to have different DNA packaging strategies, many of which were experimentally validated, to generate a large-scale molecular phylogeny (see Supplementary Fig. S5 online). The results of this analysis suggest that most of the 18 phages from our study have a headful packaging strategy, although one phage (AS7) has short direct terminal repeats (DTRs) and four (3, Asp37, 32 and 59.1) use 5' protuberant *cos* ends. These findings support the view that a diverse phage population infects strains of *A. salmonicida*.

However, it also complicates their phylogenetic analysis because the sequences do not share a sufficient number of valuable sites to infer robust relative phylogenetic positions among the phages without introducing a clear functional bias. Even by using a Bayesian phylogeny approach with the site-heterogeneous model CAT (Lartillot & Philippe 2004), an approach known to reduce artefacts due to long branch attraction (Lartillot *et al.* 2007), we recovered a tree topology with poor statistical support where the podophage AS7 was basal to the clade comprised of phages with medium and large genomes (see Supplementary Fig. S6A online). We assessed the potential saturation of the phylogenetic matrix by plotting the uncorrected p-distance with the pairwise distances in branch lengths from the tree (Borowiec *et al.* 2015). Since the resulting plot clearly showed a non-linearity, we concluded that any true evolutionary signals were obfuscated by saturation and homoplasy (see Supplementary Fig. S6B online). In order to bypass this issue, we encoded the pan-genome matrix generated by the previous analysis in binary (i.e. presence/absence of gene clusters) and then performed hierarchical clustering (Figure 34). The nodes within this tree were well supported (Approximately Unbiased (AU) p-value greater than 95), with some exceptions. With this approach, the phage genomes clustered based on their size (small: 45,329 bp ± 2,502; medium: 169,191 bp ± 6,036; large: 227,947 bp ± 7,641). By coupling this tree with a sequence alignment (Figure 34), we were able to classify the phages into six groups (I to VI), with the fourth group further divided into subgroups A and B. We supported this grouping by combining the tree with a resulting identity matrix based on BLASTP (see Supplementary Fig. S7 online). Interestingly,

phages also clustered together based on their predicted mode of DNA packaging (Figure 34). These results show that even if phages infect the same host, they may exhibit considerable genomic diversity.

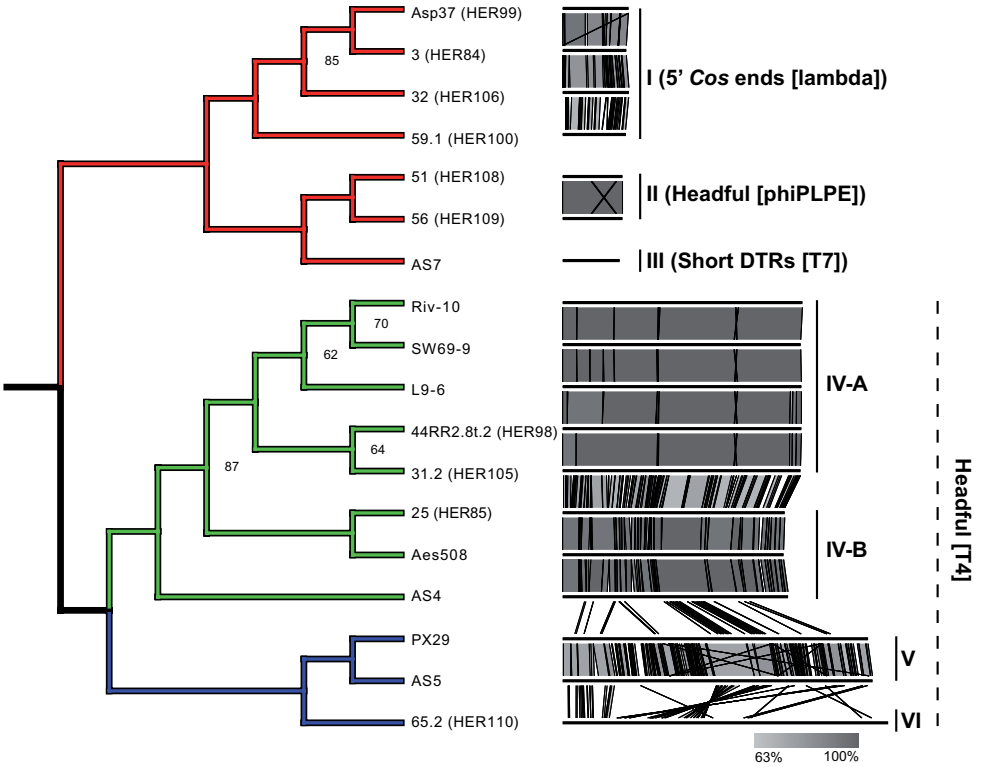

Figure 34. Clustering based on the gene repertoire.

Phages having a small, medium and large size genome are in red, green and blue, respectively. The AU (Approximately Unbiased) p-value is indicated at each node when inferior to 100. Alignment of genomes and the proposed grouping are coupled to the tree. The inferred mode of DNA packaging is indicated for each cluster.

The group III contained the only *Aeromonas* phage of the *Podoviridae* family. Our phylogeny clustered, *Aeromonas viruses 25, Aes508,* and *AS4* into the same subgroup (IV-B), indicating that they are closely related, and likely in the same species. In contrast the ICTV has classified these three phages as different species albeit in the same genus (*Secunda5virus*). Moreover, *Aeromonas viruses 31.2* and *44RR2.8t.2*, which are also considered different species by the ICTV, clustered in subgroup IV-A along with the three new phages isolated in this study (Riv-10, SW69-9, and L9-6). As shown in Figure 34 and with a focus on the group IV-A (see Supplementary Fig. S8 online), the new isolated phages share genomic features, even if they were isolated from various sites within the Province of Quebec. It is also interesting to note that the three new phages from the Province of Quebec are as genetically distant between them (~97.8% of whole genome

identity) as *Aeromonas virus 31.2* is from *44RR2.8t.2* (97.2% of whole genome identity). The latter were isolated in France and Ontario, respectively (see Supplementary Fig. S9) and are the most similar phage genome sequences available in GenBank. Phages Asp37, 3, 32 and 59.1 were clustered in group I, while phages 51 and 56 were combined in group II. Phages PX29 and AS5 were found in group V and finally, phage 65.2 was placed in group VI. Clearly, ICTV uses a very stringent approach to speciate phages, which otherwise seem highly similar at the genomic level. A 95% DNA sequence identity (BLASTN algorithm) was apparently chosen by the ICTV as the criterion for demarcation of species in the *Secunda5virus* genus.

Because no gene clusters were shared by all the genomes, we identified the core-genome for each group and verified the number of shared core-clusters among them. We were unable to identify a core cluster among the small genome phages (I, II and III) or shared between the small and the medium size phage genomes (IV-A and IV-B), underlining the extreme sequence heterogeneity among these viruses (see Supplementary Fig. S10 online). Additionally, we could not identify a core gene cluster among the small genomes and group V, a group that contains phages with larger genomes. There is a small core-cluster, however, shared between the genome of phages AS7 (III) and 65.2 (VI). This is surprising given that AS7 belongs to the *Podoviridae* family while 65.2 is a myophage. The shared gene encodes a hypothetical protein without any known function. A BLASTP analysis uncovered an orthologous gene in the genome of CC2, a phage that infects *Aeromonas hydrophila* (Shen *et al.* 2012). But again, this putative phage protein has yet to be assigned a function.

The medium and large size genomes are less heterogeneous in their gene repertoire and thus allowed us to identify a larger core genome (Figure 35A). Our analysis identified 37 clusters that were shared amongst medium and large genomes. These genes were grouped by functional categories accordingly to another study (Figure 35B)(Miller *et al.* 2003). More than half (~66%) of the coding sequences (CDSs) can be grouped into two categories: non-structural (i.e. DNA replication, recombination, repair, packaging), and structural (capsid, tail etc.) proteins. Interestingly, 16% of the CDSs could not be assigned to a

functional category. These CDSs included four hypothetical proteins, a putative nicotinamide phosphoribosyl transferase and a lysozyme.

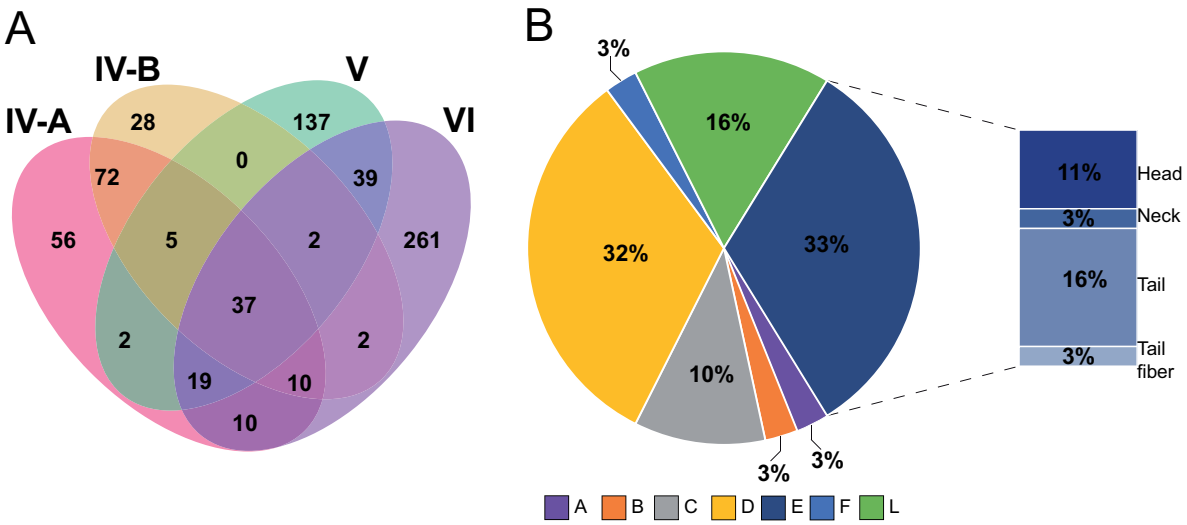

Figure 35. Shared core-clusters analysis.
The shared core-clusters between medium (IV-A and IV-B) and large (V and VI) genomes are represented as a Venn diagram (**A**). The 37 core-clusters shared by the medium- and large-size genomes were grouped by functional categories (**B**). The categories are: (A) Transcription, (B) Translation, (C) Nucleotide metabolism, (D) DNA replication, recombination, repair, packaging, and processing, (E) Virion proteins, (F) Chaperonins/assembly catalysts and (L) Others.

Genes under positive selection

Our next objective was to investigate whether the orthologous genes that we had identified in the previous analysis were under positive selection (also known as diversifying or Darwinian selection). A standard procedure to quantify the selection of a gene is by calculating the d*N*/d*S* ratio, d*S* being the synonymous substitution rate (assumed to be neutral) and d*N* being the non-synonymous substitution rate (an indicator of positive selection since the amino acid composition of the encoded protein was modified) (Mugal *et al.* 2014). When two phages co-infect a bacterial cell, their genomes may exchange genetic segments through recombination (Pérez-Losada *et al.* 2015). Recombination can drive genome evolution but also bias in the detection of selection by increasing the number of false positively selected sites (Pérez-Losada *et al.* 2015). We evaluated the recombination events and partitioned each gene based on where these events may have taken place. We then used an algorithm optimized to infer positive selection from the recombining coding sequences identified in the previous analysis.

Our results identified five phage genes that were significantly ($p < 0.05$) under positive selection (Table 12). The gene under positive selection with the smallest *p*-value was *ndd*, a gene that encodes a protein implicated in the disruption of the bacterial nucleoid (Snustad *et al.* 1974). The second most significant gene under positive selection was gene *6*, encoding the baseplate protein gp6. This protein is likely present in multiple copies and forms a continuous ring around the central hub while playing a critical role in the assembly and function of the baseplate (Taylor *et al.* 2016; Yap *et al.* 2016). During the infection of an *Escherichia coli* cell by the myophage T4, six long-tail fibers interact reversibly to the cell-surface of the host, followed by the attachment of short-tail fibers that bind to additional receptors on the cell surface in an irreversible fashion. The attachment of the short-tail fibers triggers a conformation change of the baseplate from a dome-shaped to a star-shaped (Kostyuchenko *et al.* 2003). Recent studies have shown that gp6 is one of the key proteins in the signal transmission from the short-tail fibers to the central region of the baseplate (Taylor *et al.* 2016; Yap *et al.* 2016) during this conformational shift. Gene *6* was found in medium and large size genomes, with the exception of phage AS4. Deeper investigation led to the identification of a *6-like* gene within the genome of AS4, but with multiple frameshifts. It is tempting to speculate that these frameshifts may be the result of sequencing artefacts given the vital role of gp6. It is not clear why gene *6* is under positive selection, but if gp6 is implicated in host infection as it appears, mutations to this gene may alter attachment kinetics of the phage to the cell surface, leading to changes in host specificity.

Tableau 12. Genes under positive selection.

Gene name	Protein	*p* value	Clusters
ndd	Host nucleoid disruption protein	0,0096557	IV-A, IV-B
6	gp6 base plate wedge component	0,0126069	IV-A, IV-B[a], V, VI
N/A	Hypothetical protein[b]	0,0143135	I
44	gp44 clamp-loader subunit	0,0212075	IV-A, IV-B, V, VI
60plus39	Topoisomerase II large subunit	0,04291	IV-A, IV-B, V, VI

a : The gene contains multiple frameshifts for AS4 and was consequently not added to the analysis.
b : *de novo* predicted by the present study.

The last three genes under positive selection code for a hypothetical protein, the clamp-loader subunit gp44 and a topoisomerase II large subunit. It is worth noting that the gene encoding the hypothetical protein is the only gene from phages with small genomes found

to be under positive selection. Additionally, the topoisomerase II large subunit is the product of two genes (*39* and *60*) in phage T4 (Huang *et al.* 1985) and thus may be a genomic region under disproportionate positive selection.

Host range of the phages

Temperate phages are able to integrate their genomes into the chromosome of their host. In some instances, these phage-encoded genes are used by the host to its own advantage. We investigated each of the 18 genomes for the presence of an integrase gene, the hallmark of temperate phages. A gene coding for an integrase was found in all the genomes of phages in group I (3, Asp37, 59.1, and 32). This suggests that these phages are likely capable of lysogeny and are therefore not good candidates for phage therapy. The genomes of phages 51 and 56, both from the group II, appeared to harbour a gene encoding for a truncated integrase, however we have low confidence in the gene's identity. The podophage AS7 (group III) is the only phage having a small genome that does not harbour a gene encoding for an integrase. No genes coding for antibiotic resistance and/or virulence factors were identified in any genome.

We assessed the host range of the 12 phages with a panel of 65 *A. salmonicida* isolates (Figure 36). To investigate the specificity of these phages, isolates from subspecies other than *salmonicida* were added: *smithia*, *masoucida*, *pectinolytica* as well as three Indian strains suspected of being part of a new *A. salmonicida* subspecies (Vincent *et al.* 2016). The resulting host range patterns coupled to a clustering (heatmap) allowed us to classify the phages into three groups.

The first group contained phages (phages 59.1 (genomic cluster I) and 56 (II)) with the narrowest host range, where only few bacterial isolates (<5 out of 65) were sensitive to the phages based on spot tests (Figure 36). As indicated previously, the genome of phage 59.1 possesses an integrase gene while it is not clear if phage 56 has a functional integrase gene. Neither of these phages was reported as temperate by the original studies that characterized them[15,39, 58]. However, the bioinformatics tool PHACTS (McNair *et al.* 2012), which predict the lifestyle of a phage, indicated that these two phages are likely temperate.

The second group, composed of phages 32 (group I), 3 (group I), 65.2 (group VI) and Asp37 (group I), displayed an intermediate host range where 10 to 30 bacterial isolates were sensitive to the phages (Figure 36). Phages 32, 3 and Asp37 have a gene encoding for an integrase, consequently with the potential to be temperate under some conditions. Even though we were unable to identify a gene coding for an integrase in the phage 65.2 genome, it has the highest GC% discrepancy (37%) with the genome GC% of its host (58.5%), raising the possibility that this phage is less efficient at lysing its host.

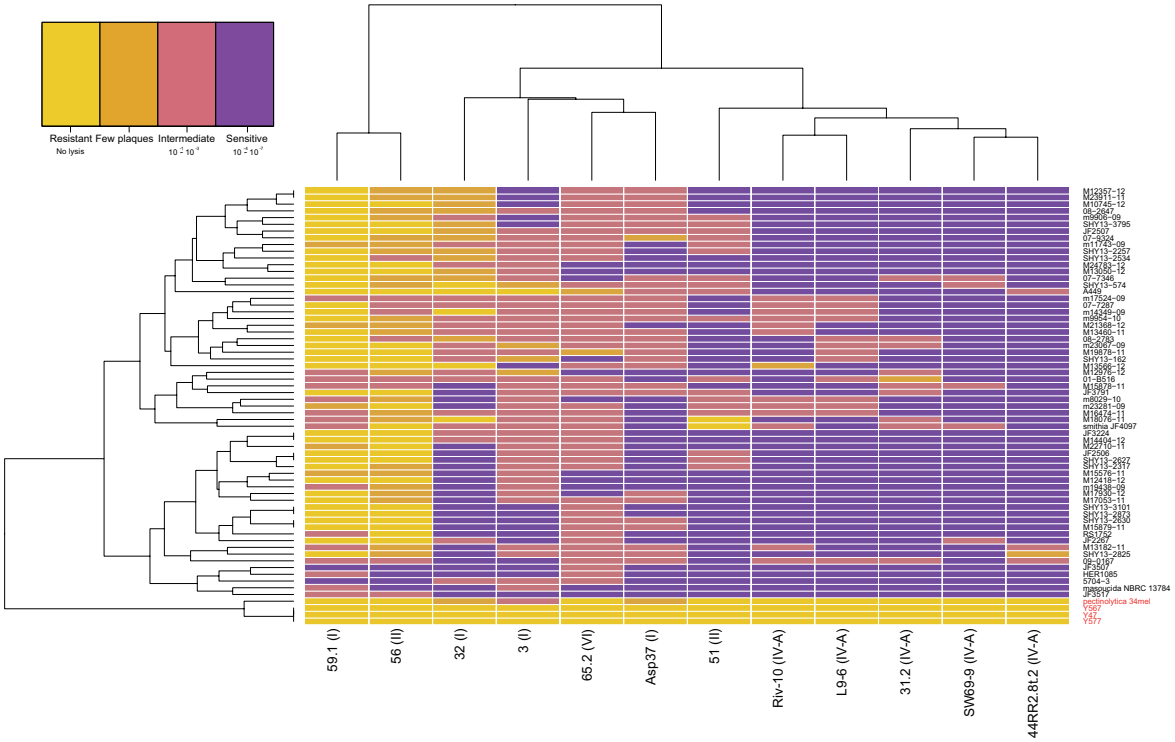

Figure 36. Clustering and heatmap based on a panel of 65 *A. salmonicida* isolates challenged with 12 phages.

The genomic cluster of each phage is indicated in parentheses. The mesophilic isolates are shown in red while the psychrophilic ones are in black. High and low lytic activities are represented in purple and yellow, respectively. In addition, the dilution ranges obtained by spot tests used to encode the matrix are indicated below the legend.

The third group included phages within the genomic cluster group IV-A and phage 51 (cluster II). Members of this group had the broadest host range (> 44 out of 65) (Figure 36). Consequently, phages from this group have the most potential from a phage therapy perspective. For example, phage 44RR2.8t.2 infected 57 out of the 65 *A. salmonicida* strains. Similarly, the newly isolated phage SW69-9 infected 56 out of the 65 strains. The host range of phages 44RR2.8t.2 and SW69-9 was overlapping and as such, all

A. salmonicida subsp. *salmonicida* strains could be infected by one of the two phages or both (Figure 36).

Surprisingly, phage 51 was able to lyse 41 strains, even if the original study reported it to be temperate (Popoff & Vieu 1970). The tool PHACTS also predicted the lifestyle of this phage as temperate, but with an uncertain probability (0.515 ± 0.042). This was even more surprising because the other phage within the cluster II, phage 56, had a very limited host range (lysed 4 strains). A global alignment of both phage genomes (51 and 56) resulted in only four non-synonymous SNPs. The first two were located in genes coding for hypothetical proteins, each having a predicted conserved unknown domain (DUF2213 and DUF2184). While the last two SNPs are in a gene encoding for a tail or truncated integrase protein. One SNP led to change from a methionine to a valine and the other one from a proline to leucine. Further work is required to determine which mutation(s) is responsible for the expanded host range.

Strains belonging to other psychrophilic subspecies (*smithia* and *masoucida*) were also sensitive to phages infecting the subspecies *salmonicida*. However, the three mesophilic strains from India (Vincent *et al.* 2016) (Y577, Y567 and Y47) and the strain of the subspecies *pectinolytica* (also having a mesophilic lifestyle (Pavan *et al.* 2000)) were insensitive to all 12 phages, at the exception of *pectinolytica*, which is sensitive to phage 3. The A-layer, which is a structure composed of a protein and lipopolysaccharides implicated in virulence (Kay *et al.* 1981), could be one of the phage receptors in *A. salmonicida* (Ishiguro *et al.* 1983). Analysis of the various bacterial genomes showed that the gene *vapA*, encoding the protein forming the A-layer, is present in psychrophilic isolates but absent in mesophilic isolates. In order to better understand host phage dynamics in mesophilic *A. salmonicida*, it could be interesting to isolate phages able to infect them and to identify the phage receptor.

It is well documented that prophages can provide resistance to infection from other phages, by superinfection exclusion (Sie) systems found in both Gram negative and positive bacteria (Labrie *et al.* 2010). At the exception of the mesophilic/psychrophilic separation, the heatmap in Figure 36 shows no clear bacterial clustering suggesting that such resistance

could be provided (see Supplementary Fig. S11 online). This is of interest because *A. salmonicida* subsp. *salmonicida* isolates usually harbour two prophages sharing structural similarities with the temperate phage ΦO18P (Beilstein & Dreiseikelmann 2008), found in *Aeromonas media*. Moreover some *A. salmonicida* subsp. *salmonicida* strains also have a new recently discovered prophage (prophage 3) and variants of a genomic island named *AsaGEI* (Emond-Rheault *et al.* 2015a, 2015b; Long *et al.* 2016). None of these mobile elements actually have a known function and we can reasonably here rule out their implication in protection against phages.

In conclusion, we investigated the genomes of 18 phages infecting *A. salmonicida* and characterized their diversity, which led to a robust classification scheme based on their genomic composition. We argue that our approach to classifying these phages will result in a more accurate characterization and classification of new *A. salmonicida* phages in the future. We also evaluated the infectivity of 12 phages, including three newly isolated phages, on a panel of 65 isolates of *A. salmonicida*. Overall, these phages showed a heterogeneous host range. Phages with overlapping and large host range were identified and hold potential to contribute to a phage cocktail to control this fish pathogen in the aquaculture industry.

Methods

Environmental sampling, phage isolation, and characterization

Skin mucus samples from furunculosis-infected fishes from four fish farms in the province of Quebec (Canada) as well as water samples (50 ml) from the same fish farms and rivers in the same region were collected and kept at 4°C. The mucus was swabbed and diluted in 5 ml of phage buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$). Each environmental sample was centrifuged for 10 min at 3200 x *g* and filtered (0.45 µm, Sarstedt, Canada). The filtrate was then mixed with the same volume of 2X TSB (EMD Millipore, Canada) and inoculated with 1% (v/v) of *A. salmonicida* subsp. *salmonicida* in exponential growth phase. The bacterial strains used were those recommended by the Felix d'Hérelle Reference Center for Bacterial Viruses (http://www.phage.ulaval.ca). All cultures were incubated at 18°C overnight and agitated at 200 RPM before being filtered again. The above amplification procedure was repeated four times or until the appearance of cell lysis

(replacing 2X TSB with 1X TSB after each round). In parallel, controls without environmental sample were inoculated with the bacterial host to compare the growth and whether lysis had occurred in challenged host cultures.

The new phages were isolated from single plaques as follows: in 3 ml of TSB soft agar (0,75%) kept at 55°C, 100 µl of filtrate and 100 µl of bacterial host (*A. salmonicida* subsp. *salmonicida* 08-2783 host of L9-6 and *A. salmonicida* subsp. *salmonicida* M15879-11 host of SW69-9 and Riv-10) were mixed and poured onto TSA plates (EMD Millipore, Canada) before being incubated at 18°C overnight. Plaques (up to 10 per sample) were picked up with sterile tips and suspended in 500 µl of phage buffer. Phages were allowed to diffuse for 30 min at room temperature before serial dilutions in phage buffer. The above plaque isolation procedure was repeated two more times. Subsequently, phages were amplified in 10 ml of TSB incubated with bacterial host at 18°C overnight and agitated at 200 RPM. Aliquots of the resulting filtrates were either stored at -80°C in 15% glycerol or stored at 4°C for up to six months until subsequent analysis.

Transmission electron microscopy was used to observe the three new phages as described elsewhere (Fortier & Moineau 2007). Briefly, 1.5 mL of phage lysate was centrifuged at 23,500 g for 1 h at 4°C and the pellet washed twice with ammonium acetate (0.1 M, pH 7.0). The resulting phage preparation was then used to prepare observation grids, which were stained with uranyl-acetate (2%) and observed with a JEOL 1230 at the microscopy platform of the Institut de Biologie Intégrative et des Systèmes (U. Laval). Capsid size and tail length were determined by measuring at least 15 different specimens.

Phage one-step growth curve assays of phages were performed in triplicate, as previously reported elsewhere (Wang *et al.* 2016). Approximately 10^9 CFU/ml of precultured cells that just reached stationary phase were harvested by centrifugation and resuspended in 900 µl of TSB. Each phage was respectively added at a multiplicity of infection (MOI) of 0.05 and allowed to adsorb for 20 minutes at 18°C. Then, infected cells were harvested by centrifugation and the pellet washed twice with 1 ml of fresh TSB. Finally, the pellet containing the infected cells was resuspended in a final dilution of 0.001 in a glass tube containing 10 ml of TSB and incubated at 18°C at 200 RPM. Every 30 min, an aliquot of

100 µl was taken to determine the phage titer, up to 210 minutes. The burst size was calculated by dividing the average phage titer after the exponential phase by the average titer before the infected cells began to release virions (Moineau *et al.* 1993). The latent period was evaluated according to the median of the exponential curve.

The DNA of the amplified phages was extracted using a standard phenol/chloroform protocol and then analyzed by restriction profiles using the enzymes DraI, SspI and MseI (NEB, Canada) according to the manufacturer recommendations at 37°C for one hour. Restricted phage DNA was run on a 1% agarose gel for 30 min at 90V. Three different restriction profiles were observed and the distinct phages were named SW69-9, L9-6 and Riv-10.

Sequencing and *de novo* assembly

Sequencing libraries were prepared from purified phage DNA using the Nextera XT DNA Library Preparation Kit and sequenced on an Illumina MiSeq apparatus. The resulting sequencing reads were *de novo* assembled by the pipeline A5-miseq version 20150522 (Coil *et al.* 2014) to obtain an initial sequencing depth. Given their small size and the high-throughput capabilities of the Illumina platform, viral genomes are usually sequenced with very high depth, causing an unnecessary complexity for subsequent *de novo* assembly (Wan *et al.* 2015). Consequently, the reads of each sequenced genome were randomly down-sampled by seqtk (https://github.com/lh3/seqtk) to obtain assemblies (also with A5-miseq version 20150522) having around 100X of sequencing depth.

We compared the resequenced phage genomes 44RR2.8t, 31, 56 and 65 by generating kmers with a length of 300 nt and then mapping them with BWA version 0.7.12-1039 (Li & Durbin 2009) to reference sequences. Mutations were called by using samtools version 0.1.19-44428cd (Li *et al.* 2009) and VarScan version 2.4.2 (Koboldt *et al.* 2012). Finally, the effect of mutations was evaluated based on SnpEff version 4.2 (Cingolani *et al.* 2012) (with the reference sequences added beforehand in the database).

Annotation, pan-genome and other bioinformatics analyses

Each genome, including those already available in GenBank, was annotated through the webserver RAST (Overbeek *et al.* 2014) by choosing the "virus" parameter. A list of all

predicted genes is available for each new phage: SW69-9, L9-6 and Riv-10 (see Supplementary Table S2 online). Annotated CDSs were downloaded and evaluated by GET_HOMOLOGUES version 2.0.16 (Contreras-Moreira & Vinuesa 2013). The sequences were clustered by using the COG and OMCL algorithms, both included in GET_HOMOLOGUES. Only the clusters found by both algorithms were used for downstream analyses (see Supplementary Fig. S12 online). The presence/absence binary matrix was evaluated by Pvclust (Suzuki & Shimodaira 2006) under the binary distance method with 10,000 bootstrap replicates through the statistical framework R (https://www.r-project.org) resulting in a clustering of the phages based on their gene repertoire. An identity matrix was calculated with the BLASTP scores among protein sequences using also GET_HOMOLOGUES.

Genes under positive selection and other analysis

We codon aligned the nucleotide sequences corresponding to each cluster found by GET_HOMOLOGUES and having at least four sequences by PRANK version 150803 (Löytynoja 2014). Alignments were then evaluated by GARD through HyPhy version 2.2.4 (Kosakovsky Pond *et al.* 2005) to find potential recombination events. Finally, positive selection was evaluated with the PARRIS algorithm, also through HyPhy version 2.2.4.

Each genome was screened for antibiotic resistance genes by the RGI tool of the webserver CARD (McArthur *et al.* 2013). Homology searches were performed locally with BLASTP between all the CDSs annotated by RAST and the curated PATRIC_VF database (Mao *et al.* 2015) which contained 1570 sequences of CDSs known to be implicated in virulence (downloaded on July 8th 2016) and also a database containing all integrase sequences available in the Protein database of the NCBI (1 347 621 sequences on July 8th, 2016).

Host range

The host range of 12 phages was determined using a set of 65 isolates of *A. salmonicida* (see Supplementary Table S3 online). Cells stocks were thawed, streaked on TSA plates and grown for three days at 18°C. Isolated colonies were inoculated in 3 ml TSB in sterile snap cap tubes and incubated overnight at 18°C and agitated at 200 RPM. For each strain, 100 µl of overnight bacterial culture was added to 3 ml of soft agar and poured onto a TSA

plate. Ten-fold serial dilutions of each phage (up to 10^{-7}) were done in phage buffer and 5 µl of each dilution was spotted onto the inoculated TSA plates. The plates were incubated at 18°C overnight and the sensitivity or insensitivity was recorded.

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Author's contributions

Conceived and designed the experiments: A.T.V., V.E.P., D.M.T., S.M., and S.J.C. Performed the experiments: A.T.V., V.E.P., A.B., and D.M.T. Analyzed the data: A.T.V., V.E.P., A.B., D.M.T., S.M., S.J.C. Contributed reagents/materials/analysis tools: A.T.V., V.E.P., and D.M.T. Wrote the paper: A.T.V., V.E.P., S.M., and S.J.C. All authors reviewed the manuscript.

Competing Interests

The authors declare no competing financial interests.

References

Beilstein F, Dreiseikelmann B. Temperate bacteriophage PhiO18P from an *Aeromonas media* isolate: characterization and complete genome sequence. *Virology* 2008;**373**:25–9.

Borowiec ML, Lee EK, Chiu JC *et al.* Extracting phylogenetic signal and accounting for bias in whole-genome data sets supports the Ctenophora as sister to remaining

Metazoa. *BMC Genomics* 2015;**16**:987.

Cingolani P, Platts A, Wang LL *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w 1118; iso-2; iso-3. *Fly (Austin)* 2012;**6**:80–92.

Coil D, Jospin G, Darling AE. A5-miseq : an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics* 2015;**31**:587-9.

Comeau AM, Tremblay D, Moineau S *et al.* Phage morphology recapitulates phylogeny: the comparative genomics of a new group of myoviruses. *PLoS One* 2012;**7**:e40102.

Contreras-Moreira B, Vinuesa P. GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis. *Appl Environ Microbiol* 2013;**79**:7696–701.

Dallaire-Dufresne S, Tanaka KH, Trudel MV *et al.* Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* 2014;**169**:1–7.

Emond-Rheault J-G, Vincent AT, Trudel MV *et al.* AsaGEI2b: a new variant of a genomic island identified in the *Aeromonas salmonicida* subsp. *salmonicida* JF3224 strain isolated from a wild fish in Switzerland. *FEMS Microbiol Lett* 2015a;**362**:fnv093.

Emond-Rheault J-G, Vincent AT, Trudel MV *et al.* Variants of a genomic island in *Aeromonas salmonicida* subsp. *salmonicida* link isolates with their geographical origins. *Vet Microbiol* 2015b;**175**:68–76.

Fortier LC, Moineau S. Morphological and genetic diversity of temperate phages in *Clostridium difficile*. *Appl Environ Microbiol* 2007;**73**:7358–66.

Huang WM, Wei LS, Casjens S. Relationship between bacteriophage T4 and T6 DNA topoisomerases. T6 39-protein subunit is equivalent to the combined T4 39- and 60-protein subunits. *J Biol Chem* 1985;**260**:8973–7.

Ishiguro EE, Ainsworth T, Harkness RE *et al.* A temperate bacteriophage specific for strains of *Aeromonas salmonicida* possessing A-layer, a cell surface virulence factor. *Curr Microbiol* 1984;**10**:199–202.

Ishiguro EE, Ainsworth T, Shaw DH *et al.* A lipopolysaccharide-specific bacteriophage for *Aeromonas salmonicida*. *Can J Microbiol* 1983;**29**:1458–61.

Ishiguro EE, Kay WW, Trust TJ. Temperate bacteriophages for *Aeromonas salmonicida*. *FEMS Microbiol Lett* 1980;**8**:247–50.

Kay WW, Buckley JT, Ishiguro EE *et al.* Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. *J Bacteriol* 1981;**147**:1077–84.

Kim JH, Son JS, Choi YJ *et al.* Complete genomic sequence of a T4-like bacteriophage, phiAS4, infecting *Aeromonas salmonicida* subsp. *salmonicida*. *Arch Virol* 2012a;**157**:391–5.

Kim JH, Son JS, Choi YJ *et al.* Complete genome sequence and characterization of a broad-host range T4-like bacteriophage phiAS5 infecting *Aeromonas salmonicida* subsp. *salmonicida*. *Vet Microbiol* 2012b;**157**:164–71.

Kim JH, Son JS, Choresca CH *et al.* Complete genome sequence of bacteriophage phiAS7, a T7-like virus that infects *Aeromonas salmonicida* subsp. *salmonicida*. *J Virol* 2012c;**86**:2894–5.

Klimenko AI, Matushkin YG, Kolchanov NA *et al.* Bacteriophages affect evolution of bacterial communities in spatially distributed habitats: a simulation study. *BMC Microbiol* 2016;**16**:31–41.

Koboldt DC, Zhang Q, Larson DE *et al.* VarScan 2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* 2012;**22**:568–76.

Kosakovsky Pond SL, Frost SDW, Muse SV. HyPhy: Hypothesis testing using phylogenies. *Bioinformatics* 2005;**21**:676–9.

Koskella B, Brockhurst MA. Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol Rev* 2014;**38**:916–31.

Kostyuchenko VA, Leiman PG, Chipman PR *et al.* Three-dimensional structure of bacteriophage T4 baseplate. *Nat Struct Biol* 2003;**10**:688–93.

Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. *Nat Rev Microbiol* 2010;**8**:317–27.

Lartillot N, Brinkmann H, Philippe H. Suppression of long-branch attraction artefacts in the animal phylogeny using a site-heterogeneous model. *BMC Evol Biol* 2007;**7 Suppl 1**:S4.

Lartillot N, Philippe H. A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Mol Biol Evol* 2004;**21**:1095–109.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;**25**:1754–60.

Li H, Handsaker B, Wysoker A *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* 2009;**25**:2078–9.

Long M, Nielsen TK, Leisner JJ *et al. Aeromonas salmonicida* subsp. *salmonicida* strains isolated from Chinese freshwater fish contain a novel genomic island and possible regional-specific mobile genetic elements profiles. *FEMS Microbiol Lett* 2016;**363**:fnw190.

Löytynoja A. Phylogeny-aware alignment with PRANK. *Methods Mol Biol* 2014;**1079**:155–70.

Mao C, Abraham D, Wattam AR *et al.* Curation, integration and visualization of bacterial virulence factors in PATRIC. *Bioinformatics* 2015;**31**:252–8.

McArthur AG, Waglechner N, Nizam F *et al.* The comprehensive antibiotic resistance

database. *Antimicrob Agents Chemother* 2013;**57**:3348–57.

McNair K, Bailey BA, Edwards RA. PHACTS, a computational approach to classifying the lifestyle of phages. *Bioinformatics* 2012;**28**:614–8.

Merrill BD, Ward AT, Grose JH *et al.* Software-based analysis of bacteriophage genomes, physical ends, and packaging strategies. *BMC Genomics* 2016;**17**:679.

Miller ES, Kutter E, Mosig G *et al.* Bacteriophage T4 genome. *Microbiol Mol Biol Rev* 2003;**67**:86–156.

Modi SR, Lee HH, Spina CS *et al.* Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature* 2013;**499**:219–22.

Moineau S, Durmaz E, Pandian S *et al.* Differentiation of two abortive mechanisms by using monoclonal antibodies directed toward lactococcal bacteriophage capsid proteins. *Appl Environ Microbiol* 1993;**59**:208–12.

Mugal CF, Wolf JBW, Kaj I. Why time matters: Codon evolution and the temporal dynamics of dN/dS. *Mol Biol Evol* 2014;**31**:212–31.

Overbeek R, Olson R, Pusch GD *et al.* The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res* 2014;**42**:D206–14.

Paterson WD, Douglas RJ, Grinyer I *et al.* Isolation and preliminary characterization of some *Aeromonas salmonicida* bacteriophages. *J Fish Res Board Canada* 1969;**26**:629–32.

Paul JH. Prophages in marine bacteria: dangerous molecular time bombs or the key to survival in the seas? *ISME J* 2008;**2**:579–89.

Pavan ME, Abbott SL, Zorzópulos J *et al. Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. *Int J Syst Evol Microbiol* 2000;**50**:1119–24.

Pérez-Losada M, Arenas M, Galán JC *et al.* Recombination in viruses: Mechanisms,

methods of study, and evolutionary consequences. *Infect Genet Evol* 2015;**30**:296–307.

Petrov VM, Ratnayaka S, Nolan JM *et al.* Genomes of the T4-related bacteriophages as windows on microbial genome evolution. *Virol J* 2010;**7**:292.

Popoff M, Vieu J-F. Bactériophages et lysotypie d'*Aeromonas salmonicida*. *Comptes rendus l'Académie des Sci* 1970;**270**:2219–22.

Popoff M. Etude sur les *Aeromonas salmonicida*. II. Characterisation des bacteriophages actifs sur les *Aeromonas salmonicida* et lysotypie. *Ann Rech Vet* 1971;**2**:35–45.

Popoff M. *Study of Aeromonas salmonicida II Characterization of the Phages Acting on Aeromonas salmonicida and Phage-Typing*. Halifax, N. S., 1973.

Prabhakaran R, Chithambaram S, Xuhua X. *Aeromonas* phages encode tRNAs for their overused codons. *Int J Comput Biol Drug Des* 2014;**7**:168–82.

Reardon S. Phage therapy gets revitalized. *Nature* 2014;**510**:15–6.

Richards GP. Bacteriophage remediation of bacterial pathogens in aquaculture: a review of the technology. *Bacteriophage* 2014;**4**:e975540.

Shen C, Liu Y, Lu C. Complete genome sequence of *Aeromonas hydrophila* phage CC2. *J Virol* 2012;**86**:10900.

Sime-Ngando T. Environmental bacteriophages: viruses of microbes in aquatic ecosystems. *Front Microbiol* 2014;**5**:1–14.

Snustad DP, Parson KA, Warner HR *et al.* Mutants of bacteriophage T4 deficient in the ability to induce nuclear disruption. *J Mol Biol* 1974;**89**:675–87.

Stern A, Sorek R. The phage-host arms race: Shaping the evolution of microbes. *BioEssays* 2011;**33**:43–51.

Suzuki R, Shimodaira H. Pvclust: An R package for assessing the uncertainty in

hierarchical clustering. *Bioinformatics* 2006;**22**:1540–2.

Taylor NMI, Prokhorov NS, Guerrero-Ferreira RC *et al.* Structure of the T4 baseplate and its function in triggering sheath contraction. *Nature* 2016;**533**:346–52.

Todd C. The presence of a bacteriophage for *B. salmonicida* in river waters. *Nature* 1933;**131**:360.

Vincent AT, Trudel MV, Freschi L *et al.* Increasing genomic diversity and evidence of constrained lifestyle evolution due to insertion sequences in *Aeromonas salmonicida*. *BMC Genomics* 2016;**17**:1–12.

Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 1996;**272**:1910–4.

Wan Y, Renner DW, Albert I *et al.* VirAmp: a galaxy-based viral genome assembly pipeline. *Gigascience* 2015;**4**:19.

Wang J Bin, Lin NT, Tseng YH *et al.* Genomic characterization of the novel *Aeromonas hydrophila* phage ahp1 suggests the derivation of a new subgroup from phiKMV-like family. *PLoS One* 2016;**11**:e0162060.

Xia X, Yuen KY. Differential selection and mutation between dsDNA and ssDNA phages shape the evolution of their genomic AT percentage. *BMC Genet* 2005;**6**:20.

Yap ML, Klose T, Arisaka F *et al.* Role of bacteriophage T4 baseplate in regulating assembly and infection. *Proc Natl Acad Sci* 2016;**113**:2654–9.

Chapitre 8 – Discussion générale

L'augmentation des bactéries résistantes aux antibiotiques est un phénomène à l'échelle mondiale et *A. salmonicida* subsp. *salmonicida* ne fait pas exception. En effet, celle-ci est bien connue par le service de diagnostic en ichthyopathologie de l'Université de Montréal puisque plusieurs de ces souches sont résistantes aux antibiotiques (Morin 2010; Lafaille 2016, 2017). Bien que des plasmides porteurs de gènes de résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida* avaient déjà été répertoriés avant la présente thèse (Piotrowska & Popowska 2015), leur importance dans le résistome de la bactérie était méconnue. Dans tous les cas, il était crucial d'investiguer le phénomène de résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida* et de développer des méthodes alternatives pour contrôler les infections causées par celle-ci.

Malgré son importance vétérinaire, ils existaient, en plus de la résistance aux antibiotiques, au moins deux autres volets négligés concernant *A. salmonicida* subsp. *salmonicida* et qui contribuaient à notre incompréhension de cette bactérie : sa taxonomie et sa diversité intra-espèce (Austin 2011; Austin & Austin 2016). Il y a actuellement cinq sous-espèces reconnues d'*A. salmonicida* : *salmonicida*, *smithia*, *achromogenes*, *masoucida* et *pectinolytica* (Martin-Carnahan & Joseph 2005). Cependant, le positionnement phylogénétique de ces sous-espèces les unes par rapport aux autres était particulièrement chaotique. Avant cette thèse, l'ensemble des études rigoureuses s'intéressant à la phylogénie d'*A. salmonicida* n'utilisait au maximum que quelques séquences de gènes domestiques (Pavan *et al.* 2000; Soler *et al.* 2004; Han *et al.* 2011). Similairement à l'utilisation du gène de l'ARNr 16S (Woo *et al.* 2008; Rajendhran & Gunasekaran 2011), les séquences de quelques gènes domestiques ne permettent pas de positionner avec robustesse des taxa qui sont très proches évolutivement (Hall *et al.* 2010), comme les différentes sous-espèces d'*A. salmonicida*. Il faut dire que ces études ont été publiées avant la démocratisation du séquençage à haut débit. Il était donc impensable d'utiliser plusieurs dizaines, voir centaines de séquences par génome pour des analyses phylogénétiques. Cette incompréhension taxonomique, couplée au fait que plusieurs études ne tenaient aucunement compte des différentes sous-espèces, rendait très difficile l'étude évolutive d'*A. salmonicida*, tant au niveau de sa répartition géographique, que de ses différentes

caractéristiques (résistance aux antibiotiques, virulence, flux de gènes, sensibilité aux phages, etc.).

Les buts de mes travaux de doctorat étaient d'étudier la résistance aux antibiotiques (les occurrences et les supports des déterminants de cette résistance) chez *A. salmonicida* subsp. *salmonicida* et d'évaluer la possibilité d'utiliser les phages comme agents thérapeutiques contre la furonculose. Afin de réaliser ces buts, il était essentiel de mieux connaître la bactérie, sa taxonomie, sa diversité intra-espèces et son histoire évolutive.

La résistance aux antibiotiques

Le laboratoire du professeur Steve Charette possède une importante collection de plus de 250 souches d'*A. salmonicida* isolées sur plusieurs années et provenant de divers endroits géographiques. L'ensemble de ces souches est caractérisé par antibiogrammes, PCR (ciblant les gènes de résistance aux antibiotiques) et profils de digestion plasmidique. Riches de ces informations, nous avons été en mesure de catégoriser les souches et de faire séquencer à haut débit l'ADN de certaines de celles-ci. Tel que décrit dans les chapitres 2, 3 et 4, ceci a permis de mettre en évidence plusieurs plasmides qui sont des vecteurs de gènes de résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida* (Table 13).

Tableau 13. Plasmides avec des gènes de résistance aux antibiotiques caractérisés dans la présente thèse

Plasmide	Taille (pb)	%GC	#CDS	CDS/kb	Gènes Abio^R	Référence
pAsa7	5 276	53.39	6	1 137	*cat*	(Vincent *et al.* 2016)
pRAS3.3	11 845	58.89	14	1 181	*tetA*	(Vincent *et al.* 2014b)
pAB5S9b	25 540	52.27	28	1 096	*tetH, floR, sul2, strA, strB*	(Vincent *et al.* 2014b)
pAsa8	110 577	54.87	132	1 193	*tetA, tetR, floR, tetG, sul1, blaPSE-1, aadA*	(Trudel *et al.* 2016)
pSN254b	152 216	52.47	180	1 182	*tetA, floR, sul1, sul2, blaCMY, aadA, strA, strB*	(Vincent *et al.* 2014b)

L'un de ces plasmides, pAsa7, n'a pour l'instant été trouvé que dans une souche d'origine Suisse. Ce petit plasmide semble être un dérivé du plasmide cryptique pAsa2, trouvé dans toutes les souches d'*A. salmonicida* subsp. *salmonicida* (Attéré *et al.* 2015) et qui possède un réplicon de type ColE1 (Boyd *et al.* 2003). Il est donc possible que ce plasmide soit le fruit d'un évènement unique. Cependant, cela ouvre aussi la porte à ce que les plasmides cryptiques, comme pAsa2, puissent être des supports pour acquérir de nouvelles fonctions, comme une résistance aux antibiotiques. Une étude menée en parallèle par notre équipe a d'ailleurs apporté de nouvelles évidences soutenant cette hypothèse sur les plasmides

cryptiques d'*A. salmonicida* subsp. *salmonicida* (Attéré *et al.* 2017). Il serait donc important de surveiller ces plasmides afin de suivre leur évolution. D'ailleurs, il a été proposé que les plasmides de type ColE1 puissent être d'importants vecteurs de gènes de résistance aux antibiotiques chez l'agent pathogène humain *Salmonella enterica* et que ceux-ci devraient être surveillés de près (Chen *et al.* 2010).

Un second plasmide d'intérêt caractérisé lors de la présente étude doctorale est pAsa8. Ce plasmide n'a été trouvé que dans deux isolats québécois : M15448-11 et M16474-11. Cependant, il est important de noter que les deux isolats proviennent de la même ferme piscicole et isolés la même année. Nous ne pouvons par conséquent pas exclure que les souches soient clonales ou que le plasmide ait été transféré horizontalement. De plus, comme pour pAsa7, ce plasmide pourrait être unique, dû aux différentes acquisitions d'éléments d'ADN mobiles qu'il a subies. En effet, le squelette de pAsa8 a aussi été trouvé dans *Aeromonas rivuli* DSM 22539, laissant croire que le plasmide peut se transférer entre espèces du genre *Aeromonas* et qu'il est présent chez des souches de différents continents, puisque la souche DSM 22539 provient de l'Allemagne (Figueras *et al.* 2011). Cependant, pAsa8, contrairement au plasmide trouvé chez *A. rivuli*, possède un transposon Tn*1721* et un intégron In104, tous deux apportant des gènes de résistance aux antibiotiques. Le transposon Tn*1721* avait déjà été répertorié chez *A. salmonicida* et est connu comme étant un important vecteur du gène *tetA*, conférant une résistance à la tétracycline (Rhodes *et al.* 2000). Le point culminant est que l'intégron In104 n'avait été répertorié que dans un îlot génomique, *Salmonella genomic island 1* (SGI1), trouvé chez *S. enterica* (Boyd *et al.* 2000) et *Proteus mirabilis* (Ahmed *et al.* 2007). Cette découverte montre que du matériel génétique peut être échangé entre des agents pathogènes humains et *A. salmonicida*, un agent pathogène chez les salmonidés.

Les plasmides pAB5S9b, pRAS3 et pSN254b, dû au fait qu'ils sont fréquemment retrouvés (Vincent *et al.* 2014b; Trudel *et al.* 2016), sont beaucoup plus problématiques que pAsa7 et pAsa8 pour l'aquaculture. En fait, pSN254b a été répertorié dans 82 % des souches québécoises ayant une résistance aux antibiotiques. Cette dominance de pSN254b est inquiétante puisque ce plasmide, tout comme pAB5S9b, cause une résistance à tous les antibiotiques approuvés par le gouvernement canadien pour une utilisation en aquaculture.

Cependant, cette présence élevée de pSN254b est pour l'instant localisée dans l'est du Canada alors que pAB5S9b est moins fréquent. Le plasmide pSN254b est très similaire au plasmide pSN254, trouvé chez *S. enterica* (Welch *et al.* 2007). Sachant que pAsa8 possède un intégron trouvé aussi chez *S. enterica*, il est de plus en plus évident que ces deux bactéries peuvent échanger directement ou indirectement (avec d'autres bactéries comme intermédiaires), du matériel génétique. Sachant que *S. enterica* est une bactérie présente dans plusieurs environnements, dont ceux aquatiques (Jokinen *et al.* 2015; Maurer *et al.* 2015), il est donc cohérent qu'elle soit en contact avec *A. salmonicida*. Il est raisonnable de se demander pourquoi est-ce qu'*A. salmonicida* et *S. enterica* partageraient entre elles plus d'éléments d'ADN mobiles qu'avec d'autres bactéries. Bien que cela demeure encore un mystère, il est important de noter qu'il y a plus de 7 500 génomes complets de *S. enterica* de déposer dans GenBank et que sa diversité est conséquemment bien couverte. Il n'est donc pas impossible d'exclure que cette surreprésentation des séquences de *S. enterica* comparativement à plusieurs autres bactéries dans les bases de données cause une fausse impression que celle-ci partage plus de matériel génétique.

Bien que des souches de plusieurs pays européens (Suisse, Norvège, France, Autriche et Écosse) aient été étudiées, plusieurs autres pays demeurent sans souches analysées. Les bactéries n'ont pas de frontières réelles, cependant, la régulation et l'utilisation des antibiotiques en agriculture et aquaculture diffèrent entre les pays et conséquemment la pression de sélection sur les souches résistantes aux antibiotiques (Van Boeckel *et al.* 2015). C'est pourquoi il serait primordial d'échantillonner des souches de plusieurs autres pays, dont ceux asiatiques et d'Amérique latine, afin de pouvoir continuer à investiguer la répartition des plasmides chez *A. salmonicida*.

Une chose est certaine à la lumière des chapitres 2, 3 et 4 : *A. salmonicida* subsp. *salmonicida* à la capacité d'interagir avec d'autres bactéries partageant la même niche écologique. Il existe un concept intéressant permettant d'évaluer la capacité d'une bactérie à acquérir du matériel génétique exogène. Ce concept, celui du pan-génome « ouvert » ou « fermé », exige de vérifier le nombre de nouveaux gènes trouvés toutes les fois qu'on pige aléatoirement un génome parmi un jeu de données (Medini *et al.* 2005). Si nous trouvons plusieurs nouveaux gènes toutes les fois qu'un nouveau génome est investigué, alors son

pan-génome est ouvert, tandis qu'il est fermé si peu de nouveaux gènes sont trouvés. Le type de pan-génome reflète le mode de vie d'une bactérie : un pan-génome ouvert se rencontre chez les espèces sympatriques ayant une grande capacité d'échanges de matériel génétique avec d'autres espèces, alors qu'un pan-génome fermé est typique des espèces allopatriques ayant une capacité limitée d'échanger des éléments génétiques (Rouli *et al.* 2015). Dans une étude complémentaire à la présente thèse, il a été possible de montrer qu'*A. salmonicida* a un pan-génome ouvert (Vincent & Charette 2017) (Figure 37). Ce résultat corrobore bien le fait que de nombreux éléments d'ADN mobiles sont acquis par cette bactérie, tant au niveau des plasmides (Vincent *et al.* 2014b, 2016; Attéré *et al.* 2015; Tanaka *et al.* 2016, 2017; Trudel *et al.* 2016) que des îlots génomiques et prophages (Reith *et al.* 2008; Emond-Rheault *et al.* 2015a, 2015b). Sachant que le chromosome de la souche de référence génomique d'*A. salmonicida* subsp. *salmonicida* A449 a environ 4 000 gènes codant pour des protéines, il est impressionnant de constater qu'en analysant 26 génomes, il est possible de trouver environ 6 500 groupes de gènes orthologues, soit 62 % plus de gènes que le chromosome à lui seul. Ce résultat intéressant montre que de nouveaux gènes demeurent à découvrir chez *A. salmonicida* et que sa plasticité génomique peut faire d'elle une bactérie avec une grande capacité d'adaptation.

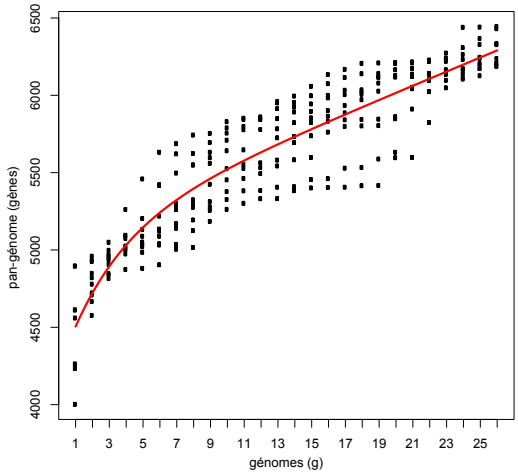

Figure 37. Estimation de la taille du pan-génome (nombre total de gènes) d'*A. salmonicida* en fonction du nombre de génomes échantillonnés.
Pour éviter un biais, les génomes ont été échantillonnés au hasard dix fois. Cette figure est dérivée d'une étude publiée (Vincent & Charette 2017).

Taxonomie et évolution de l'espèce *salmonicida*

Un défi de taille à la présente étude doctorale a été le manque de consensus dans la littérature concernant les positionnements phylogénétiques des différentes sous-espèces de

l'espèce *salmonicida* (*salmonicida*, *smithia*, *achromogenes*, *masoucida* et *pectinolytica*). Une phylogénie robuste est un outil puissant afin d'inférer les liens évolutifs entre les différents taxa et trouver des corrélations entre les métadonnées. Il est d'autant plus important d'avoir une vision claire et panoramique de l'évolution d'*A. salmonicida* qu'un des volets de la présente étude est d'investiguer les phages d'*A. salmonicida* subsp. *salmonicida*. Or, plusieurs phages sont connus pour être très spécifiques (Koskella & Meaden 2013). Afin d'évaluer cette spécificité pour les phages de la sous-espèce *salmonicida*, il fallait clarifier les liens entre les sous-espèces. De plus, certaines d'entre elles ont des caractéristiques très différentes, comme *pectinolytica* qui a la capacité de pousser à 37°C (Pavan *et al.* 2000). Bien que jamais étudié, il était raisonnable de croire que cette capacité de croître à 37°C était sous-jacente d'une importante diversité, tant au niveau génomique que métabolique.

Avant mes travaux, il était connu que la température pouvait jouer un rôle prépondérant dans l'évolution d'*A. salmonicida*, plus particulièrement pour les souches de la sous-espèce *salmonicida*. Il a été démontré que ces souches perdaient leur virulence lorsqu'elles étaient cultivées à des températures avoisinant 26-27°C (Ishiguro *et al.* 1981). Il a par la suite été montré que certaines séquences d'insertion étaient plus actives lorsque les souches étaient cultivées à de telles températures et qu'elles pouvaient s'insérer dans le gène *vapA*, encodant une protéine impliquée dans la couche de surface A, un important facteur de virulence (Gustafson *et al*. 1994) et le seul récepteur phagique répertorié pour être reconnu par les phages infectant cette espèce (Ishiguro *et al.* 1983). Similairement, le locus du système de sécrétion de type trois, situé sur le grand plasmide pAsa5, peut être éliminé par recombinaison de séquences d'insertion IS*AS11* lorsque la souche est cultivée à une température proche de 25°C (Tanaka *et al.* 2012, 2017). Bien que des séquences d'insertion soient impliquées comme éléments de recombinaison, des recombinases jouent des rôles importants dans de tels mécanismes (Renkawitz *et al*. 2014). Un survol de l'annotation de la souche de référence A449 de la sous-espèce *salmonicida* révèle au moins huit gènes codant pour des recombinases putativement fonctionnelles. Afin de mieux comprendre l'instabilité génomique des souches psychrophiles d'*A. salmonicida*, il serait pertinent d'investiguer la structure et surtout la dynamique moléculaire de ces enzymes selon différentes températures.

Bien que ces découvertes sur l'implication des séquences d'insertion sur l'instabilité génomique d'*A. salmonicida* subsp. *salmonicida* ont permis d'en apprendre plus sur sa virulence, les mécanismes évolutifs fondamentaux entre les sous-espèces psychrophiles (*salmonicida*, *smithia*, *achromogenes* et *masoucida*) et celle mésophile (*pectinolytica*), demeuraient inconnus. C'est dans l'objectif de découvrir les caractéristiques uniques aux souches mésophiles et psychrophiles d'*A. salmonicida* que l'ADN de trois souches indiennes et mésophiles (Y47, Y567 et Y577) a été séquencé et analysé dans le chapitre 5. Étant donné que seule la sous-espèce *pectinolytica* avait été répertoriée et analysée, il était inconnu si d'autres *A. salmonicida* pouvaient être mésophiles. En plus d'apporter une preuve que d'autres *A. salmonicida* peuvent être mésophiles, nous avons mis en évidence au moyen d'une phylogénie moléculaire robuste que les souches mésophiles forment un groupe phylogénétique basal aux souches psychrophiles (Figure 38), supportant ainsi l'hypothèse d'un ancêtre commun partagé directement entre les souches mésophiles. Le fait que les souches mésophiles soient basales à celles psychrophiles est intéressant étant donné que les autres espèces du genre *Aeromonas* sont aussi mésophiles, montrant par le fait même que la psychrophilie d'*A. salmonicida* est un phénotype dérivé dans l'évolution par rapport à la mésophilie.

De plus, les valeurs d'identité nucléotidiques suggèrent que les trois souches indiennes sont distinctes de la sous-espèce *pectinolytica*, et même entre elles. Il est donc maintenant clair qu'il existe une importante diversité au niveau même des souches mésophiles d'*A. salmonicida*. Plusieurs caractéristiques connues pour varier entre les bactéries mésophiles et psychrophiles, comme le pourcentage G+C, la composition en acides aminés des protéines ainsi que le répertoire en ARNt, ont été investiguées. Aucune différence significative dans ces caractéristiques n'a été répertoriée. Il faut dire que l'étude de la psychrophilie et de la mésophilie chez les bactéries se fait souvent en comparant des espèces de genres différents (Yang *et al.* 2015). Il est donc difficile de discriminer les évènements évolutifs propres à la dichotomie psychrophile/mésophile de ceux inhérents à d'autres types d'adaptation et aussi provenant d'évolution neutre et de dérive génétique (Lynch *et al.* 2016). Cependant, une caractéristique importante des souches mésophiles d'*A. salmonicida* est de pouvoir croître aussi efficacement que les souches psychrophiles à des températures aussi basses que 7°C. Ce résultat est crucial puisqu'il démontre que les

souches psychrophiles ne sont pas strictement psychrophiles par adaptation, mais bien qu'elles ont perdu leur capacité de croître à 37°C, ce qui est cohérent avec leur position dérivée dans l'arbre phylogénétique.

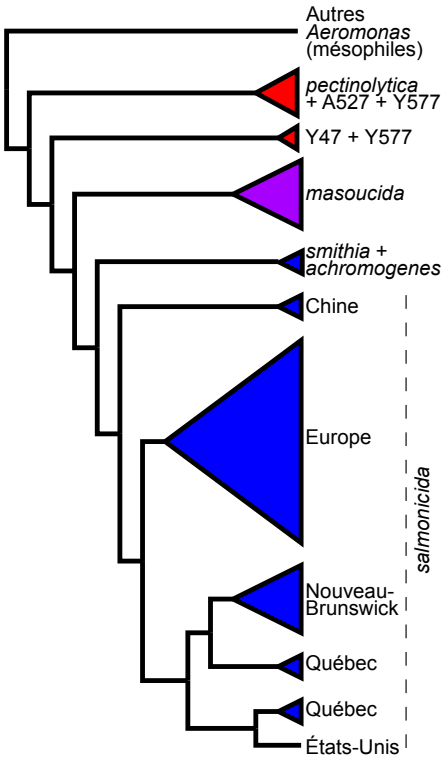

Figure 38. Schématisation des relations phylogénétiques entre les différents taxa d'*A. salmonicida*. La grosseur des triangles représente le nombre de souches analysées pour chacun des groupes. De plus, la couleur de chacun des triangles indique si les souches sont mésophiles (rouge), intermédiaires (mauve) ou psychrophiles (bleu).

Sachant l'implication des séquences d'insertion dans l'instabilité génomique des souches psychrophiles, il était raisonnable de vérifier si leur nombre et leur type étaient similaires dans les génomes des souches mésophiles. Il a été possible dans le chapitre 5 de déterminer que les génomes des souches mésophiles avaient moins de séquences d'insertion et que celles présentes sont différentes des séquences d'insertion trouvées dans les génomes des souches psychrophiles. À ce moment, l'ensemble des séquences génomiques provenant de souches mésophiles était morcelé en plusieurs dizaines de contigs. Puisque les séquences d'insertion sont répétées, celles-ci sont l'une des principales causent des bris de contigage lors de l'assemblage *de novo* de génomes d'*A. salmonicida* (Vincent *et al.* 2014a). Nous n'avions conséquemment qu'une représentation floue du répertoire réel en séquences d'insertion des souches mésophiles.

Dans l'optique d'investiguer en détail les séquences d'insertion des génomes des souches d'*A. salmonicida* mésophiles, le génome d'une nouvelle souche indienne et mésophile, A527, a été séquencé avec la technologie PacBio dans le chapitre 6 de la présente thèse. Le séquençage PacBio peut générer de très longues lectures, permettant de contourner le problème occasionné par les séquences répétées lors de l'assemblage *de novo* (Phillippy 2017). Ce génome complet a permis de confirmer avec plus de certitude que le répertoire en séquences d'insertion est différent entre les souches d'*A. salmonicida* psychrophiles et celles mésophiles. Cependant, le génome de la souche A527 a presque autant de séquences d'insertion que celui de la souche psychrophile A449, de la sous-espèce *salmonicida*. Il est donc maintenant plus clair que ce qui peut être le plus déterminant dans le contexte mésophilie/psychrophilie n'est pas la quantité, mais les différents types de séquences d'insertion. Par exemple, il a été possible de mettre en évidence que certaines séquences d'insertion ayant été répertoriées chez la sous-espèce *salmonicida*, comme les séquences d'insertion IS*AS1*, IS*AS2* et IS*AS11*, connues comme étant des éléments pouvant altérer le génome d'une souche lorsqu'elle est cultivée au-dessus de sa température optimale (Gustafson *et al*. 1994; Tanaka *et al.* 2012), sont exclusives aux souches psychrophiles. Lorsque d'autres génomes complets d'*A. salmonicida* seront fermés, tant du côté des souches mésophiles que psychrophiles, il sera possible de continuer les investigations et d'ajouter de la robustesse à cette théorie.

Avec nos connaissances actuelles sur *A. salmonicida* et ses séquences d'insertion, il est possible de déduire un scénario évolutif (Figure 39). La façon la plus parcimonieuse d'envisager l'apparition de la psychrophilie chez *A. salmonicida* implique que la population initiale de cette bactérie était mésophile, comme toutes les autres espèces du genre *Aeromonas*. Cependant, un évènement inconnu a fait en sorte que la pression de croître à 37°C a diminué pour une sous-population d'*A. salmonicida*. Cet évènement, qui peut être un goulot d'étranglement dans la population, a permis la prolifération de certains types de séquences d'insertion sans qu'il n'y ait de conséquences négatives sur cette sous-population. À cause sans doute d'une raison multifactorielle (types de séquences d'insertion, endroits où elles se sont insérées, compatibilité avec les recombinases, etc.), les souches psychrophiles sont bloquées dans cet état afin de préserver leur intégrité génomique. Finalement, il est exclu d'envisager que les souches psychrophiles se soient

spécialisées afin d'infecter les poissons d'eau froide, comme les salmonidés, puisque plusieurs espèces mésophiles, telles qu'*A. hydrophila* et *A. veronii*, peuvent causer des infections similaires à ces mêmes hôtes (Janda & Abbott 2010). Ceci suggère, encore une fois, que la psychrophilie chez *A. salmonicida* doit être perçue comme une contrainte et non pas comme une adaptation.

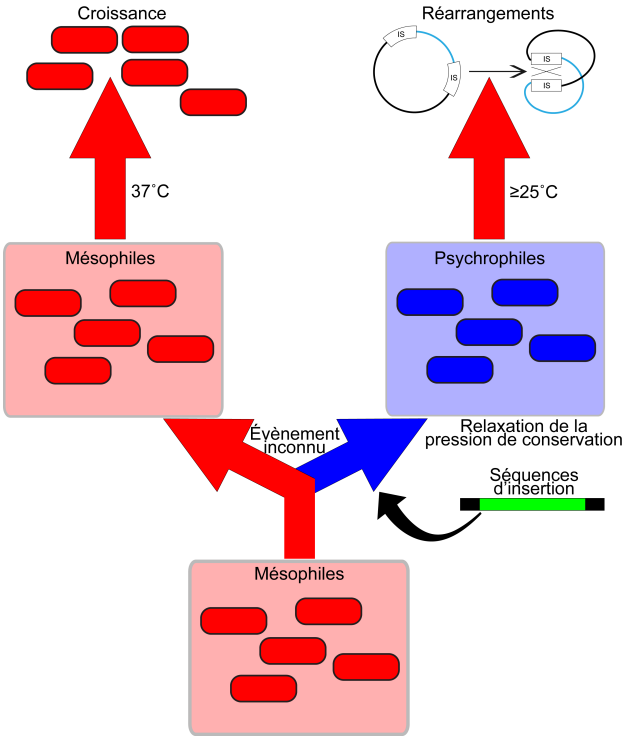

Figure 39. Chemin évolutif proposé pour expliquer la dichotomie psychrophile/mésophile observée chez *A. salmonicida* en relation avec les séquences d'insertion.

Dans un autre ordre idée, les chapitres 5 et 6 ont permis de démontrer qu'il existe des regroupements phylogénétiques selon les emplacements géographiques où les souches ont été isolées. Avant la présente étude doctorale, les souches d'*A. salmonicida* subsp. *salmonicida* étaient considérées comme homogènes (Belland & Trust 1988; Hänninen *et al.* 1995; García *et al.* 2000; O'hIci *et al.* 2000) avec une structure populationnelle clonale (McCormick *et al.* 1990; Umelo & Trust 1998). Les souches européennes et celles canadiennes forment deux groupes bien distincts et fortement supportés statistiquement (Figure 38). En fait, il est même possible de séparer les souches des provinces du Québec de celles du Nouveau-Brunswick. Finalement, l'ADN de deux isolats de la Chine a récemment été séquencé et leurs séquences génomiques déposées dans GenBank (Long *et al.* 2016). Ces deux souches sont basales à toutes les autres de la sous-espèce *salmonicida*. Tel que discuté dans l'introduction, il a été possible de démontrer des corrélations entre les

emplacements géographiques où les souches ont été isolées et le type d'*AsaGEI* (Emond-Rheault *et al.* 2015a, 2015b), un ilot génomique, ainsi que dans la propension à perdre certains petits plasmides (Attéré *et al.* 2015). Il ne fait, par conséquent, aucun doute que les souches d'*A. salmonicida* subsp. *salmonicida* sont plus hétérogènes que précédemment soupçonné, ce tant au niveau de l'architecture génomique, du contenu plasmidique ou même des mutations (SNP et InDel). À la lumière de ces résultats, il faut donc considérer les souches d'*A. salmonicida* comme des entités différentes, avec des caractéristiques propres.

Diversité des phages infectant *A. salmonicida*

Dans les chapitres 2, 3 et 4, il a été possible d'établir l'ampleur de la résistance aux antibiotiques chez *A. salmonicida* subsp. *salmonicida*. Dans les chapitres 5 et 6, les relations évolutives et taxonomiques ont été éclaircies. Le chapitre 7 avait pour but d'investiguer la diversité, tant au niveau microbiologique que génomique, des phages infectant *A. salmonicida* subsp. *salmonicida*. La collection Félix d'Hérelle de l'Université Laval avait déjà neuf phages spécifiques à *A. salmonicida* (http://www.phage.ulaval.ca). Une campagne d'échantillonnage en milieu naturel et en pisciculture a permis d'amplifier trois nouveaux phages contre cette bactérie, nous permettant ainsi d'avoir un total de 12 phages. Le séquençage et l'analyse de ces 12 phages, combinés à six génomes de phages d'*A. salmonicida* disponibles sur GenBank, ont permis de mettre en évidence six groupes de phages génétiquement distincts (Figure 40). Ces groupes correspondent aux différentes stratégies d'empaquetage de l'ADN utilisées par les phages ainsi que selon la taille de leur génome.

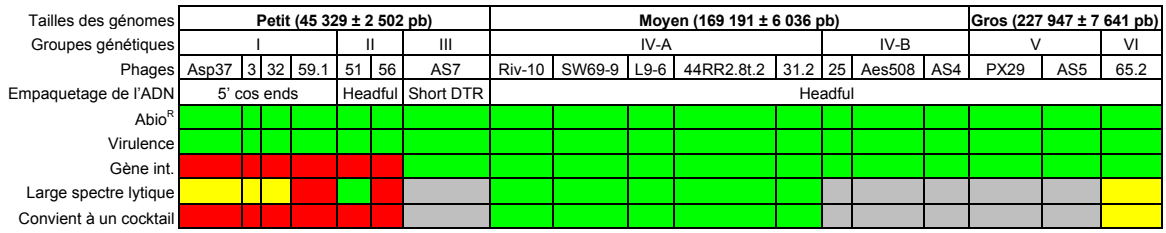

Figure 40. Récapitulatif de la classification et de la caractérisation des phages d'*A. salmonicida* étudiés dans la présente thèse.

Les couleurs des rectangles représentent si les phénotypes ou génotypes sont positifs (vert), moyens (jaune), négatifs (rouge) ou non testés (gris) pour un contexte thérapeutique. Ainsi, afin d'être vert dans la catégorie « Convient à un cocktail », le phage ne doit avoir aucun carré rouge.

Deux des principales limitations à l'utilisation des phages dans un contexte thérapeutique sont (1) l'étroitesse du spectre lytique et (2) la génération de bactéries mutantes insensibles aux phages. Cependant, il est connu que l'utilisation d'un cocktail de plusieurs phages complémentaires peut minimiser ces deux problèmes (Chan & Abedon 2012). Puisqu'il a été possible de mettre en lumière six groupes de phages génétiquement différents, il était raisonnable de partir avec comme *a priori* la possibilité d'un cocktail avec environ six phages (un phage de chacun des groupes). Cependant, il est fortement recommandé d'utiliser des phages exclusivement lytiques afin d'éviter l'intégration du génome phagique dans le génome de la bactérie et ainsi prévenir la possibilité d'une conversion lysogénique (Fortier & Sekulovic 2013; Abedon *et al.* 2017). Les intégrases sont des enzymes clés dans le processus de lysogénie (Nash 1981; Feiner *et al.* 2015). Les génomes de tous les phages des groupes I et II portent au moins un gène codant pour une intégrase, ceux-ci seront donc à exclure d'un potentiel cocktail. Il faut aussi utiliser des phages n'ayant pas de gènes codant pour des facteurs de virulence ou conférant une résistance aux antibiotiques. Aucun génome analysé lors de la présente thèse ne présente de tels gènes. Finalement, la largeur du spectre lytique est aussi un facteur déterminant pour l'utilisation d'un phage dans un contexte thérapeutique. Un phage capable de lyser plusieurs souches de la bactérie ciblée est bien sûr à prioriser. Malheureusement, seuls les génomes déposés sur GenBank étaient disponibles pour les phages des groupes III, IV-B et V. Les spectres lytiques de ces phages n'ont donc pas été analysés pour la présente thèse. Les phages des groupes I et II ont des spectres lytiques plus étroits alors que ceux des groupes IV-A et VI ont des spectres lytiques larges. Couplé au fait que les génomes des phages des groupes I et II possèdent des gènes pour des intégrases, ceux-ci seraient à éviter dans un contexte thérapeutique. À priori, les phages des groupes IV-A et VI seraient de bons candidats.

Plusieurs défis restent cependant à surmonter avant d'envisager l'application d'un cocktail de phages contre *A. salmonicida*. Il a été possible d'identifier des phages candidats à un cocktail. Malgré cela, ces phages doivent être testés *in vivo* avec des poissons afin de vérifier leur efficacité et leur innocuité. Bien que les phages infectent exclusivement les bactéries, des réponses immunitaires de l'hôte (les poissons dans notre cas) sont possibles (Khan Mirzaei *et al.* 2016), entre autres, s'il y a des débris bactériens présents dans le cocktail de phages (Abedon 2014; Dufour *et al.* 2016). Un complément à la présente étude

serait donc la mise au point d'un protocole de production et de purification à haut-débit de phages d'*A. salmonicida*. Bien sûr, des protocoles et des tentatives d'optimisation des procédés ont déjà été réalisés (Bourdin *et al.* 2014; Lipinski *et al.* 2016), permettant ainsi de partir avec une base connue. Il faut aussi s'assurer de l'influence des facteurs environnementaux, comme la température, le pH, la salinité et le niveau de matière organique, sur les phages candidats (Silva *et al.* 2014). De plus, la méthode d'administration demeure à trouver et à optimiser. Plusieurs méthodes ont été répertoriées pour administrer des phages en aquaculture (Gon Choudhury *et al.* 2017) : administration par la prise de nourriture, par injection intramusculaire ou intra-péritonéale, par intubation anale, par des bains contenant des phages ou simplement par le relâchement des phages dans les bassins. Malheureusement, l'étude d'*A. salmonicida* dans un tel contexte exige un laboratoire aquatique avec un niveau de confinement deux, qui n'est pas actuellement accessible à l'Université Laval, et une expertise dans la manutention des poissons.

Comme indiqué précédemment, la génération de bactéries mutantes résistantes aux phages peut être un problème majeur dans un contexte thérapeutique. Cependant, contrairement aux antibiotiques, les phages peuvent aussi évoluer afin de réinfecter les souches bactériennes mutantes (Samson *et al.* 2013). Dans la littérature scientifique, ce processus d'évolution continue des bactéries et des phages est souvent désigné comme une course à l'armement. Il est donc important de connaître la dynamique évolutive entre les phages et la bactérie d'intérêt qui est ciblée, en l'occurrence, *A. salmonicida* subp. *salmonicida*. Cette dynamique évolutive n'a pas été étudiée dans la présente thèse, la cause étant l'incapacité à générer des bactéries mutantes aux phages testés. En fait, les quelques bactéries mutantes générées, après de nombreux essais, perdent leur phénotype de résistance, retournant au phénotype sauvage après quelques générations, soit sensible aux phages. Les mêmes observations ont été décrites par l'une des premières études ayant investigué l'utilisation des phages contre *A. salmonicida* subsp. *salmonicida* (Imbeault *et al.* 2006b). Le seul récepteur bactérien connu est la protéine VapA (Ishiguro *et al.* 1983), une protéine impliquée dans la couche de surface A (Kay *et al.* 1981), un facteur de virulence. Puisqu'aucun système CRISPR-Cas n'a été répertorié chez *A. salmonicida*, il n'est pas clair si la bactérie doit altérer VapA, ou une autre structure membranaire, pour se protéger des phages. Malgré le fait que l'incapacité à générer des bactéries insensibles aux phages

empêche d'approfondir nos connaissances fondamentales sur la dynamique évolutive entre *A. salmonicida* et ses phages, il s'agit d'une excellente nouvelle pour une application thérapeutique. Encore une fois, il serait important de vérifier *in vivo* avec des poissons si *A. salmonicida* conserve cette difficulté à muter pour se protéger des phages qui l'attaquent.

Finalement, dans les chapitres 5 et 6, il a été possible de démontrer qu'il existe des regroupements phylogénétiques selon les origines géographiques des souches. Dans le chapitre 7, aucune corrélation entre les origines géographiques des souches et leur sensibilité aux phages n'a été trouvée. De même, il n'y a aucune corrélation entre la sensibilité aux phages et la présence de l'ilot génomique *AsaGEI*. Ces résultats très prometteurs laissent croire qu'un cocktail de phages élaboré au Québec pourrait être efficace ailleurs dans le monde, notamment en Europe où la furonculose est bien présente (Studer *et al*. 2013; Bartkova *et al.* 2017).

Il a aussi été possible de démontrer qu'il existe une étroite relation entre la sensibilité aux phages et la capacité des souches à croître à 37°C (souches mésophiles) ou pas (souches psychrophiles). Afin de vérifier la largeur du spectre lytique des différents phages, en plus de multiples souches de la sous-espèce *salmonicida*, des souches d'autres sous-espèces psychrophiles (*smithia* et *masoucida*) et mésophiles (*pectinolytica*, Y577, Y567 et Y47) ont été analysées. Les phages isolés à partir de souches de la sous-espèce *salmonicida* lysent sans distinction les souches testées de *smithia* et *masoucida,* toutes deux psychrophiles. Cependant, les souches mésophiles sont insensibles aux différents phages.

Il est connu que les souches des sous-espèces psychrophiles d'*A. salmonicida* possèdent le gène *vapA*, contrairement aux souches de la sous-espèce mésophile *pectinolytica* (Merino *et al.* 2015; Gulla *et al.* 2016). De plus, nous avons montré que les souches mésophiles Y577, Y567 et Y47 ne possèdent pas *vapA*. Sachant que le gène *vapA* encode le seul récepteur connu des phages chez *A. salmonicida* (Ishiguro *et al.* 1983), il était donc attendu que les souches mésophiles soient insensibles aux différents phages testés, à moins que ces derniers reconnaissent un autre récepteur à la surface des cellules des souches mésophiles. Afin d'augmenter nos connaissances fondamentales, il serait pertinent d'isoler des phages pouvant infecter les souches mésophiles d'*A. salmonicida*, afin de comparer leur diversité génomique et microbiologique avec les phages étudiés dans la présente thèse et ainsi

espérer pouvoir continuer d'en apprendre plus sur la dichotomie psychrophile/mésophile d'*A. salmonicida*.

Conclusion et perspectives

Afin de bien comprendre le phénomène de la résistance aux antibiotiques, il est important de considérer les bactéries comme formant un réseau, un tout cohérent et dynamique, et non pas comme des individus isolés et statiques. L'un des nœuds de ce réseau, qui est la bactérie *A. salmonicida* subsp. *salmonicida* a été investigué. Il a été possible de mettre en lumière qu'*A. salmonicida* subsp. *salmonicida* a la capacité d'échanger du matériel génétique, principalement par les plasmides, avec d'autres bactéries. Compte tenu du grand nombre de plasmides répertoriés et que plusieurs de ceux-ci sont retrouvés dans des genres ou des espèces bactériennes différents, il est raisonnable de penser qu'*A. salmonicida* subsp. *salmonicida* pourrait avoir une place importante au sein du réseau (Figure 41). Les bactéries ayant une position centrale sont d'une importance capitale puisqu'elles peuvent jouer un rôle de relais entre des bactéries qui ne peuvent échanger directement du matériel génétique.

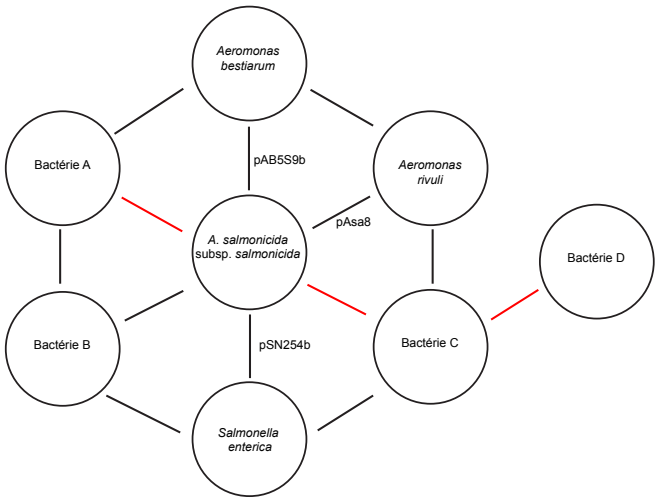

Figure 41. Schématisation d'un réseau d'interaction impliquant différentes bactéries connues et hypothétiques.

Le chemin en rouge illustre un exemple de comment les bactéries A et D pourraient échanger indirectement entre elles de l'information génétique. Les plasmides pSN254, pAB5S9b et pAsa8 sont indiqués pour montrer une partie putative du réseau d'interactions mis en lumière lors de la présente thèse et impliquant *A. salmonicida* subsp. *salmonicida*.

L'un des problèmes majeurs avec les antibiotiques est que l'utilisation d'un de ces composés, souvent à large spectre, sur une bactérie donnée va modifier le réseau d'interactions dans lequel la bactérie ciblée participe. Cette réaction en chaîne est

imprévisible et difficile à contrôler. C'est l'une des raisons pour laquelle la thérapie par les phages est attrayante. La grande spécificité des phages à une bactérie permet de minimiser l'impact du traitement sur le réseau microbien et ainsi avoir une action localisée et ciblée.

Certaines méthodes scientifiques, comme la métagénomique, se développent et permettent d'étudier avec une vision panoramique le réseau d'interactions entre les différentes bactéries (Parks *et al.* 2017). Il sera crucial dans les prochaines années d'investiguer avec ces méthodes la répartition d'*A. salmonicida* et son réseau d'interactions. Finalement, la présente thèse a en partie levé le voile sur une immense diversité potentielle en montrant que plusieurs souches d'*A. salmonicida* pouvaient être mésophiles. Cela ouvre la porte à échantillonner des environnements qui initialement n'étaient pas considérés propices afin d'y trouver cette bactérie.

Références

Abedon ST. Phage therapy: eco-physiological pharmacology. *Scientifica (Cairo)* 2014;**2014**:581639.

Abedon ST, García P, Mullany P *et al.* Editorial: Phage therapy: Past, present and future. *Front Microbiol* 2017;**8**:981.

Abedon ST, Kuhl SJ, Blasdel BG *et al.* Phage treatment of human infections. *Bacteriophage* 2011;**1**:66–85.

Abuladze T, Li M, Menetrez MY *et al.* Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. *Appl Environ Microbiol* 2008;**74**:6230–8.

Ackermann H-W. Phage classification and characterization. In: Clokie MRJ, Kropinski AM (eds.). *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions*. Totowa, NJ: Humana Press, 2009, 127–40.

Ackermann H-W. Ruska H. Visualization of bacteriophage lysis in the hypermicroscope. Naturwissenschaften 1940; 28. *Bacteriophage* 2011;**1**:183–5.

Ackermann H-W, Dauguet C, Paterson WD *et al. Aeromonas* bacteriophages: Reexamination and classification. *Ann l'Institut Pasteur / Virol* 1985;**136**:175–99.

Actis LA, Tolmasky ME, Crosa JH. Bacterial plasmids: replication of extrachromosomal genetic elements encoding resistance to antimicrobial compounds. *Front Biosci* 1999;**4**:D43–62.

Adams C, Austin B, Meaden P *et al.* Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl Environ Microbiol* 1998;**64**:4194–201.

Adams MJ, Lefkowitz EJ, King AMQ *et al.* 50 years of the International Committee on Taxonomy of Viruses: progress and prospects. *Arch Virol* 2017;**162**:1441–6.

Adriaenssens E, Brister JR. How to name and classify your phage: An informal guide. *Viruses* 2017;**9**:E70.

Ahmed AM, Hussein AIA, Shimamoto T. *Proteus mirabilis* clinical isolate harbouring a new variant of *Salmonella* genomic island 1 containing the multiple antibiotic resistance region. *J Antimicrob Chemother* 2007;**59**:184–90.

Allen HK, Trachsel J, Looft T *et al.* Finding alternatives to antibiotics. *Ann N Y Acad Sci* 2014;**1323**:91–100.

Aminov RI. A brief history of the antibiotic era: Lessons learned and challenges for the future. *Front Microbiol* 2010;**1**:134

Aoki T, Egusa S, Kimura T *et al.* Detection of R factors in naturally occurring *Aeromonas salmonicida* strains. *Appl Microbiol* 1971;**22**:716–7.

Aoki T, Egusa S, Yada C *et al.* Studies of drug resistance and R factors in bacteria from pond-cultured salmonids. *Jpn J Microbiol* 1972;**16**:233–8.

Aoki T, Kitao T, Iemura N *et al.* The susceptibility of *Aeromonas salmonicida* strains isolated in cultured and wild salmonids to various chemotherapeutics. *Bull Japan Soc Sci Fish* 1983;**49**:17–22.

Aoki T, Mitoma Y, Crosa JH. The characterization of a conjugative R-plasmid isolated from *Aeromonas salmonicida*. *Plasmid* 1986;**16**:213–8.

Attéré SA, Vincent AT, Paccaud M *et al.* The role for the small cryptic plasmids as moldable vectors for genetic innovation in *Aeromonas salmonicida* subsp. *salmonicida*. *Front Genet* 2017;**8**:211.

Attéré SA, Vincent AT, Trudel MV *et al.* Diversity and homogeneity among small plasmids of *Aeromonas salmonicida* subsp. *salmonicida* linked with geographical origin. *Front Microbiol* 2015;**6**:1274.

Aunsmo A, Guttvik A, Midtlyng PJ *et al.* Association of spinal deformity and vaccine-

induced abdominal lesions in harvest-sized Atlantic salmon, *Salmo salar L*. *J Fish Dis* 2008a;**31**:515–24.

Aunsmo A, Larssen RB, Valle PS *et al.* Improved field trial methodology for quantifying vaccination side-effects in farmed Atlantic salmon (*Salmo salar L.*). *Aquaculture* 2008b;**284**:19–24.

Austin B. Taxonomy of bacterial fish pathogens. *Vet Res* 2011;**42**:20.

Austin B, Austin DA. Aeromonadaceae representative (*Aeromonas salmonicida*). In: Austin B, Austin DA (eds.). *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*. Cham: Springer International Publishing, 2016, 215–321.

Bai J, Kim Y-T, Ryu S *et al.* Biocontrol and rapid detection of food-borne pathogens using bacteriophages and endolysins. *Front Microbiol* 2016;**7**:474.

Bartkova S, Leekitcharoenphon P, Aarestrup FM *et al.* Epidemiology of danish *Aeromonas salmonicida* subsp. *salmonicida* in fish farms using whole genome sequencing. *Front Microbiol* 2017;**8**:2411.

Bast L, Daly JG, Degrandis SA *et al.* Evaluation of profiles of *Aeromonas salmonicida* as epidemiological markers of furunculosis infections in fish. *J Fish Dis* 1988;**11**:133–45.

Beilstein F, Dreiseikelmann B. Temperate bacteriophage PhiO18P from an *Aeromonas media* isolate: characterization and complete genome sequence. *Virology* 2008a;**373**:25–9.

Belland RJ, Trust TJ. DNA:DNA reassociation analysis of *Aeromonas salmonicida*. *J Gen Microbiol* 1988;**134**:307–15.

Belland RJ, Trust TJ. *Aeromonas salmonicida* plasmids: Plasmid-directed synthesis of proteins in vitro and in *Escherichia coli* minicells. *Microbiology* 1989;**135**:513–24.

Berdy J. Thoughts and facts about antibiotics: Where we are now and where we are

heading. *J Antibiot* 2012;**65**:385–95.

Bergh O, Børsheim KY, Bratbak G *et al.* High abundance of viruses found in aquatic environments. *Nature* 1989;**340**:467–8.

Vanden Bergh P, Frey J. *Aeromonas salmonicida* subsp. *salmonicida* in the light of its type-three secretion system. *Microb Biotechnol* 2014;**7**:381–400.

Van Boeckel TP, Brower C, Gilbert M *et al.* Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci* 2015;**112**:5649–54.

Bouchard JD, Moineau S. Homologous recombination between a lactococcal bacteriophage and the chromosome of its host strain. *Virology* 2000;**270**:65–75.

Bourdin G, Schmitt B, Guy LM *et al.* Amplification and purification of T4-Like *Escherichia coli* phages for phage therapy: From laboratory to pilot scale. *Appl Environ Microbiol* 2014;**80**:1469–76.

Boyd DA, Peters GA, Ng LK *et al.* Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica Typhymurium* DT104. *FEMS Microbiol Lett* 2000;**189**:285–91.

Boyd J, Williams J, Curtis B *et al.* Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. *Plasmid* 2003;**50**:131–44.

Bradley DE, Aoki T, Kitao T *et al.* Specification of characteristics for the classification of plasmids in incompatibility group U. *Plasmid* 1982;**8**:89–93.

Bratbak G, Heldal M, Norland S *et al.* Viruses as partners in spring bloom microbial trophodynamics. *Appl Environ Microbiol* 1990;**56**:1400–5.

Brown D. Antibiotic resistance breakers: Can repurposed drugs fill the antibiotic discovery void? *Nat Rev Drug Discov* 2015;**14**:821–32.

Brüssow H. Prophage genomics. In: Calendar R (ed.). *The Bacteriophages*. Second edi. Oxford University Press, USA, 2006, 20.

Brüssow H. What is needed for phage therapy to become a reality in Western medicine? *Virology* 2012;**434**:138–42.

Brüssow H, Canchaya C, Hardt W-D. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev* 2004;**68**:560–602.

Burr SE, Stuber K, Frey J. The ADP-ribosylating toxin, AexT, from *Aeromonas salmonicida* subsp. *salmonicida* is translocated via a yype III secretion pathway. *J Bacteriol* 2003;**185**:6583–91.

Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol* 2006;**8**:1137–44.

Cabello FC, Godfrey HP, Buschmann AH *et al.* Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. *Lancet Infect Dis* 2016;**16**:e127–33.

Campbell EA, Korzheva N, Mustaev A *et al.* Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* 2001;**104**:901–12.

Casjens S. Prophages and bacterial genomics: What have we learned so far? *Mol Microbiol* 2003;**49**:277–300.

Centers for Diseases Control and Prevention. *Antibiotic Resistance Threats in the United States*. 2013;1-112

Chan BK, Abedon ST. Phage therapy pharmacology. Phage cocktails. *Adv Appl Microbiol* 2012;**78**:1–23.

Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. *Future Microbiol* 2013;**8**:769–83.

Chang Q, Wang W, Regev-Yochay G *et al.* Antibiotics in agriculture and the risk to human

health: How worried should we be? *Evol Appl* 2015;**8**:240–7.

Chanishvili N. Chapter 1 - Phage Therapy—History from Twort and d'Herelle through soviet experience to current approaches. In: Research MŁ and WSBT-A in V (ed.). *Bacteriophages, Part B*. Vol 83. Academic Press, 2012, 3–40.

Chen CY, Lindsey RL, Strobaugh TP *et al.* Prevalence of ColE1-like plasmids and kanamycin resistance genes in *Salmonella enterica* serovars. *Appl Environ Microbiol* 2010;**76**:6707–14.

Colwell RR, Macdonell MT, De J. Proposal to recognize the family *Aeromonadaceae* fam. nov. *Int J Microbiol* 1986;**36**:473–7.

Costa TRD, Felisberto-Rodrigues C, Meir A *et al.* Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* 2015;**13**:343–59.

Czaplewski L, Bax R, Clokie M *et al.* Alternatives to antibiotics - a pipeline portfolio review. *Lancet Infect Dis* 2016;**16**:239–51.Daher RK, Filion G, Tan SGE *et al.* Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions. *Vet Microbiol* 2011;**152**:353–60.

Dallaire-Dufresne S, Tanaka KH, Trudel MV *et al.* Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol* 2014;**169**:1–7.

Deng Y, Bao X, Ji L *et al.* Resistance integrons: class 1, 2 and 3 integrons. *Ann Clin Microbiol Antimicrob* 2015;**14**:45.

Drulis-Kawa Z, Majkowska-Skrobek G, Maciejewska B *et al.* Learning from bacteriophages - advantages and limitations of phage and phage-encoded protein applications. *Curr Protein Pept Sci* 2012;**13**:699–722.

Dublanchet A, Bourne S. The epic of phage therapy. *Can J Infect Dis Med Microbiol* 2007;**18**:15–8.

Duff DCB. The oral immunization of trout against *Bacterium salmonicida*. *J Immunol* 1942;**44**:87–94.

Dufour N, Henry M, Ricard J-D *et al.* Commentary: Morphologically distinct *Escherichia coli* bacteriophages differ in their efficacy and ability to stimulate cytokine release in vitro. *Front Microbiol* 2016;**7**:1029.

Ellis EL, Delbrück M. The growth of bacteriophage. *J Gen Physiol* 1939;**22**:365–84.

Embody GC, Hayford CO. The advantage of rearing brook trout fingerlings from selected breeders. *Trans Am Fish Soc* 1925;**55**:135–48.

Emmerich R, Weibel E. Über eine durch Bakterien verursachte Infektionskrankheit der Forellen. *Allg Fischerei-Zeitung* 1890;**15**:73-77-92.

Emond-Rheault J-G, Vincent AT, Trudel MV *et al.* Variants of a genomic island in *Aeromonas salmonicida* subsp. *salmonicida* link isolates with their geographical origins. *Vet Microbiol* 2015a;**175**:68–76.

Emond-Rheault J-G, Vincent AT, Trudel MV *et al.* AsaGEI2b: a new variant of a genomic island identified in the *Aeromonas salmonicida* subsp. *salmonicida* JF3224 strain isolated from a wild fish in Switzerland. *FEMS Microbiol Lett* 2015b;**362**:fnv093.

Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. *Perspect Medicin Chem* 2014;**6**:25–64.

FAO. *Global Aquaculture Production Statistics Database Updated to 2013*. 2015.

FAO. Fishery and Aquaculture Statistics. Global production by production source 1950-2015 (FishstatJ). 2017.

Fehr D, Casanova C, Liverman A *et al.* AopP, a type III effector protein of *Aeromonas salmonicida*, inhibits the NF-kappaB signalling pathway. *Microbiology* 2006;**152**:2809–18.

Feiner R, Argov T, Rabinovich L *et al.* A new perspective on lysogeny: prophages as active

regulatory switches of bacteria. *Nat Rev Microbiol* 2015;**13**:641–50.

Fiers W, Contreras R, Duerinck F *et al.* Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. *Nature* 1976;**260**:500–7.

Figueras MJ, Alperi A, Beaz-Hidalgo R *et al. Aeromonas rivuli* sp. nov., isolated from the upstream region of a karst water rivulet. *Int J Syst Evol Microbiol* 2011;**61**:242–8.

Fleming A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. *Br J Exp Pathol* 1929;**10**:226–36.

Floss HG, Yu T-W. Rifamycin - mode of action, resistance, and biosynthesis. *Chem Rev* 2005;**105**:621–32.

Fortier L-C, Sekulovic O. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence* 2013;**4**:354–65.

Gao L, Qi J. Whole genome molecular phylogeny of large dsDNA viruses using composition vector method. *BMC Evol Biol* 2007;**7**:41.

García JA, Larsen JL, Dalsgaard I *et al.* Pulsed-field gel electrophoresis analysis of *Aeromonas salmonicida* ssp. *salmonicida*. *FEMS Microbiol Lett* 2000;**190**:163–6.

Girons IS, Bourhy P, Ottone C *et al.* The LE1 bacteriophage replicates as a plasmid within *Leptospira biflexa*: Construction of an *L. biflexa-Escherichia coli* shuttle vector. *J Bacteriol* 2000;**182**:5700–5.

Gon Choudhury T, Tharabenahalli Nagaraju V, Gita S *et al.* Advances in bacteriophage research for bacterial disease control in aquaculture. *Rev Fish Sci Aquac* 2017;**25**:113–25.

Goossens H, Ferech M, Vander Stichele R *et al.* Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. *Lancet* 2005;**365**:579–87.

Gulla S, Lund V, Kristoffersen AB *et al. vapA* (A-layer) typing differentiates *Aeromonas*

salmonicida subspecies and identifies a number of previously undescribed subtypes. *J Fish Dis* 2016;**39**:329–42.

Gustafson CE, Chu S, Trust TJ. Mutagenesis of the paracrystalline surface protein array of *Aeromonas salmonicida* by endogenous insertion elements. *J Mol Biol* 1994;**237**:452–63.

Hackett JL, Lynch WH, Paterson WD *et al.* Extracellular protease, extracellular haemolysin, and virulence in *Aeromonas salmonicida*. *Can J Fish Aquat Sci* 1984;**41**:1354–60.

Hall BG, Ehrlich GD, Hu FZ. Pan-genome analysis provides much higher strain typing resolution than multi-locus sequence typing. *Microbiology* 2010;**156**:1060–8.

Han H-J, Kim D-Y, Kim W-S *et al.* Atypical *Aeromonas salmonicida* infection in the black rockfish, *Sebastes schlegeli* Hilgendorf, in Korea. *J Fish Dis* 2011;**34**:47–55.

Hänninen ML, Ridell J, Hirvelä-Koski V. Phenotypic and molecular characteristics of *Aeromonas salmonicida* subsp. *salmonicida* isolated in southern and northern Finland. *J Appl Bacteriol* 1995a;**79**:12–21.

Hatfull GF. Bacteriophage genomics. *Curr Opin Microbiol* 2008;**11**:447–53.

Hatfull GF, Hendrix RW. Bacteriophages and their genomes. *Curr Opin Virol* 2011;**1**:298–303.

Hayden MS, Ghosh S. NF-κB, the first quarter-century: Remarkable progress and outstanding questions. *Genes Dev* 2012;**26**:203–34.

d'Herelle F. Sur un microbe invisible antagoniste des bacilles dysentériques. *Comptes rendus l'Académie des Sci* 1917;**165**:373–5.

Hershey AD, Chase M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J Gen Physiol* 1952;**36**:39–56.

Högberg LD, Heddini A, Cars O. The global need for effective antibiotics: Challenges and

recent advances. *Trends Pharmacol Sci* 2010;**31**:509–15.

Hollis A, Ahmed Z. Preserving antibiotics, rationally. *N Engl J Med* 2013;**369**:2474–6.

Holmes AH, Moore LSP, Sundsfjord A *et al.* Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet* 2016;**387**:176–87.

Hyman P, Abedon ST. Bacteriophage host range and bacterial resistance. *Adv Appl Microbiol* 2010;**70**:217–48.

Imbeault S, Parent S, Blais J-F *et al.* Utilisation de bactériophages pour contrôler les populations de *Aeromonas salmonicida* résistantes aux antibiotiques. *Rev des Sci l'eau* 2006a;**19**:275.

Imbeault S, Parent S, Lagacé M *et al.* Using bacteriophages to prevent furunculosis caused by *Aeromonas salmonicida* in farmed brook trout. *J Aquat Anim Health* 2006b;**18**:203–14.

Inglis V, Yimer E, Bacon EJ *et al.* Plasmid-mediated antibiotic resistance in *Aeromonas salmonicida* isolated from Atlantic salmon, *Salmo salar L.*, in Scotland. *J Fish Dis* 1993;**16**:593–9.

Ishiguro EE, Ainsworth T, Harkness RE *et al.* A temperate bacteriophage specific for strains of *Aeromonas salmonicida* possessing A-layer, a cell surface virulence factor. *Curr Microbiol* 1984;**10**:199–202.

Ishiguro EE, Ainsworth T, Shaw DH *et al.* A lipopolysaccharide-specific bacteriophage for *Aeromonas salmonicida*. *Can J Microbiol* 1983;**29**:1458–61.

Ishiguro EE, Kay WW, Ainsworth T *et al.* Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. *J Bacteriol* 1981;**148**:333–40.

Ishiguro EE, Kay WW, Trust TJ. Temperate bacteriophages for *Aeromonas salmonicida*. *FEMS Microbiol Lett* 1980;**8**:247–50.

Janda JM, Abbott SL. The genus *Aeromonas*: Taxonomy, pathogenicity, and infection. *Clin*

Microbiol Rev 2010;**23**:35–73.

Jokinen CC, Koot J, Cole L *et al.* The distribution of *Salmonella enterica* serovars and subtypes insurface water from five agricultural regions across Canada. *Water Res* 2015;**76**:120–31.

Kay WW, Buckley JT, Ishiguro EE *et al.* Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. *J Bacteriol* 1981;**147**:1077–84.

Khan Mirzaei M, Haileselassie Y, Navis M *et al.* Morphologically distinct *Escherichia coli* bacteriophages differ in their efficacy and ability to stimulate cytokine release in vitro. *Front Microbiol* 2016;**7**:437.

Kim JH, Choresca CH, Shin SP *et al.* Biological control of *Aeromonas salmonicida* subsp. *salmonicida* infection in rainbow trout (*Oncorhynchus mykiss*) using *Aeromonas* Phage PAS-1. *Transbound Emerg Dis* 2013;**62:**81-6.

Klumpp J, Loessner M. New research on bacteriophages and food safety. *Advances in Microbial Food Safety*. Elsevier Science, 2013, 321.

Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol* 2010;**8**:423–35.

Koskella B, Meaden S. Understanding bacteriophage specificity in natural microbial communities. *Viruses* 2013;**5**:806–23.

Krantz GE, Reddecliff JM, Heist CE. Immune response of trout to *Aeromonas salmonicida*. Part I. Development of agglutinating antibodies and protective immunity. *Progress Fish-Culturist* 1964;**26**:3–10.

L'Abée-Lund TM, Sørum H. Functional Tn*5393*-like transposon in the R plasmid pRAS2 from the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida* isolated in Norway. *Appl Environ Microbiol* 2000;**66**:5533–5.

L'Abée-Lund TM, Sørum H. Class 1 integrons mediate antibiotic resistance in the fish

pathogen *Aeromonas salmonicida* worldwide. *Microb Drug Resist* 2001;**7**:263–72.

L'Abée-Lund TM, Sørum H. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. *Plasmid* 2002;**47**:172–81.

Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. *Nat Rev Microbiol* 2010;**8**:317–27.

Lafaille A. *Rapport Des Activités En Ichtyopathologie*. 2016: 1-23

Lafaille A. *Rapport Des Activités En Ichtyopathologie*. 2017: 1-24

Lewis K. Platforms for antibiotic discovery. *Nat Rev Drug Discov* 2013;**12**:371–87.

Lipinski T, Gamian A, Zuziak E *et al.* Purified bacteriophage, its preparation and application. 2016. Patent US9255251.

Liu Y-Y, Wang Y, Walsh TR *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016;**16**:161–8.

Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. *Bacteriophage* 2011;**1**:111–4.

Loftie-Eaton W, Rawlings DE. Comparative biology of two natural variants of the IncQ-2 family plasmids, pRAS3.1 and pRAS3.2. *J Bacteriol* 2009;**191**:6436–46.

Loftie-Eaton W, Rawlings DE. Evolutionary competitiveness of two natural variants of the IncQ-like plasmids, pRAS3.1 and pRAS3.2. *J Bacteriol* 2010;**192**:6182–90.

Long M, Nielsen TK, Leisner JJ *et al. Aeromonas salmonicida* subsp. *salmonicida* strains isolated from Chinese freshwater fish contain a novel genomic island and possible regional-specific mobile genetic elements profiles. *FEMS Microbiol Lett* 2016;**363**:fnw190.

Lu TK, Koeris MS. The next generation of bacteriophage therapy. *Curr Opin Microbiol* 2011;**14**:524–31.

Luria S, Delbrück M. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 1943;**28**:491–511.

Lwoff A, Horne R, Tournier P. A System of viruses. *Cold Spring Harb Symp Quant Biol* 1962;**27**:51–5.

Lynch M, Ackerman MS, Gout J-F *et al.* Genetic drift, selection and the evolution of the mutation rate. *Nat Rev Genet* 2016;**17**:704–14.

MAPAQ. *Pêches et Aquaculture Commerciales Au Québec En Un Coup D'oeil*. 2016:1-52

Martin-Carnahan A, Joseph S. *Aeromonadales* ord. nov. In: Brenner D, Krieg N, Staley J, et al. (eds.). *Bergey's Manual® of Systematic Bacteriology SE - 12*. Springer US, 2005, 556–87.

Martin MJ, Thottathil SE, Newman TB. Antibiotics overuse in animal agriculture: A call to action for health care providers. *Am J Public Health* 2015;**105**:2409–10.

Martinez JL. Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* 2009;**157**:2893–902.

Mateus L, Costa L, Silva YJ *et al.* Efficiency of phage cocktails in the inactivation of *Vibrio* in aquaculture. *Aquaculture* 2014;**424–425**:167–73.

Maurer JJ, Martin G, Hernandez S *et al.* Diversity and persistence of *Salmonella enterica* strains in rural landscapes in the southeastern United States. *PLoS One* 2015;**10**: e0128937

McCarthy DH. Detection of *Aeromonas salmonicida* antigen in diseased fish tissue. *J Gen Microbiol* 1975a;**88**:384–6.

McCarthy DH. Fish Furunculosis. *Aquac Res* 1975b;**6**:13–8.

McCormick WA, Stevenson RM, MacInnes JI. Restriction endonuclease fingerprinting analysis of Canadian isolates of *Aeromonas salmonicida*. *Can J Microbiol* 1990;**36**:24–32.

McGann P, Snesrud E, Maybank R *et al. Escherichia coli* harboring *mcr-1* and blaCTX-M on a novel IncF plasmid: First report of *mcr-1* in the United States. *Antimicrob Agents Chemother* 2016;**60**:4420–1.

McKinley EB. The bacteriophage in the treatment of infections. *Arch Intern Med* 1923;**32**:899–910.

Medini D, Donati C, Tettelin H *et al.* The microbial pan-genome. *Curr Opin Genet Dev* 2005;**15**:589–94.

Merino S, De Mendoza E, Canals R *et al.* Functional genomics of the *Aeromonas salmonicida* lipopolysaccharide O-antigen and A-layer from typical and atypical strains. *Mar Drugs* 2015;**13**:3791–808.

Midtlyng PJ. Vaccination against furunculosis. *Fish Vaccination*. John Wiley & Sons, Ltd, 2014, 185–99.

Moreira D, López-García P. Ten reasons to exclude viruses from the tree of life. *Nat Rev Microbiol* 2009;**7**:306–11.

Morin R. L'utilisation des antibiotiques pour combattre la furonculose chez l'omble de fontaine génère de l'antibiorésistance chez *Aeromonas salmonicida*. *L'aquicole* 2010;**15**.

Munita JM, Arias CA. Mechanisms of antibiotic resistance. *Microbiol Spectr* 2016;**4**:1-24

Najimi M, Balado M, Lemos ML *et al.* Genetic characterization of pAsa6, a new plasmid from *Aeromonas salmonicida* subsp. *salmonicida* that encodes a type III effector protein AopH homolog. *Plasmid* 2009;**61**:176–81.

Najimi M, Lemos ML, Osorio CR. Distribution of small plasmids in *Aeromonas salmonicida* subsp. *salmonicida* strains isolated from NW Spain and Portugal: evidence of clonality in strains isolated from turbot, *Psetta maxima (L.)*. *J Fish Dis* 2008;**31**:469–72.

Nash HA. Integration and excision of bacteriophage λ: The mechanism of conservative site specific recombination. *Annu Rev Genet* 1981;**15**:143–67.

Nielsen B, Olsen J., Larsen JL. Plasmid profiling as an epidemiological marker within *Aeromonas salmonicida*. *Dis Aquat Organ* 1993;**15**:129–35.

O'hIci B, Olivier G, Powell R. Genetic diversity of the fish pathogen *Aeromonas salmonicida* demonstrated by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses. *Dis Aquat Organ* 2000;**39**:109–19.

O'Neil J. *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations*. 2016:1-84.

De Paepe M, Hutinet G, Son O *et al.* Temperate phages acquire DNA from defective prophages by relaxed homologous recombination: The role of Rad52-like recombinases. *PLoS Genet* 2014;**10**:e1004181.

Parks DH, Rinke C, Chuvochina M *et al.* Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. *Nat Microbiol* 2017;**2**:1533–42.

Paterson WD, Douglas RJ, Grinyer I *et al.* Isolation and preliminary characterization of some *Aeromonas salmonicida* bacteriophages. *J Fish Res Board Canada* 1969;**26**:629–32.

Paul JH. Prophages in marine bacteria: dangerous molecular time bombs or the key to survival in the seas? *ISME J* 2008;**2**:579–89.

Pavan ME, Abbott SL, Zorzópulos J *et al. Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase- positive subspecies isolated from a heavily polluted river. *Int J Syst Evol Microbiol* 2000;**50**:1119–24.

Pereira C, Silva YJ, Santos AL *et al.* Bacteriophages with potential for inactivation of fish pathogenic bacteria: Survival, host specificity and effect on bacterial community structure. *Mar Drugs* 2011;**9**:2236–55.

Petrov VM, Ratnayaka S, Nolan JM *et al.* Genomes of the T4-related bacteriophages as windows on microbial genome evolution. *Virol J* 2010;**7**:292.

Phillippy AM. New advances in sequence assembly. *Genome Res* 2017;**27**:xi–xiii.

Piddock LJ V. The crisis of no new antibiotics-what is the way forward? *Lancet Infect Dis* 2012;**12**:249–53.

Piotrowska M, Popowska M. Insight into the mobilome of *Aeromonas* strains. *Front Microbiol* 2015;**6:**494.

Plunkett G, Rose DJ, Durfee TJ *et al.* Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* O157:H7: Shiga toxin as a phage late-gene product. *J Bacteriol* 1999;**181**:1767–78.

Popoff M. Etude sur les *Aeromonas salmonicida*. II. Characterisation des bacteriophages actifs sur les *Aeromonas salmonicida* et lysotypie. *Ann Rech Vet* 1971;**2**:35–45.

Prabhakaran R, Chithambaram S, Xuhua X. *Aeromonas* phages encode tRNAs for their overused codons. *Int J Comput Biol Drug Des* 2014;**7**:168–82.

Proctor LM, Fuhrman JA. Viral mortality of marine bacteria and cyanobacteria. *Nature* 1990;**343**:60–2.

Public Health Agency of Canada. *Canadian Antimicrobial Resistance Surveillance System*. 2016:1-118.

Queenan K, Häsler B, Rushton J. A One Health approach to antimicrobial resistance surveillance: is there a business case for it? *Int J Antimicrob Agents* 2016;**48**:422–7.

Raaijmakers JM, Mazzola M. Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu Rev Phytopathol* 2012;**50**:403–24.

Rajendhran J, Gunasekaran P. Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. *Microbiol Res* 2011;**166**:99–110.

Reardon S. Antibiotic alternatives rev up bacterial arms race. *Nature* 2015;**521**:402–3.

Reith ME, Singh RK, Curtis B *et al.* The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* 2008;**9**:427.

Renkawitz J, Lademann CA, Jentsch S. Mechanisms and principles of homology search during recombination. *Nat Rev Mol Cell Biol* 2014;**15**:369–83.

Rhodes G, Huys G, Swings J *et al.* Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: Implication of Tn*1721* in dissemination of the tetracycline resistance determinant Tet A. *Appl Environ Microbiol* 2000;**66**:3883–90.

Rouli L, Merhej V, Fournier P-E *et al.* The bacterial pangenome as a new tool for analysing pathogenic bacteria. *New Microbes New Infect* 2015;**7**:72–85.

Samson JE, Magadán AH, Sabri M *et al.* Revenge of the phages: defeating bacterial defences. *Nat Rev Microbiol* 2013;**11**:675–87.

Sandaa RA, Enger O. Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. *Appl Environ Microbiol* 1994;**60**:4234–8.

Sandaa RA, Enger Ø. High frequency transfer of a broad host range plasmid present in an atypical strain of the fish pathogen *Aeromonas salmonicida*. *Dis Aquat Organ* 1996;**24**:71–5.

Sanger F, Air GM, Barrell BG *et al.* Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* 1977;**265**:687–95.

Sanger F, Coulson AR, Hong GF *et al.* Nucleotide sequence of bacteriophage lambda DNA. *J Mol Biol* 1982;**162**:729–73.

Sarhan WA, Azzazy HME. Phage approved in food, why not as a therapeutic? *Expert Rev*

Anti Infect Ther 2015;**13**:91–101.

Schwarz S, Kehrenberg C, Doublet B *et al.* Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev* 2004;**28**:519–42.

Silva YJ, Costa L, Pereira C *et al.* Influence of environmental variables in the efficiency of phage therapy in aquaculture. *Microb Biotechnol* 2014;**7**:401–13.

Sime-Ngando T. Environmental bacteriophages: viruses of microbes in aquatic ecosystems. *Front Microbiol* 2014;**5**:1–14.

Smith HO, Welcox KW. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. *J Mol Biol* 1970;**51**:379–91.

Smith IW. The classification of "*Bacterium salmonicida*." *J Gen Microbiol* 1963;**33**:263–74.

Snieszko SF, Bullock GL. Resistance to ulcer disease and furunculosis in eastern brook trout. *USDI, Fish Wildl Fish Bull* 1957;**125**:555–64.

Snieszko SF, Dunbar CE, Bullock GL. Resistance to ulcer disease and furunculosis in eastern brook trout, *Salvelinus Fontinalis*. *Progress Fish-Culturist* 1959;**21**:111–6.

Soler L, Yáñez MA, Chacon MR *et al.* Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes. *Int J Syst Evol Microbiol* 2004;**54**:1511–9.

Sorum H. Mobile drug resistance genes among fish bacteria. *APMIS* 1998;**106**:74–6.

Sørum H, L'Abée-Lund TM, Solberg A *et al.* Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 2003;**47**:1285–90.

Srivastava S. Plasmids: Their biology and functions. *Genetics of Bacteria*. India: Springer India, 2013, 125–51.

Stuber K, Burr SE, Braun M *et al.* Type III secretion genes in *Aeromonas salmonicida*

subsp. *salmonicida* are located on a large thermolabile virulence plasmid. *J Clin Microbiol* 2003;**41**:3854–6.

Studer N, Frey J, Vanden Bergh P. Clustering subspecies of *Aeromonas salmonicida* using IS*630* typing. *BMC Microbiol* 2013;**13**:13–36.

Sulakvelidze A, Alavidze Z. Bacteriophage therapy. *Antimicrob Agents Chemother* 2001;**45**:649–59.

Summers WC. The strange history of phage therapy. *Bacteriophage* 2012;**2**:130–3.

Sutcliffe JG. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harb Symp Quant Biol* 1979;**43**:77–90.

Suttle CA. Marine viruses--major players in the global ecosystem. *Nat Rev Microbiol* 2007;**5**:801–12.

Tanaka KH, Dallaire-Dufresne S, Daher RK *et al.* An insertion sequence-dependent plasmid rearrangement in *Aeromonas salmonicida* causes the loss of the type three secretion system. *PLoS One* 2012;**7**:e33725.

Tanaka KH, Vincent AT, Emond-Rheault J-G *et al.* Plasmid composition in *Aeromonas salmonicida* subsp. *salmonicida* 01-B526 unravels unsuspected type three secretion system loss patterns. *BMC Genomics* 2017;**18**:528.

Tanaka KH, Vincent AT, Trudel MV *et al.* The mosaic architecture of *Aeromonas salmonicida* subsp. *salmonicida* pAsa4 plasmid and its consequences on antibiotic resistance. *PeerJ* 2016;**4**:e2595.

Todd C. The presence of a bacteriophage for *B. salmonicida* in river waters. *Nature* 1933;**131**:360.

Tomás JM. The main *Aeromonas* pathogenic factors. *ISRN Microbiol* 2012;**2012**:1–22.

Toranzo AE, Barja JL, Colwell RR *et al.* Characterization of plasmids in bacterial fish pathogens. *Infect Immun* 1983;**39**:184–92.

Toranzo AE, Santos Y, Nunez S *et al.* Biochemical and serological characteristics, drug resistance and plasmid profiles of spanish isolates of *Aeromonas salmonicida*. *Fish Pathol* 1991;**26**:55–60.

Trudel MV., Vincent AT, Attéré SA *et al.* Diversity of antibiotic-resistance genes in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida*: dominance of pSN254b and discovery of pAsa8. *Sci Rep* 2016;**6**:35617.

Trudel MV, Tanaka KH, Filion G *et al.* Insertion sequence *AS5* (IS*AS5*) is involved in the genomic plasticity of *Aeromonas salmonicida*. *Mob Genet Elements* 2013;**5**:1–7.

Twort FW. An investigation on the nature of ultra-microscopic viruses. *Lancet* 1915;**186**:1241–3.

Umelo E, Trust TJ. Physical map of the chromosome of *Aeromonas salmonicida* and genomic comparisons between *Aeromonas* strains. *Microbiology* 1998;**144**:2141–9.

Verheust C, Pauwels K, Mahillon J *et al.* Contained use of bacteriophages : Risk assessment and biosafety recommendations. *Appl Biosaf* 2010;**15**:32–44.

Vincent AT, Boyle B, Derome N *et al.* Improvement in the DNA sequencing of genomes bearing long repeated elements. *J Microbiol Methods* 2014a;**107**:186–8.

Vincent AT, Charette SJ. Phylogenetic analysis of the fish pathogen *Aeromonas salmonicida* underlines the dichotomy between European and Canadian strains for the *salmonicida* subspecies. *J Fish Dis* 2017;**40**:1241–7.

Vincent AT, Derome N, Boyle B *et al.* Next-generation sequencing (NGS) in the microbiological world: How to make the most of your money. *J Microbiol Methods* 2017;**138**:60–71.

Vincent AT, Emond-Rheault J-G, Frenette M *et al.* Antibiotic resistance due to an unusual ColE1-type replicon plasmid in *Aeromonas salmonicida*. *Microbiology* 2016;**162**:942–53.

Vincent AT, Trudel MV, Paquet VE *et al.* Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug-resistance, interspecies exchanges, and plasmid reshaping. *Antimicrob Agents Chemother* 2014b;**58**:7367–74.

Wayne A, Mccarthy R, Lindenmayer J. Therapeutic antibiotic use patterns in dogs: Observations from a veterinary teaching hospital. *J Small Anim Pract* 2011;**52**:310–8.

Weiss B, Richardson CC. Enzymatic breakage and joining of deoxyribonucleic acid, I. Repair of single-strand breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. *Proc Natl Acad Sci U S A* 1967;**57**:1021–8.

Welch TJ, Fricke WF, McDermott PF *et al.* Multiple antimicrobial resistance in plague: An emerging public health risk. *PLoS One* 2007;**2**:e309.

Woo PCY, Lau SKP, Teng JLL *et al.* Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin Microbiol Infect* 2008;**14**:908–34.

World Health Organization. *Global Action Plan on Antimicrobial Resistance*. 2015:1-28

Wright A, Hawkins CH, Änggård EE *et al.* A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa* ; a preliminary report of efficacy. *Clin Otolaryngol* 2009;**34**:349–57.

Wu J-L, Lin H-M, Jan L *et al.* Biological control of fish bacterial pathogen, *Aeromonas hydrophila*, by bacteriophage AH 1. *Fish Pathol* 1981;**15**:271–6.

Yang L-L, Tang S-K, Huang Y *et al.* Low temperature adaptation is not the opposite process of high temperature adaptation in terms of changes in amino acid composition. *Genome Biol Evol* 2015;**7**:3426–33.

Zhu W, Wang J, Zhu Y *et al.* Identification of three extra-chromosomal replicons in *Leptospira pathogenic* strain and development of new shuttle vectors. *BMC Genomics* 2015;**16**:90.