UNIVERSITY OF INSUBRIA

Aeromonas spp.: model for monitoring the impact and diffusion of antibiotic compounds in water environments.

Ph.D. School:

Biological and Medical Sciences

Ph.D. Program in:

"ANALYSIS, PROTECTION AND MANAGEMENT OF BIODIVERSITY"

-XXVIII Course-

Federica Mauri

Work carried out at the:

Laboratory of Applied Microbiology, Bellinzona

Tutors:

Prof. Paola Barbieri

Dr. Antonella Demarta

December 2015

1. Purpose 5
2. Introduction 6
2.1. The worldwide problem of antibiotic resistance 6
2.2. Swiss situation and strategies 7
2.3. Antibiotics as a pollutants 8
2.4. The aquatic environment: role in spread of resistance 9
2.5. Antibiotic Resistance genes and horizontal gene transfer 10
2.5.1. Conjugation 12
2.5.2. Transformation 12
2.5.3. Bacteriophages 14
2.5.4. Transduction 16
2.5.5. Plasmids 17
2.5.6. Integrons and gene cassettes 18
2.5.7. Transposon tn21 and tn21-like 19
2.6. Aeromonas spp. 20
2.6.1. Taxonomy 21
2.6.2. Role of Aeromonas spp. in the aquatic environment 21
2.6.3. Diseases 22
2.7. Fecal coliforms 23
3. References 24
4. First manuscript submitted to the *Science to the total environment* 32
4.1. Introduction 34
4.2. Materials and Methods 35
4.2.1. Sampling areas and isolation of strains 35
4.2.2. Antibiotic susceptibility test 36
4.2.3. Dot-blot hybridization 36
4.2.4. Southern blot hybridization 36
4.2.5. Gene cassettes identification 37
4.2.6. Conjugation experiments 37
4.3. Results 38
4.3.1. Species distribution in the samples 38
4.3.2. Antibiotic susceptibility 38
4.3.3. Plasmids, MOB typing and conjugation experiments 40
4.3.4. Distribution and putative location of the IntI1 gene, and characterization of the associated gene cassettes. 41

4.3.5. Distribution and putative location of the tnpA gene 42
4.4. Discussion and Conclusions 45
4.5. Acknowledgements 50
4.6. References 51
5. Second manuscript submitted to the *Applied and Environmental Microbiology* 57
5.1. Abstract 58
5.2. Introduction 59
5.3. Materials and Methods 60
5.3.1. Bacterial strains 60
5.3.2. DNA and plasmid extraction 63
5.3.3. PCR detection of specific antibiotic resistance genes 63
5.3.4. Natural transformation assays 64
5.3.5. Electroporation 65
5.4. Results and Discussion 66
5.5. Acknowledgments 72
5.6. References 73
6. Third manuscript submitted to the *Future Microbiology* 77
6.1. Abstract 78
6.2. Introduction 79
6.3. Material and methods 80
6.3.1. Bacterial strains 80
6.3.2. Lytic bacteriophages 80
6.3.3. Induction of lysogenic bacteriophages using mitomycin C 81
6.3.4. Bacteriophage host range 81
6.3.5. Phage concentration by PEG/NaCl 81
6.3.6. DNA extraction from bacteria 82
6.3.7. DNA extraction from bacteriophages 82
6.3.8. Screening of antibiotic resistance genes by PCR 83
6.4. Results 84
6.4.1. Host range of lytic bacteriophages 84
6.4.2. Antibiotic resistance determinants in the DNA of lytic bacteriophages 86
6.4.3. Host range of lysogenic bacteriophages 86
6.4.4. Antibiotic resistance determinants in the DNA of lysogenic bacteriophages 87
6.4.5. Morphology 87
6.5. Discussion and conclusions 88

6.6. Acknowledgments 90

6.7 References 91

7. Transduction 96

7.1. Preliminary attempts for the demonstration of phage transduction between Aeromonas strains.. 98

7.1.1 Material and methods 98

7.1.2 Preliminary Results. 99

7.1.3. Outlooks for the future 99

7.2 Acknowledgments 100

7.3 References 101

8. Conclusions 104

9. Ringraziamenti 106

1. Purpose

Antibiotic resistant bacteria and antibiotic resistance genes cause increasing problems in clinical setting and are worldwide considered emerging environmental contaminants, but little is known about their fate in the receiving environment and how natural microbial populations may be affected. In the environment, antibiotic resistant bacteria (e.g. pathogen) may die but their resistance determinants may become part of the environmental gene pool via horizontal gene transfer to environmental bacteria. From this localization, resistance genes may move back to human and animal bacteria via food and drinking water. Natural environment, especially water, is considered a "hot spot" for spread of antibiotics resistance in this scenario *Aeromonas* spp. were considered the model of hydric microorganisms exposed to the actions of residual antibiotic compounds and to the aquatic resistome, whereas Faecal Coliforms represented the allochthonous population contaminating the aquatic environment. In order to investigate how these two bacterial populations could be influenced by potential antibiotic pollution, we want to examine the presence, the distribution and the transferability of transposons, integrons, and plasmids in *Aeromonas spp.* and Faecal Coliforms isolated from different aquatic environments submitted to a diverse degree of antibiotic contamination. In this work, we want to highlight if there are common genetic determinants in the two populations and check the potential transferability of these genetic determinants. Conjugation, natural transformation and transduction are the three mechanisms of horizontal antibiotic resistance genes transfer among bacteria. Additionally, we want to investigate if *Aeromonas* could also be a good donor in mating experiments with other bacteria and if it is able to naturally acquired free DNA from the environment and if *Aeromonas* DNA could be incorporated by natural competent bacteria. Finally we want to study lytic and lysogenic bacteriophages of *Aeromonas* spp., investigate the presence of resistance genes on phage DNA and evaluate if phage can transfer these resistance genes by transduction experiments.

2. Introduction

2.1. The worldwide problem of antibiotic resistance.

The increase of antibiotic resistance genes, represent a serious threat to public health in the United States and in Europe, where it was estimated that 25,000 people die each year due to multi-resistant bacteria infections (Blair et al. 2014). "Combating antibiotic-resistant bacteria is a national security priority, so there is hereby established the Task Force for Combating Antibiotic-Resistant Bacteria" [1]. This is an extract of an executive order released from the Office of the Press Secretary of Withe House. Although discovery of antibiotics was one of the best goal of scientific world, over the last year antibiotic resistance become a global problem. The use and mainly the ab-use of antibiotic therapy led to development of multi-resistant bacteria that cause diseases very difficult to treat (Gillings 2013; Alekshun et al. 2007). In order to fight against these super-bugs the healthcare resources will be consumed with a considerable economic loss. The World health organization (WHO), the World Organization for Animal Health (OIE) and the European Commission[2], as well as countries such as the UK, France and the USA are developing a global action to control the diffusion of antibiotic resistant bacteria (figure 1).

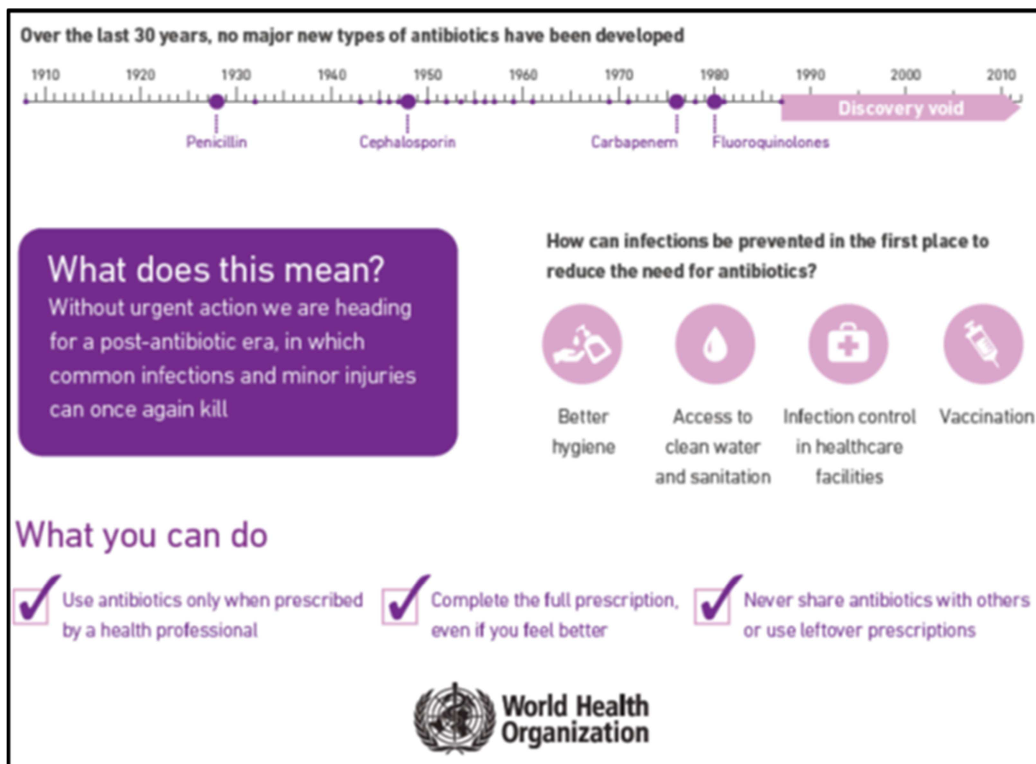


Figure 1 What should be done according World Health Organization in order to prevent infection and avoid spread of antibiotic resistance. Hygiene, vaccination and good clinical practical can prevent the need of antibiotics. Correct use of antibiotic avoid develop of super-bugs with several antibiotic resistances.

The main goals of the WHO are to elaborate standard strategies for surveillance of antimicrobials resistance favoring the collaboration between global networks involved in control and prevention of antibiotic resistance; and to make all people aware about the problem, giving a guideline for the correct usage of antibiotics (WHO Antimicrobial resistance: global report on surveillance 2014).

[1] http://www.whitehouse.gov/the-press-office/2014/09/18/executive-order-combating-antibiotic-resistant-bacteria.

2 COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT AND THE COUNCIL (Action plan against the rising threats from Antimicrobial Resistance)

2.2. Swiss situation and strategies

Within a national program of research (PNR49 2001-2006) two centers for antibiotic resistance surveillance have been created, Anresis.ch5 and ARCH-Vet6 which were responsible to collect data about resistances and antibiotic consumption in human and veterinary medicine. Antibiotics consumption in outpatient in Switzerland are the lowest observed in Europe (Czekalski et al. 2015), nevertheless the nosocomial infections are about 70,000 for year and 2000 lead to patients decease. The percentage of these nosocomial infections, caused by antibiotic resistance bacteria, still unknown. On July 2013 the federal department of the interior (DPI) and the federal department of economy, training and research (DEFR), have commissioned the federal office of public health (OFSP); the federal office of food safety and veterinary practice (OSAV); the federal office of agriculture (OFAG) and the federal office of environment (OFEV) to elaborate a global strategy to fight against antibiotic resistance in Switzerland. Since antimicrobial resistance genes are present in animal and human pathogens or commensals, as well as in microorganism present in the environment, they might be eventually transmissible by mobile genetic elements such as plasmids, transposable elements and phages, and hence contribute to the emergence and diffusion of

antimicrobial resistance in clinical isolates. For this reason the Swiss strategy addressing the issue with a one-health approach that recognize linkages among humans, animals and their environments in the context of human health. The main objectives of this strategy are: i) implement the knowledge about spread of antibiotic resistance favoring research project; ii) increase prevention measures and favor alternative therapies in order to decrease antibiotic consumption; iii) enforce the directives about the correct use of antibiotic therapy not only in human but also in veterinary and agriculture (Figure 2).

Figure 2 Swiss strategies to fight against antibiotic resistance. One-health approach: linkages among humans, animals and their environments in the context of human health.

2.3. Antibiotics as a pollutants

Pollutant is a substance introduced into the environment that has undesired effects, or adversely affects the usefulness of a resource. A pollutant may cause damage by changing the growth rate of organisms, or by interfering with human health. Pollutants may be classified by various criteria: (1) by the origin: whether they are natural or synthetic. (2) By the effect: on an organ, specie, or on entire ecosystem. (3) By the properties: mobility, persistence, toxicity. (4) By the controllability: ease or difficult of removal[3]. Before the 1998 the antibiotics were used without restrictions for preventing and treating infection in humans, animals and agriculture, for promoting growth of livestock and in aquaculture where they were released directly in the water. Now the European Union has limited the antibiotics use in veterinary and in agricultural field in order to prevent rise of bacteria resistant to antibiotics also used in clinical settings. A large percentage of antibiotics (20-80%) is excreted by humans and animals (urine and feces) and released in waste water and sewage (Andersson and Hughes 2014). Waste water treatment plants reduce the concentration of antibiotics, although low concentration of compound are released in the environment. It was been demonstrated

that continuous exposure at sub-lethal dose of antibiotics, concentrations found in the environment (Gullberg et al. 2011), induce selection of bacteria that are able to survive in this condition (Martinez 2009[a]; Gillings 2013; Andersson and Hughes 2014). This selective pressure act by increasing the mutation rate and inducing compensatory mutation in the resistome that is a complex of all genes involved in phenotype of resistance. In environmental bacteria that naturally have resistance genes, the continuous exposure at low concentration of antibiotic contributes to fixation of preexisting mutation (Björkman 2000; Andersson and Hughes 2014). According to the above criteria antibiotics can be considered environmental pollutants; they are natural or synthetic compounds release in the environment and they cause changing in natural microbiome with an impact on humans' health.

[3]http://www.businessdictionary.com

2.4. The aquatic environment: role in spread of resistance

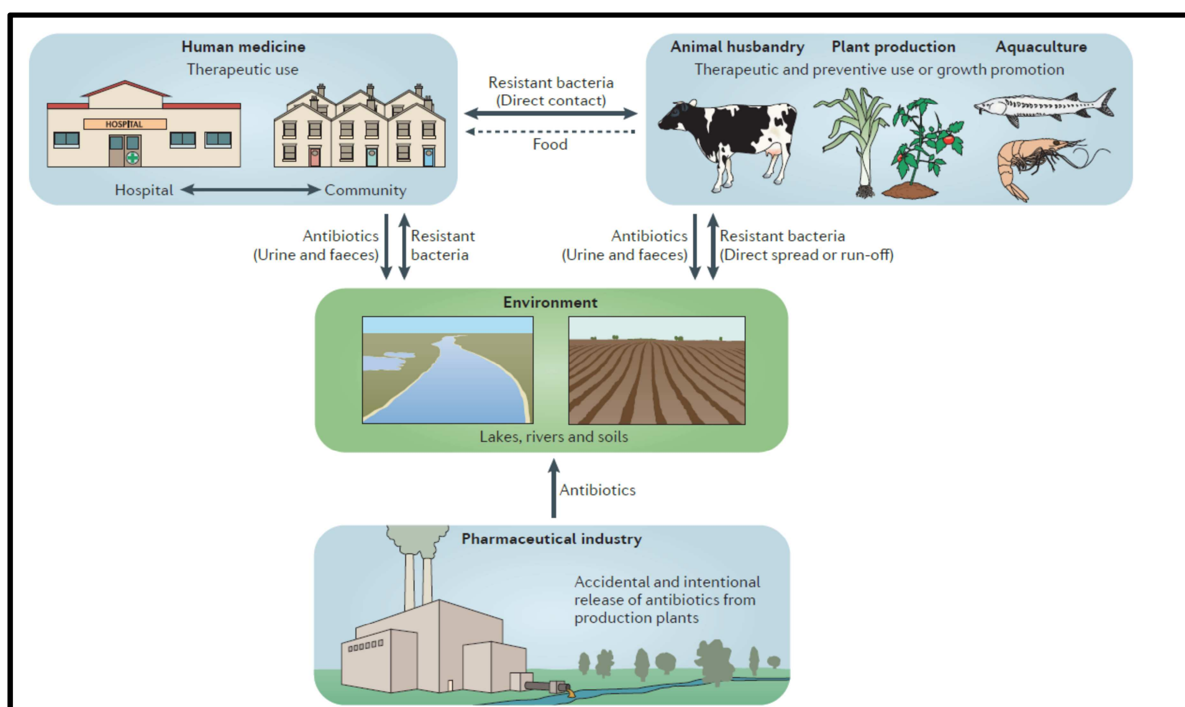


Figure 3. Activities involved in environmental spread of antibiotic resistant bacteria and antibiotic resistance genes (Andersson and Hughes 2014).

The aquatic environment, considered an ideal medium for bacterial life, is divided into four water types: freshwater, seawater, sewage and potable water. Freshwater includes: groundwater that is poor of nutrient but rich of minerals derived from rocks; and surface waters (rivers and alpine or eutrophic lakes) that range from oligotrophic to hypereutrophic states. Superficial waters are influenced by human activities such as treated and untreated sewage, hospital waste water, aquaculture and agriculture discharges (figure 3). Through these settings different kind of pollutants, including antibiotics, reach water environment where affect the natural bacteria population. So water environment is a good ecosystem for spread of antibiotic resistance (Baquero et al. 2008; Taylor et al 2011; Marti et al 2014).

2.5. Antibiotic Resistance genes and horizontal gene transfer

Antibiotic resistance is a process that allows bacteria to survive in presence of antibiotics. Bacteria have become resistant by different mechanism on the basis of the antibiotics target (Van Hoek 2011; Blair et al. 2014). The main mechanisms of resistance are illustrated in figure 4: permeability changes in the bacterial cell wall which restricts antimicrobial access to target sites; active efflux of the drug through pumps; enzymatic inactivation of antibiotic (modification and degradation); acquisition of pathways different from those inhibited by the antibiotic and modification or overproduction of targets.

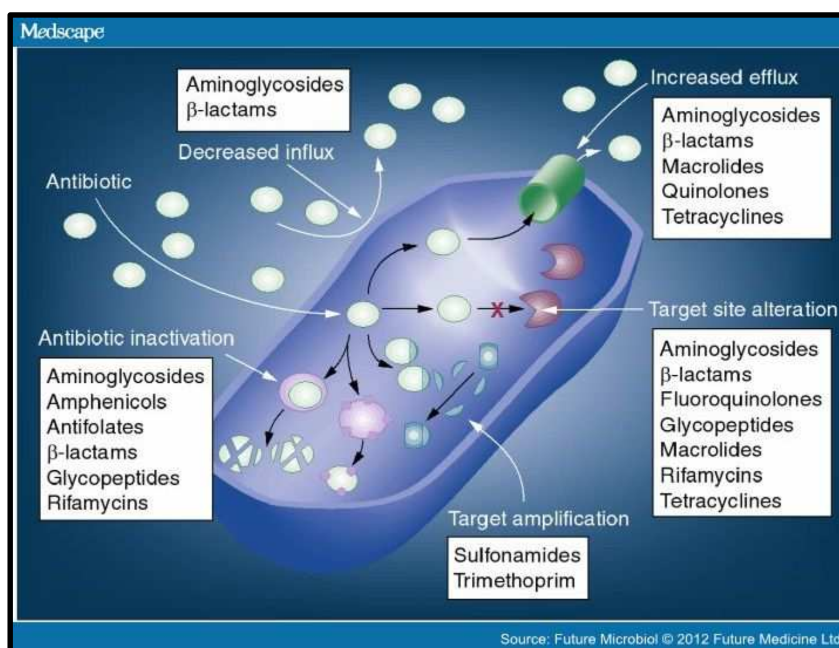


Figure 4: Main antibiotics classes and mechanisms of resistance (McManus MC. 1997).

Bacteria can be intrinsically resistant to certain antibiotics; this kind of resistance does exist before the discovery of antibiotics and is frequent in environmental opportunistic pathogens that have adapted themselves to live in their natural habitat. Human consumption of antibiotics has led to a selective pressure that is an evolutionary system by which bacteria develop resistance through cumulative mutation or horizontal gene acquisition. Mutations are acquired, as result of adaptation, during antibiotic treatment or in contact with sub lethal doses, whereas horizontal gene transfer (HGT) requires a contact between donor (such as environmental bacteria) and recipient (Martinez 2009[b]). Several genes are involved in antibiotic resistance and many are located on mobile elements (plasmid, integrons and transposon) and can be spread by HGT. Resistance gene frequently associated with mobile element are: *aadA* gene confers resistance to streptomycin and spectinomycin codifying for an adenyltransferase that inactivates the antibiotics (Hollingshead and Vapnek 1985); *catB* gene confers resistance to chloramphenicol by antibiotic modification catalyzed by an acetyltransferase (Shaw 1983); betalactamase proteins that inactivate beta-lactam compounds are codify by different genes bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{TEM}, $\mathrm{bla}_{\mathrm{OXA}}$ (Van Hoek 2011); *sulI* and *dfr* genes confer resistance to sulfamethoxazole and trimethoprim respectively by modification of antibiotics targets. *sulI* gene codify for a drug resistant dihydropteroate syntetase and *dfr* genes codify for an alternative dihydrofolate reductase (Radstrom et al. 1991; Brolund et al. 2010). Horizontal gene transfer is a process by which DNA (from one cell of free DNA) is physically moved into another cell, this transfer does not required cells division. DNA can be stably integrated into the recipient cell by self-replication, homologous or illegitimate recombination, transposition and site- specific recombination (Stokes and Gillings 2011). Three mechanisms are involved in HGT (figure 5): conjugation, transformation and transduction (Frost et. al 2005).

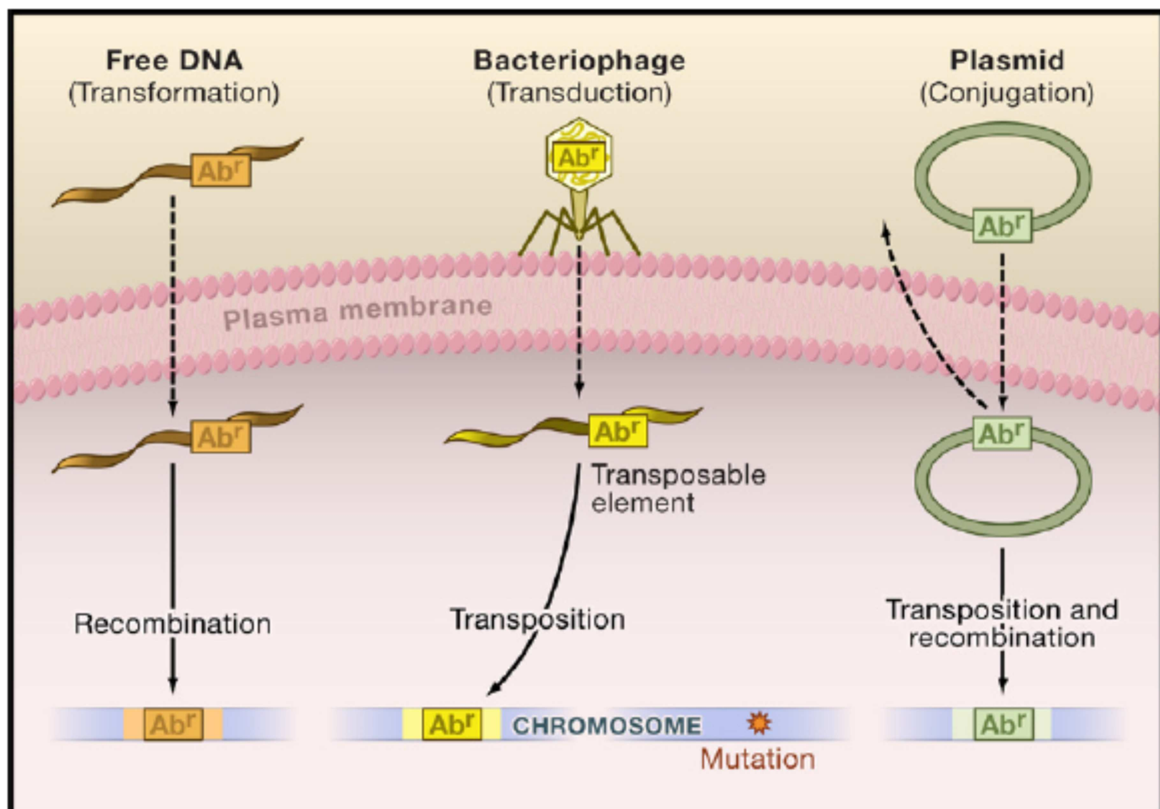


Figure 5 Horizontal gene transfer mechanisms (Alekshun et al. 2007)

2.5.1. Conjugation

Bacterial conjugation is a mechanism by which a donor can transfer genetic material to a recipient with direct contact between the two cells. The conjugation machinery consist of three main components: type IV secretion system (transferosome) that allows the formation of conjugative pilus; the relaxome that promotes the DNA incorporation at origin of transfer (*oriT*) and the T four coupling protein that keep together the other entities (Filutowicz et al. 2008). Conjugation is mainly associated to plasmids acquisition (Thomas and Nielsen 2005).

2.5.2. Transformation

Transformation is a stable genetic change due to the acquisition of exogenous necked DNA. This mechanism not needs the contact between donor and recipient cells but bacteria have to be in a particular physiological state called competence (Thomas and Nielsen 2005). Historically first transformation experiment was performed by Frederick Griffith (1877–1941) in the 1928. He discovered that heat-killed cells of virulent (S) strains of *Pneumococcus* were able to transform

living non-encapsulate (R) strains when they were injected together in a mouse. The animal succumbed to the infection and his blood contained virulent strains (McCarty et al. 1944). There are two kinds of transformation: artificial and natural. Artificial transformation consists of a chemical method for insert foreign DNA, plasmid or ligation products into a bacterial cell (Froger et al. 2007). Bacteria are treated with calcium chloride solution and exposed to heat or electric shock. Calcium chloride is positive charged so bind DNA on the cell surface then the shock lead to pore formation and DNA incorporation (Dagert et al 1979). Some bacterial species are naturally able to bind and internalize foreign DNA under specific condition (natural competence); this mechanism is called natural transformation and depends on expression of specific proteins (Lorenz et al. 1994). The first step is the DNA uptake and involves type IV pili; the passage of DNA through the cytoplasmic membrane is aided by membrane-anchored dsDNA binding protein; finally the exogenous DNA is incorporated into recipient by homologous recombination (Johnsborg et al. 2007; Seitz et al. 2013).The competence machinery is not expressed all the time during bacteria life but there are specific environmental signals (figure 6). During starvation condition, for example when a culture reaches stationary phase of growth, some bacteria die because of decrease of nutrients and release DNA that can be "eated" from live bacteria as a source of nourishment. These process could be also regulated by quorum sensing that is a cell to cell communication through small molecules that bacteria released into the environment when cell density increase. Bacteria that sensing these molecules become competent and start to incorporate free DNA. Another situation that leads to competent state is genotoxic stress induced by UV light or antibiotics that cause DNA damage that could be repaired using external homologous DNA (Solomon and Grossman 1996; Blokesch 2012; Seitz et al. 2013). There are few studies on natural competence as a mechanism for acquisition of antibiotic resistance genes but must be an explanation for spread of non-conjugative transposon, integrons or gene cassettes among different species. Domingues and colleagues demonstrated that *Acinetobacter baylyi* reference strains *BD413* is able to acquire integrons and transposons by natural transformation. Donor DNA derived from different species: *Pseudomonas aeruginosa, Salmonella enterica, Citrobacter freundii, Enterobacter cloacae, Eschrichia coli* and *Escherichia fergusonii* and transformation frequency was about 10^{-9} - 10^{-7} (Domingues et al. 2012). Another natural competent

organism also associated with human and animal diseases is *Campylobacter jejuni*. It was demonstrated that this bacteria can acquired erythromycin resistance by natural transformation with a high frequency and the transformants maintain the resistance stably also in absence of antibiotic (Kim et al. 2006). Natural competence could be considered an evolutionary process that allows bacteria to react and adapt themselves to different stimuli.

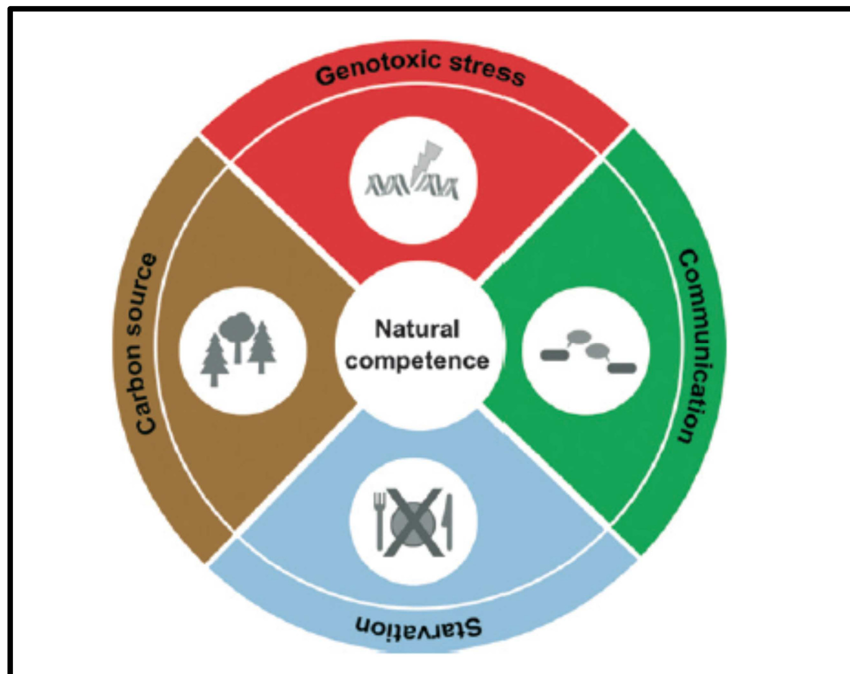


Figure 6 Most frequent hints involved in natural competence development. Genotoxic stress like UV-irradiation or antibiotics exposure; quorum sensing that allow communication between bacteria, depletion of nutrients and use of different carbon source (e.g. chitin substrate. All these signals cause up-regulation of genes involved in DNA up-take.

2.5.3.Bacteriophages

Viruses are non-living genetic element that completely relies on their host for replication. They have a nucleic acid that could be either DNA (double or single strand) or RNA and that is enclosed by a protein shell called capsid. Viral replication cycle are divided in five steps: attachment of the virion to host cell membrane by specific receptor; injection of viral DNA into the host; synthesis of virus nucleic acid and proteins by host machinery; assembly of capsid and maturation of new virus particles; release of mature virions (lysis). Bacterial viruses are named bacteriophages or phages and are classified according to different factors: type of genetic

material, life cycles and morphology. Bacteriophages T2, T4; T3, T7; Mu and lambda are the well characterized phages and infect well known bacteria such as *Escherichia Coli* and *Salmonella.* These phages possess an icosahedral head plus a helical tail and have linear dsDNA genomes. Tailed phages are the most abundant and are divided in three main morphological families: *Myoviridae, Siphoviridae, Podoviridae.* There are also ssDNA and RNA phages (figure 7).

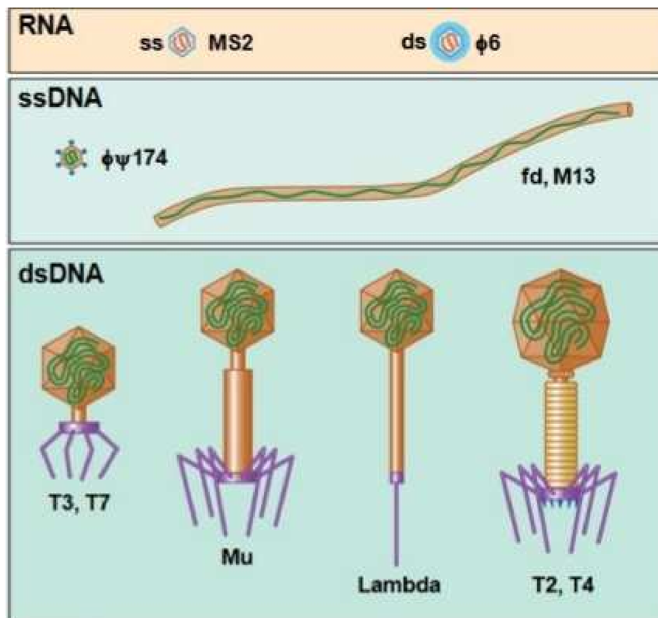


Figure 7 Main type of bacteriophages: DNA and structure (Madiganet al. 2012 Brock).

According to their life cycle bacteriophages are divided in virulent and temperate (figure 8): in virulent (or lytic) mode phage kills the host and mature virions are released, whereas temperate phage (or lysogen) integrates their DNA (prophage) in the host genome and replicates it in synchrony during cell division. In certain condition that can be natural (UV light) or of human origin (antibiotics), temperate phages may revert to the lytic cycle (Madigan et al. 2012; Orlova 2012; Muniesa et al. 2013).

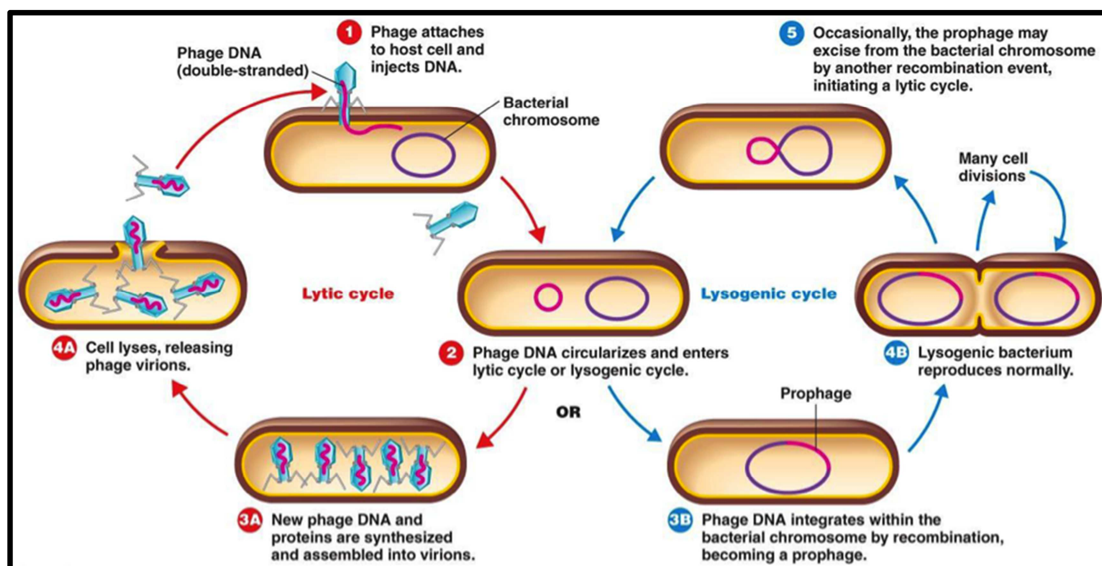


Figure 8. Bacteriophages life cycle. Lytic cycle lead to cells distruption and releasing of virions; in the lysogenic cycle phage DNA is integrated in the host chromosome and replicates with it.

Bacteriophages were first discovered in 1915 by Frederick William Twort in England and in 1917 by Felix D'Herelle in France and before the antibiotics era were used as therapeutics against infection (Clokie et al.2011). In the last years the increase of multi-resistant and difficult to treat bacteria leads to a re-establishment of phage therapy (Kutter et al. 2015) consisting in the use of bacteriophages as a biocontrol agents of bacteria (Gill and Hyman 2010). Good results are obtained in the treatment of infection caused by *Acinetobacter*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in wound, burn patient and in lungs of patient with cystic fibrosis. Phages are also used in food safety and veterinary to prevent food-borne diseases caused by *Escherichia Coli, Campylobacter, Listeria, Salmonella and Vibrio cholera* (Beheshti et al. 2015; Kutter et al. 2015). Phages distribution rely on presence of their hosts so they are the most abundant and persistent entities in the environment. Phages were found in soil, sea water, fresh and ground water, waste water and sewage and also in human and animal faeces (Baggi et al. 2001; Clokie et al.2011; Muniesa et al. 2013), for this reason and because of their role in evolution of bacteria genome, phages could potentially be involved in the horizontal gene transfer (Muniesa et al. 2011).

2.5.4.Transduction

Transduction is a horizontal gene transfer mechanism by which genetic material is transfer from a donor to a recipient using bacteriophage as a vehicle. Since donor and recipient don't need to be in the same place or in the same time, bacteriophage could potentially transfer DNA among different biomes. There are two types of transduction one is generalized transduction that consists in transfer of genetic material from donor, previously infected by phage, to recipient. Donor DNA, derived from any part of the genome (generalized), is packaged into phage head and when the phage particles infect a host, DNA is injected into cytoplasm. Inside the recipient cell, genetic material is stable inherited into the chromosome by homologous recombination. The second type is specialized transduction in which few genes can be transferred, only those integrated near the prophage attachment site. In the generalized transduction both lytic and lysogenic bacteriophages can transfer

DNA, on the contrary only temperate phages can do specialized transduction. The size of DNA incorporated in phage is limited by the capsid and can reach 100 kb so any kind of DNA can be transferred: chromosomal DNA, plasmid, integrons. These genetic elements can carry antibiotic resistance genes so bacteriophages could be involved in spread of resistance in the environment. Transduction is a rare event but, the huge number of phage distributed in different environments and they capability to survive for a long time, increase the possibility to keep in contact with the hosts (Thierauf et al. 2009; Muniesa et al. 2013[a]; Muniesa et al. 2013[b]; Balcazar 2014). There are a lot of evidences that demonstrates the role of bacteriophages in transfer of resistance genes. Colomer-Lluch and colleagues have found *bla* genes in DNA of bacteriophages isolated from urban sewage and from rivers; these genes are responsible for the resistance to β-lactamases. (Colomer-Lluch et al. 2011). Mazaheri et al. have shown that temperate bacteriophages isolated from enterococci can transfer resistance gene, conferring tetracycline and gentamycin resistance by transduction (Mazaheri et al. 2010).

2.5.5.Plasmids

Plasmids are circular double-stranded DNA molecules that vary from a few to several kilobases pairs in length. They contain the machinery essential for their maintenance and replication into recipient cell. Many plasmids contain genes that are useful also to their host (antibiotic resistance genes, heavy metal resistance genes, virulence factor). First classification was between F plasmid (fertility) that is able to be transmitted and can be integrated in host cell; and R plasmid (resistance) that confers resistance to one or more antibiotics and enables the host cell to transfer these resistances to other cells (Couturier et al.1988; Meynell et al. 1968). Now the methods for plasmids' characterization are based on their capability to be mobilizable. One method consisted in the incompatibility that is the inability of two plasmids to coexist and replicate in the same cell (Couturier et al.1988); second method, closely related to the first, is the mobility. Figure 9 shows the relaxase machinery (A) involved in the DNA uptake that is composed by relaxase protein, origin of transfer (*oriT*), T4 coupling protein (T4CP) and type IV secretion system (T4SS). Relaxase protein codifying by a

group of genes called *MOB* genes (Smillie et al. 2010) recognizes the *oriT* and allows the plasmids to be transmissible (conjugative or mobilizable) (B).

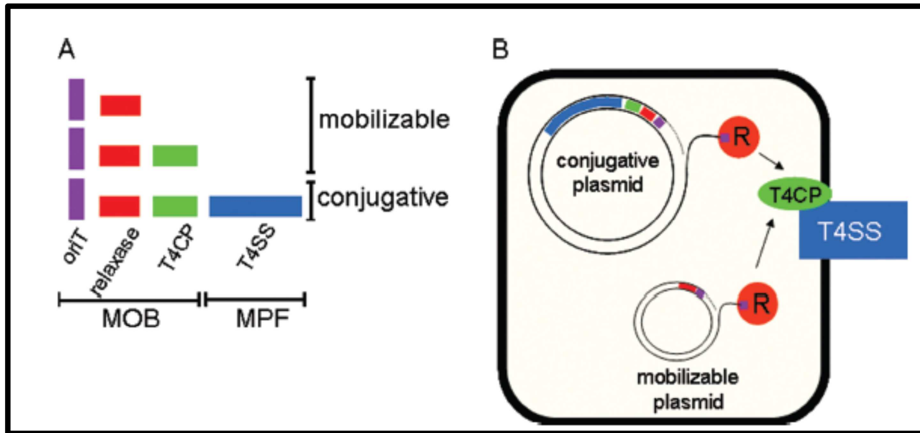


Figure 9 schematic representations of relaxase apparatus All mobilizable plasmids carried an origin of transfer and a relaxase protein; to be conjugative they need the T4 coupling protein (T4CP) and type IV secretion system (T4SS) (Smillie et al. 2010)

The most important resistance determinants carried by plasmids are: integron, gene cassettes and transposons.

2.5.6. Integrons and gene cassettes

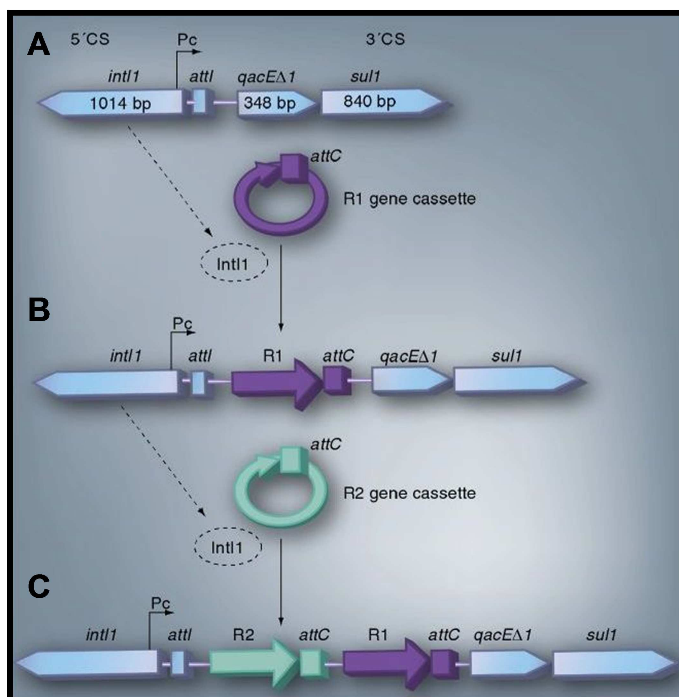


Figure 10 Class 1 integron and acquisition of gene cassettes. A. basic structure of the class 1 integrons. The 5'CS region includes the intI1, the attI and the promoter region (Pc), while the 3'CS includes the qacEΔ1 (quaternary ammonium resistance) and the sul1 (sulphonamide resistance) genes. B-C, A

class 1 integron after insertion of resistance gene cassettes (R1 and R2) (Zhao and Hu, 2011).

Integrons are mobile genetic elements composed by two main segments 5’ and 3’ conserved segment (CS) separated by a variable region (figure 10). At 5’CS there are the *attI* site where gene cassettes can be integrated by site-specific recombination; the integrase, codified by *intI* gene that mediates the recombination and a promotor *Pc*; 3’CS region include a partial deleted *qac* gene (*qacEΔ1*) fused to a *sul1* gene, that confer resistance to the quaternary ammonium compounds and to sulphonamides (Zhao and Hu, 2011). Class I integrons are the most frequently associated to the diffusion of resistance genes in humans, animals and natural environment. Gene cassettes consist in a single gene followed by a short and specific sequence called 59 base element (59-be) that represents the site of recombination. During the gene cassettes incorporation, Integrase catalyzes the recombination between attI and 59be. At attI site other gene cassettes can be integrated with no known limit of number (Hall and Collis 1998).

2.5.7.Transposon tn21 and tn21-like

Transposon tn21 family is involved in the world dissemination of antibiotic resistance genes; it could be self-replicative or inserted in a plasmid.

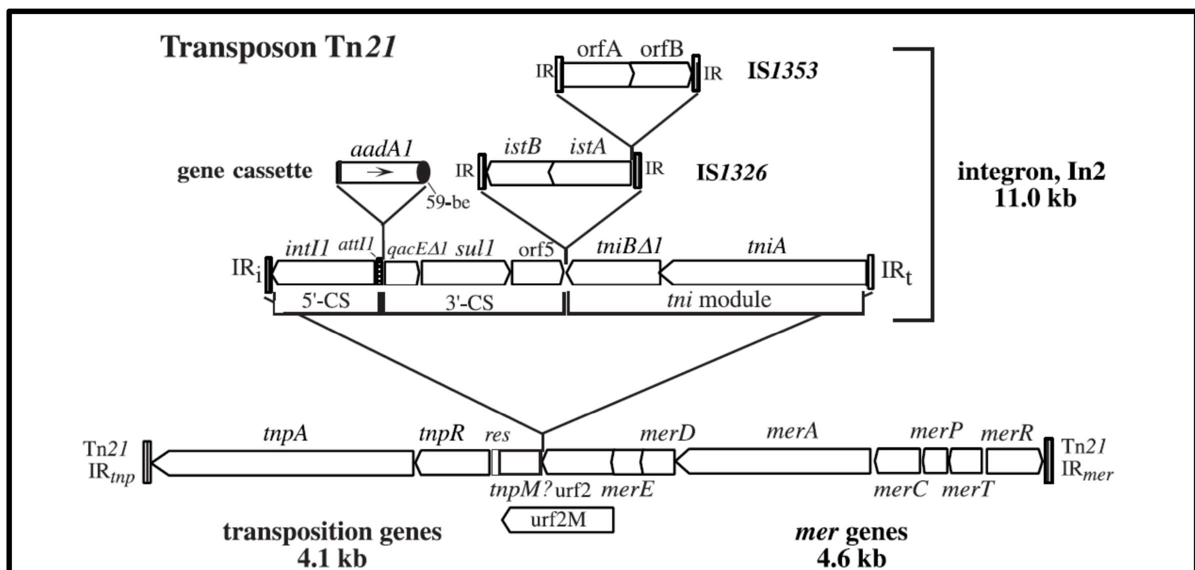


Figure 11. Transposon tn21 structure (Liebert 1999).

The basal structure of transposon is composed by class I integron with *aadA1* and *sulI* gene cassettes; genes for transposase *tnpA* and resolvase *tnpR* and *mer* genes for resistance to mercury (figure 11) (Liebert et al. 1999). Mechanism of replication consists of two steps: the replicative integration catalyzed by transposase and the resolution catalyzed by resolvase. In the first steps a cleavage of each strand of donor and recipient occurs, so both DNA are single stranded and ligated together. DNA replication makes this single stranded region double stranded using existing DNA as template. To separate donor and recipient molecules the resolvase executes a site-specific recombination at *res* sites that lead to a new replicon with one copy of the transposon and the original donor replicon, which retains its copy (Shapiro et al. 1979).

2.6. Aeromonas spp.

Aeromonas spp. is gram negative, oxidase-positive and facultative anaerobic bacillus that is autochthonous to aquatic environments. It has been isolated from brackish, fresh estuarine, marine, chlorinated and unchlorinated waters (Janda and Abbott 2010). *Aeromonas* spp. is an opportunistic pathogen and was also isolated from cold and worm blooded animals including humans (Carnahan and Altwegg 1996). Aeromonads belong to natural bacterial population that could be affected by antibiotic pollution in the environment.

2.6.1.Taxonomy

The name *Aeromonas* were proposed in 1936. First classification included only three motile and one non-motile species. In the 1981 the modern taxonomy was started and the DNA-DNA hybridization technique has allowed the discover of new species. In the nineties molecular method were introduced and new *Aeromonas* taxa were recognized, main molecular technique were: 16S rRNA, AFLP (amplified fragment length polymorphism) and sequence analysis of housekeeping genes *gyrB*, *rpoD, rpoB, recA, dnaJ, cpn60.* Recently a MALDI mass spectrometry analysis was introduced in order to discover new *Aeromonas* species. Table 1 shows the *Aeromonas* species identified until now and their source.

Species	Source of the type strain
A.hydrophila	Tin of milk with fishy odour
A.salmonicida	Salmon
A.sobria	Fish
A.media	Effluent water from a fish farm
A.veronii	Sputum of a drowning victim
A.caviae	Epizootic of young guinea pig
A.eucrenophila	Freshwater fish
A.schubertii	Forehead adscess injury
A.jandaei	Faeces of male with diarrhoea
A.trota	Human faeces
A.allosaccharophila	Diseased eel
A.encheleia	Healthy eel
A.bestiarum	Diseased fish
A.popoffii	Drinking water production plant
A.simiae	Monkey faeces
A.molluscorum	Bivalve molluscs (wedge-shells)
A.bivalvium	Bivalve molluscs (cockles)
A.tecta	Faeces of a child with diarrhoea
A.piscicola	Diseased salmon
A.fluvialis	River water
A.taiwanensis	Burn wound
A.sanarellii	Elbow wound
A.diversa	Leg wound
A.rivuli	Karst water rivulet
A.australiensis	Treated effluent used fof irrigation
A.cavernicola	Water of a brook cavern
A.dhakensis	Faeces of a child with diarrhoea

Table 1. Species included in genus *Aeromonas* (Figuera and Beaz-Hidalgo 2015).

2.6.2. Role of Aeromonas spp. in the aquatic environment

Aeromonas spp. could be a good model for monitoring the influence of antibiotic pollution in water environment not only for their ability to adapt in different aquatic environment (polluted and

unpolluted) but also for the increasing number of resistant strains (Figueira et al. 2011). In the last years were isolated, from environment but also from clinical settings, *Aeromonas* strains resistant to one or more antibiotics commonly used in human and animal therapy (Blasco et al. 2008). Most of isolates also carried genes of antibiotic resistance located on mobile elements (plasmids) (Alcaide et al. 2010; Majumdar et al. 2011; Girlich et al. 2011; Kadlec et al. 2011; Moura et al. 2012). All these features confer to *Aeromonas* the role of reservoir and/or vector of antibiotic resistance in the aquatic environment (Figueira et al. 2011). Multi-drugs resistant *Aeromonas* spp. could be introduced in community and affect human health. Possible sources of contamination are: potable water, food, animals, and contact with superficial water (Janda and Abbott 2010). Another health problem is the transferability of resistance genes from *Aeromonas spp.* to different pathogenic bacteria also present in the environment such as Enterobacteriaceae (Moura et al. 2007).

2.6.3.Diseases

Aeromonas spp. it has been considered the etiological agent of fish diseases, two major groups of illnesses are recognized according to the species that infect the animals. *Aeromonas salmonicida* caused fish foruncolosis that consists in an acute septicemia with small hemorrhages at the bases of fins or in a chronical infection of older fishes. Mesophilic species (*Aeromonas hydrophila*) cause motile *aeromonas* septicemia (hemorrhagic septicemia). The infections cause fishes die-offs with a consistent economic loss (Janda and Abbott 2010). *Aeromonas spp.* is also associated to human diseases.

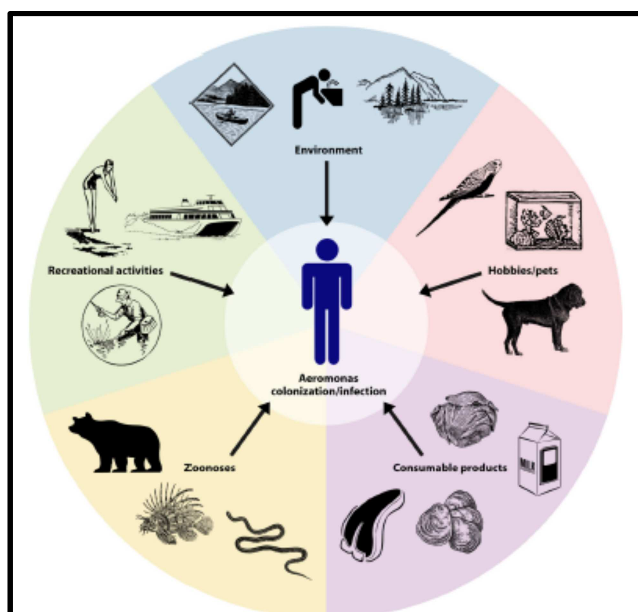


Figure 12 Possible sources that allow humans to get in contact with *Aeromonas.* Aquatic environment (lakes and rivers but also swimming pool) and food are the main causes of contamination; also animal contact could lead to *Aeromonas* infection.

The first type of infection is gastroenteritis caused mainly by *Aeromonas hydrophila, veronii* and *caviae;* second kind of infection is wound infections following a penetrating or abrasion injury that occurs in superficial water on in soil, usually *Aeromonas* in not the bacteria primarily isolated from wound; Aeromonads are also associated to septicaemia mainly in immunocompromised patient with mortality of 25-50%; there was a small cases of respiratory infections caused directly by *Aeromonas* although it is not be considered a respiratory pathogen, these infections were caused by inhalation of water during swimming; other infections affect eyes, bone and joint (Parker and Shaw 2011).

2.7. Fecal coliforms

Fecal coliforms are considered the indicator of fecal contamination of water because they are easily to isolate. They are gram-negative bacilli, oxidase-negative, able to multiply in the presence of bile salts and to ferment lactose with acid and gas production. This group includes bacteria of fecal origin (*Escherichia Coli*) and genera not derived from faces (*Enterobacter, Klebsiella* and *Citrobacter*) (Doyle and Erickson 2006; Ballesté et al. 2010). Fecal Coliforms were isolated from aquatic environment mainly those that are exposed to human activities (waste water treatment plants or hospitals waste waters) and also these strains carried genetic determinants of resistance (plasmids and integrons) (Moura et al. 2007). In this scenario fecal coliforms represent not only resistant bacteria that reach and pollute water settings, but also bacterial population that could acquire resistance genes from environmental microbiota.

3. References

1. Alcaide E, Blasco MD and Esteve C. 2010. "Mechanisms of Quinolone Resistance in Aeromonas Species Isolated from Humans, Water and Eels." *Research in Microbiology* 161 (1): 40–5
2. Alekshun M.N. and Stuart B.L. 2007. "Molecular Mechanisms of Antibacterial Multidrug Resistance." *Cell* 128 (6): 1037–50.
3. Andersson D I and Hughes D. 2014. "Microbiological Effects of Sub-lethal Levels of Antibiotics." *Nature Reviews. Microbiology* 12 (7): 465–78.
4. Baggi F., Demarta A. and Peduzzi R. 2001. "Persistence of Viral Pathogenes and Bacteriophages during Sewage Treatment: Lack of Correlation with Indicator Bacteria." *Res Microbiol* 152: 743–751.
5. Balcazar J.L. 2014. "Bacteriophages as Vehicles for Antibiotic Resistance Genes in the Environment." *PLoS Pathogens* 10 (7) (July): e1004219.
6. Ballesté E, Bonjoch X, Belanche L and Blanch AR. 2010. "Molecular Indicators Used in the Development of Predictive Models for Microbial Source Tracking." *Applied and Environmental Microbiology* 76 (6): 1789–1795
7. Baquero F, Martínez JL and Cantón R. 2008. "Antibiotics and Antibiotic Resistance in Water Environments." *Current Opinion in Biotechnology* 19 (3) (June): 260–5.
8. Beheshti M.K., Delfan A.S. and Salmanizadeh S. 2015. "Isolation and Identification of Two Novel Escherichia Coli Bacteriophages and Their Application in Wastewater Treatment and Coliform's Phage Therapy." *Jundishapur Journal of Microbiology* 8 (3).
9. Björkman J. 2000. "Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance." *Science* 287 (5457) (February 25): 1479–1482.
10. Blasco M D, Esteve C and Alcaide E. 2008. "Multiresistant Waterborne Pathogens Isolated from Water Reservoirs and Cooling Systems." *Journal of Applied Microbiology* 105 (2) (August): 469–75.

11. Blair J.M.A., Webber M.A., Baylay A.J., Ogbolu D.O. and Piddock L.J.V. 2014. "Molecular Mechanisms of Antibiotic Resistance." *Nature Reviews. Microbiology* 13 (1): 42–50.
12. Blokesch M. 2012. "A Quorum Sensing-Mediated Switch Contributes to Natural Transformation of Vibrio Cholerae." Mobile Genetic Elements 2 (5) (September 1): 224–227.
13. Brolund A, Martin S, Gunnar K and Malin G. 2010 "Molecular Characterisation of Trimethoprim Resistance in Escherichia Coli and Klebsiella Pneumoniae during a Two Year Intervention on Trimethoprim Use." *PLoS ONE* 5, no. 2: 1–5. doi:10.1371/journal.pone.0009233.
14. Carnahan A.M and Altwegg M. Taxonomy. 1996 In: *The genus Aeromonas*, Edited by Austin B., et al. (John Wiley & Sons Ltd, Chichester). 1-38.
15. Clokie M.Rj, Millard A.D., Letarov A.V. and Heaphy S. 2011. "Phages in Nature." *Bacteriophage* 1 (February): 31–45.
16. Colomer-Lluch M., Jofre J., and Muniesa M. 2011. "Antibiotic Resistance Genes in the Bacteriophage DNA Fraction of Environmental Samples." *PloS One* 6 (3) (January): e17549.
17. Couturier M., Bex F., Bergquist P.L., and Maas W.K. 1988. "Identification and Classification of Bacterial Plasmids." *Microbiological Reviews* 52 (3) (September): 375–95.
18. Czekalski N., Sigdel R., Birtel J, Blake M and Bürgmann H. 2015. "Does Human Activity Impact the Natural Antibiotic Resistance Background? Abundance of Antibiotic Resistance Genes in 21 Swiss Lakes." *Environment International* 81: 45–55.
19. Dagert M. Ehrlich S. (1979). "Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells". Gene 6 (1): 23–28.

20. Domingues S., Harms K., Fricke W.F., Johnsen P.J., da Silva G.J. and Nielsen K.M. 2012. "Natural Transformation Facilitates Transfer of Transposons, Integrons and Gene Cassettes between Bacterial Species." *PLoS Pathogens* 8 (8) (January): e1002837.
21. Doyle MP and Erickson MC. 2006. "Closing the Door on the Fecal Coliform Assay." *Microbe* 1 (4): 162–163.
22. Figueira V, Vaz-Moreira I, Silva M and Manaia CM. 2011. "Diversity and Antibiotic Resistance of Aeromonas Spp. in Drinking and Waste Water Treatment Plants." *Water Research* 45 (17) (November 1): 5599–611.
23. Figuera MJ ans Beaz-Hidalgo R. *Aeromonas* infections in humans. In: *Aeromonas*, Edited by Joerg Graf. (Caister Academic Press). 4: 65-108, 2015.
24. Filutowicz M., Burgess R., Gamelli R.L, Heinemann J., Kurenbach B., Rakowski S., and Shankar R. 2008. "Bacterial Conjugation-Based Antimicrobial Agents." *Plasmid* 60 (1) (July): 38–44.
25. Froger A., and Hall J.E. 2007. "Transformation of Plasmid DNA into E. Coli Using the Heat Shock Method." *Journal of Visualized Experiments : JoVE* (6): 253.
26. Frost L.S., Leplae R., O Summers A., and Toussaint A. 2005. "Mobile Genetic Elements: The Agents of Open Source Evolution." *Nature Reviews. Microbiology* 3 (9) (September): 722–32.
27. Gill Jason J and Hyman P. 2010. "Phage Choice, Isolation, and Preparation for Phage Therapy." Current Pharmaceutical Biotechnology 11 (1): 2–14. doi:10.2174/138920110790725311.
28. Gillings M.R. 2013. "Evolutionary Consequences of Antibiotic Use for the Resistome, Mobilome and Microbial Pangenome." *Frontiers in Microbiology* 4 (4): 1-10
29. Girlich D, Poirel L and Nordmann P. 2011. "Diversity of Clavulanic Acid-Inhibited Extended-Spectrum B-Lactamases in Aeromonas Spp. from the Seine River, Paris, France." *Antimicrobial Agents and Chemotherapy* 55 (3) (March): 1256–61.

30. Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D and Andersson DI. 2011. “Selection of Resistant Bacteria at Very Low Antibiotic Concentrations.” *PLoS Pathogens* 7 (7): 1–9.

31. Hall R.M., and Collis C.M. 1998. “Antibiotic Resistance in Gram-Negative Bacteria: The Role of Gene Cassettes and Integrons.” *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy* 1 (2) (January): 109–19.

32. Hollingshead S and D Vapnek. 1985 “Nucleotide Sequence Analysis of a Gene Encoding a Streptomycin/spectinomycin Adenylyltransferase.” *Plasmid* 13, no. 1: 17–30.

33. Janda J M and Abbott S L. 2010. “The Genus Aeromonas: Taxonomy, Pathogenicity, and Infection.” *Clinical Microbiology Reviews* 23 (1) (January): 35–73.

34. Johnsborg O., Eldholm V., and Håvarstein L.S. 2007. “Natural Genetic Transformation: Prevalence, Mechanisms and Function.” *Research in Microbiology* 158 (10) (December): 767–78.

35. Kadlec K, von Czapiewski E, Kaspar H, Wallmann J, Brenner Michael G, Steinacker U and Schwarz S. 2011. “Molecular Basis of Sulfonamide and Trimethoprim Resistance in Fish-Pathogenic Aeromonas Isolates.” *Applied and Environmental Microbiology* 77 (20) (October): 7147–50.

36. Kim J., Carver D.K. and Kathariou S. 2006. “Natural Transformation-Mediated Transfer of Erythromycin Resistance in Campylobacter Coli Strains from Turkeys and Swine Natural Transformation-Mediated Transfer of Erythromycin Resistance in Campylobacter Coli Strains from Turkeys and Swine.” Applied and Environmental Microbiology 72, no. 2 (2006): 1316–1321. doi:10.1128/AEM.72.2.1316.

37. Kutter E.M., Kuhl S.J. and Abedon S.T. 2015. “Re-Establishing a Place for Phage Therapy in Western Medicine” 10: 685–688.

38. Liebert C.R.M.H., and Summers O. 1999. “Transposon Tn21, Flagship of the Floating Genome.” *Microbiology and Molecular Biology Reviews : MMBR* 63 (3) (September): 507–22.

39. Lorenz M.G., and W. Wackernagel. 1994. “Bacterial Gene Transfer by Natural Genetic Transformation in the Environment.” *Microbiological Reviews* 58 (3) (September): 563–602.
40. Madigan M., Martinki J., Stahl D., Clark D. 2012. “Viruses and Virology.” In: *Biology of microorganisms BROCK Thirteen edition, Benjamin cummings publishers*: chapter 9 265-282.
41. Majumdar T, Das B, Bhadra RK, Dam B and Mazumder S. 2011. “Complete Nucleotide Sequence of a Quinolone Resistance Gene (qnrS2) Carrying Plasmid of Aeromonas Hydrophila Isolated from Fish.” *Plasmid* 66 (2) (July): 79–84.
42. Marti E, Variatza E and Balcazar JL. 2014. “The Role of Aquatic Ecosystems as Reservoirs of Antibiotic Resistance.” *Trends in Microbiology* 22 (1) (January): 36–41.
43. Martinez J.L. 2009[a]. “Environmental Pollution by Antibiotics and by Antibiotic Resistance Determinants.” *Environmental Pollution (Barking, Essex : 1987)* 157 (11): 2893–902.
44. Martinez J.L. 2009[b]. “The Role of Natural Environments in the Evolution of Resistance Traits in Pathogenic Bacteria.” *Proceedings. Biological Sciences / The Royal Society* 276 (1667) (July 22): 2521–30.
45. Mazaheri N.F., Barton R.M.D., and Heuzenroeder M.W. 2011. “Bacteriophage-Mediated Transduction of Antibiotic Resistance in Enterococci.” *Letters in Applied Microbiology* 52 (6) (June): 559–64
46. Mccarty M. 1944. “Studies on the chemical nature of the substance inducing transformation of pneumococcal types” *The Journal of Experimental Medicine* 79 (6): 137–58
47. McManus M.C. Mechanisms of bacterial resistance to antimicrobial agents. Am. J. Health Syst. Pharm. 54: 1420–1433 (1997).
48. Muniesa M., Imamovic L. and Jofre J. 2011. “Bacteriophages and Genetic Mobilization in Sewage and Faecally Polluted Environments.” *Microbial Biotechnology* 4 (6) (November): 725–34. doi:10.1111/j.1751-7915.2011.00264.x.

49. Muniesa M., Colomer-Lluch M. and Jofre J. 2013[a]. "Potential Impact of Environmental Bacteriophages in Spreading Antibiotic Resistance Genes." *Future Microbiology*: 739–751.

50. Muniesa M., Colomer-Lluch M. and Jofre J. 2013[b]. "Could Bacteriophages Transfer Antibiotic Resistance Genes from Environmental Bacteria to Human-Body Associated Bacterial Populations?" *Mobile Genetic Elements* 3 (4) (July 1): e25847.

51. Meynell E.G, Meynell G., and Datta N. 1968. "Phylogenetic Relationships of Drug-Resistance Factors and Other Transmissible Bacterial Plasmids." *Bacteriological Reviews* 32 (1) (March): 55–83.

52. Moura A, Henriques I, Ribeiro R and Correia A. 2007. "Prevalence and Characterization of Integrons from Bacteria Isolated from a Slaughterhouse Wastewater Treatment Plant." *The Journal of Antimicrobial Chemotherapy* 60 (6) (December): 1243–50.

53. Moura A, Oliveira C, Henriques I, Smalla K and Correia A. 2012. "Broad Diversity of Conjugative Plasmids in Integron-Carrying Bacteria from Wastewater Environments." *FEMS Microbiology Letters* 330 (2) (May): 157–64.

54. Orlova E.V. (2012). Bacteriophages and Their Structural Organisation, Bacteriophages, Dr. Ipek Kurtboke (Ed.), ISBN: 978-953-51-0272-4, InTech, Available from: http://www.intechopen.com/books/bacteriophages/bacteriophages-and-their-structural-organisation-

55. Parker JL and Shaw JG. 2011. "Aeromonas Spp. Clinical Microbiology and Disease." *The Journal of Infection* 62 (2) (February): 109–18.

56. Radstrom P, Swedberg G and Skold O. 1991 "Genetic Analyses of Sulfonamide Resistance and Its Dissemination in Gram-Negative Bacteria Illustrate New Aspects of R Plasmid Evolution." *Antimicrobial Agents and Chemotherapy* 35, no. 9: 1840–1848. doi:10.1128/AAC.35.9.1840.

57. Seitz P., and Blokesch M. 2013. "Cues and Regulatory Pathways Involved in Natural Competence and Transformation in Pathogenic and Environmental Gram-Negative Bacteria." *FEMS Microbiology Reviews* 37 (3) (May): 336–63.

58. Shapiro J. 1979. "Molecular Model for the Transposition and Replication of Bacteriophage Mu and Other Transposable Elements." *Proceedings of the National Academy of Sciences of the United States of America* 76 (4): 1933–1937.

59. Shaw W V. 1983 "Chloramphenicol Acetyltransferase: Enzymology and Molecular Biology." *CRC Critical Reviews in Biochemistry* 14, no. 1: 1–46. doi:10.3109/10409238309102789.

60. Smillie C., Garcillán-Barcia M.P., Francia M.V., Rocha E.P.C, and de la Cruz F. 2010. "Mobility of Plasmids." *Microbiology and Molecular Biology Reviews : MMBR* 74 (3) (September): 434–52.

61. Stokes H.W., and Gillings M.R. 2011. "Gene Flow, Mobile Genetic Elements and the Recruitment of Antibiotic Resistance Genes into Gram-Negative Pathogens." *FEMS Microbiology Reviews* 35 (5) (September): 790–819.

62. Taylor NGH, Verner-Jeffreys DW and Baker-Austin C. 2011. "Aquatic Systems: Maintaining, Mixing and Mobilising Antimicrobial Resistance?" *Trends in Ecology and Evolution* 26 (6): 278–284.

63. Thierauf A., Perez G. and Maloy S. 2009. Generalized transduction. In: *Bacteriophages methods and protocols*, Edited by Martha R.J. Clokie and Andrew M. Kropinski (Humana press). 23(1):267-286.

64. Thomas C.M., and Nielsen K.M. 2005. "Mechanisms Of, and Barriers To, Horizontal Gene Transfer between Bacteria." *Nature Reviews. Microbiology* 3 (9) (September): 711–21.

65. Van Hoek, A.H.A.M., Mevius D., Guerra B., Mullany P., Roberts A.P. and Aarts H.J.M. 2011. "Acquired Antibiotic Resistance Genes: An Overview." *Frontiers in Microbiology* 2 2 (203): 1-27

66. Zhao W.H., Hu Z.Q. Epidemiology and genetics of VIM-type metallo-b-lactamases in gram-negative bacilli. *Future Microbiology*, 6 (3), 317-333, 2011.

4. First manuscript submitted to the *Science to the total environment*

Spread and characterization of genetic determinants involved in antibiotic resistance in *Aeromonas spp*. and Faecal Coliforms isolated from different aquatic environments.

Alessandro Carnelli[#], Federica Mauri[#], and Antonella Demarta*

SUPSI laboratory of applied microbiology via Mirasole, 22A 6500 Bellinzona, Switzerland;

***Corresponding author Tel: +41 (0)918146076; E-mail: antonella.demarta@supsi.ch**

[#] These authors contributed equally to the study.

Keywords

Aeromonas spp., Faecal Coliforms, integrons, transposons, aquatic environment

Objectives: To characterize and to investigate the presence and the spread of transposons, integrons and plasmids involved in the antibiotic resistance of *Aeromonas* spp. and Faecal Coliforms isolated from different aquatic environments

Methods: Aeromonads and Faecal Coliforms were isolated from different aquatic environment (river, hospital waste water, waste water treatment plant and alpine lake), and their resistance phenotype and plasmid profile were determined. All strains were screened by Dot blot hybridization with specific probes for the presence of integrase, transposase and MOB subfamilies genes. The putative plasmid location of these genetic determinants was investigated by Southern blot on plasmid extracts. Conjugation experiments were carried out on selected strains.

Results: We recovered a total of 231 *Aeromonas* strains and 250 Faecal Coliforms. The majority of the strains were resistant to cefoxitin (43%), streptomycin (32%) and sulfamethoxazole (25%). Strains carrying plasmids and showing three or more antibiotic resistances were particularly abundant in the wastewater treatment plant. The 25% of the plasmids evidenced in *Aeromonas* strains belonged to the MOB_{P13} and MOB_{P14} subfamilies. In Faecal Coliforms the majority of plasmids belonged to the MOB_{F12}, MOB_{P12}, MOB_{H11}, and MOB_{H121}. Strains carrying the Integrase I gene were found in all kind of waters. The majority of *intI1*-carrying *Aeromonas* were collected from the activated sludge of the wastewater treatment plant (ca. 12% of the strains) whereas *intI1*-carrying Faecal Coliforms from the hospital wastewater (ca. 26%). The *aadA1* gene cassette was the most frequent resistance gene identified followed by the *dfr* genes. The *tnpA* gene was evidenced in the 14% of the strains. Class 1 integrons, and in a lesser extent Tn21-like transposons, were often associated to plasmids which were identified as transmissible by MOB identification.

Conclusions: Our investigations pointed out that both *Aeromonas* and Faecal Coliforms populations are influenced by the antibiotic contamination of the environment. We confirmed that *Aeromonas* spp. can represent a model for studying the incidence and the diffusion of resistance to antibiotics in the aquatic environment, and that they are a reservoir of resistant determinants.

4.1. Introduction

Antibiotic resistant bacteria and antibiotic resistance genes are considered as worldwide emerging environmental contaminants. They are dispersed mainly in the aquatic environment from hospital and community waste water, animal farms, agricultural lands, and waste water treatment plants. Their fate in the receiving environment and their effects on natural microbial populations are little known and poorly studied. In the environment, antibiotic resistant bacteria (e.g. pathogens) may be inactivated but their resistance determinants may become part of the environmental gene pool, may spread horizontally, and may move back to human and animal bacteria via food and drinking water[1,2].

Aeromonas are oxidase positive, Gram-negative bacilli included in the Gammaproteobacteria that are ubiquitous in all aquatic environments (brackish, fresh, estuarine, marine, chlorinated and un-chlorinated water) worldwide[3]. *Aeromonas* are commonly associated to fish diseases but they play also a role as opportunistic pathogens in cold and warm- blood animals[4]. In humans, they can cause life-threatening infections[5]. The term of Faecal Coliforms is referred to Gram-negative non-sporulating bacilli, aerobic or facultative anaerobic, oxidase-negative bacteria[6]. This non taxonomic group includes genera of bacteria that originate mainly in human and animal feces. In particular, *E. coli* represents the most specific indicator of faecal contamination of the aquatic environment[7].

The most important genetic elements involved in the spread of resistance gene are class I integrons, mobile elements consisting of an integrase gene (*IntI*) able to integrate gene cassettes by site-specific recombination mechanisms (*attI*)[8]. The association of integrons with mobile genetic elements such as transposons (in particular of the Tn*21* subfamily) greatly enhances their diffusion by horizontal gene transfer[9].

Transposons belonging to the Tn*21* group of the Tn*3* family are mobile genetic elements that randomly insert into the bacterial genome by the action of the transposase codified by the *tnpA* gene[10]. The Tn*21* transposons group, that can carry integrons as well as other types of resistance determinants such as heavy metal resistance operons, are the mobile structures most implicated in the global dissemination of antibiotic resistance determinants among bacteria[11].

Integrons and transposons can be found on the chromosome as well as on plasmids. The identification and characterization of a plasmid allow obtaining information on its physiology and mode of transmission. The relaxase or MOB is the most informative unit of the plasmid backbone[12] regarding its possible mobilization[13]; in fact, the relaxase recognizes the origin of transfer (*oriT*) and catalyzes the initial and the final steps in the conjugation process. The MOBs characterization allows including in each family plasmids belonging to different incompatibility groups on the basis of their relaxase sequence [17]. Integrons and transposons can be transferred by conjugation depending on the type of plasmid on which they are located [8, 11].

In this study, bacteria of the genus *Aeromonas* were considered the model of hydric microorganisms exposed to the actions of residual antibiotic compounds and to the aquatic resistome, whereas Faecal Coliforms represented the allochthonous population contaminating the aquatic environment.

In order to investigate how these two bacterial populations could be influenced by potential antibiotic pollution, we investigate the presence, the distribution and the transferability of transposons, integrons, and plasmids in *Aeromonas spp.* and Faecal Coliforms isolated from different aquatic environments submitted to a diverse degree of antibiotic contamination.

4.2. Materials and Methods

4.2.1. Sampling areas and isolation of strains

Water samples were collected in 2011 in five areas located in Ticino (South part of Switzerland) and consisted in water of a river before (b-WWTP) and after waste water treatment plant (a-WWTP), activated sludge of a waste water treatment plant (WWTP), hospital wastewater (HWW) and water from an alpine lake (SW) located at 2000 m.a.s in the Swiss Alps.

Logarithmic dilutions (from 10^{2}ml to 10^{-2}ml) of the water samples were prepared in 0.1% peptone water and filtered through 0.45 µm-pore size sterile filter (Millipore) of 47 mm diameter. Filters were placed on m-*Aeromonas* selective agar base (Biolife) supplemented with Ampicillin and on C-EC Mug agar to isolate *Aeromonas spp.* and Faecal Coliforms, respectively. *Aeromonas spp.* selective plates were incubated at 30°C for 24h, while Faecal Coliforms C-EC Mug agar plates were

incubated at 44°C for 24 hours. Fifty colonies of *Aeromonas spp*. (yellow colonies) and fifty colonies of Faecal Coliforms (blue colonies) from each sample were randomly selected, plated onto blood agar (BD BBL) and identified to the species level using MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization – Time Of Flight, Mass Spectrometry)[14].

4.2.2.Antibiotic susceptibility test

All the isolates were tested for antibiotic susceptibility by disk diffusion method according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2014) guidelines. The antibiotics tested were: cefazolin (30µg), cefuroxime (30µg), ceftriaxone (30µg), cefoxitin (30µg), gentamicin (10µg), bactrim (23.75/1.25µg), ciprofloxacin (5µg), meropenem (10µg), polymyxin (300unit), chloramphenicol (30µg), nalidixic acid (30µg), amikacin (30µg), ampicillin (10µg), tetracycline (30µg), streptomycin (10µg), trimethoprim (5µg), sulfamethoxazole (100µg), aztreonam (30µg), tobramycin (10µg), kanamycin (30µg), netilmicin (10µg) (Becton Dickinson AG).

4.2.3.Dot-blot hybridization

Dot-Blot experiments were performed using the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche Applied Science). Integrase, transposase and MOB genes were amplified by PCR as previously described [15, 16, 17,] purified and labelled with Digoxigenin. Total DNA was extracted by Instagene Matrix (BioRad) while plasmids were obtained with the kit Plasmid DNA Purification, NucleoBond® PC100 kit (Machery-Nagel). Samples were spotted on a nylon membrane, cross-linked at 100°C for 1h and hybridized with the specific probes overnight at the optimal temperature of annealing. Immunological detection was performed according to the manufacturer instructions.

4.2.4.Southern blot hybridization

To determine the putative plasmid location of the genetic determinants investigated, plasmid extracts were loaded onto a 0.8% agarose gel with 10X gel red and separated by electrophoresis at 100 V for 2 h. Agarose gel was washed once with sterile MilliQ H_2O, put in HCL 0.25M for 30 minutes with

soft shaking, and submerged twice for 20 minutes in denaturation solution and twice for 20 minutes in neutralization solution, with gentle shaking. The blot transfer was set up as follows: gel was put atop three sheets of whatman 3MM paper soaked in SSC 20X buffer. Positively Charged Nylon Membrane, was cut to the size of the gel and placed on the DNA-containing surface. Five sheets of whatman 3MM paper, a stack of paper towels (4cm) and a 500g weight were added on the top of blot assembly. The blot was transferred overnight in transfer buffer (20x SSC). Labelling and detection were performed as described above.

4.2.5. Gene cassettes identification

Gene cassettes identification were made by PCR amplification and sequencing of the Pant-QacEΔ1 region. The PCR mix consisted in 0.75 μL of each primer (Pant F GTCGAAACGGATTAAGGCACG; qacED CAAGTCTTTGCCCATGAAGC)[15], 12.5 μL Taq PCR Master Mix (2.5 U/reaction Taq DNA polymerase; 15 mM $MgCl_2$; 200 μM of each dNTPs. Qiagen), 1 to 5 μL of samples and water to a final volume of 25 μL. PCR conditions were: 94°C for 3'; 95°C for 45", 60°C for 45", 72°C for 6' for 31 cycles; 72°C for 7'.

4.2.6. Conjugation experiments

The choice of bacterial strains to be used in conjugation experiments was made considering the presence of plasmids (at least one plasmid for the donors and no plasmids for the recipients) and the resistance phenotype. The concentration of antibiotics used for the selection of transconjugants was established according to the minimum inhibitory concentration (MIC) reported in the EUCAST tables.

Bacterial strains were grown in 2 mL of Tryptic soy broth (TSB) for 3 hours, and resuspended in 5 mL of saline solution to obtain a turbidity of 0.5 McFarland. For in solid conjugation, donor and recipient were spread on non-selective plates in a 2:1 and 4:1 ratio of donor to recipient and incubated for 24 hours at 30°C. After growth on non selective plates (for in solid conjugation), colonies were collected in 5 mL of saline solution to reach a turbidity of 3 McFarland. 100 μL of this solution were spread on selective, double selective or non-selective LB agar plates and incubated at

37°C or 30°C for 24 hours, depending on the recipient strain. For in liquid conjugations the same ratio volumes (2:1 and 4:1) were inoculated in 5 mL of LB broth, and incubated for 24 hours at 30°C. 100 µL of this solution were spread on selective, double selective or non-selective LB agar plates and incubated at 37°C or 30°C for 24 hours. Transconjugated bacteria were plated for another 24 hours at 37°C or 30°C on a double selective LB agar.

4.3. Results

4.3.1.Species distribution in the samples

We recovered a total of 231 *Aeromonas* strains. The species *A. hydrophila* represented approx. 21% of the 46 isolates from the hospital wastewater. *A. media* counted for the 57% and 46% of the species identified among the 49 strains isolated from the river before the waste water treatment plant outlet and the 46 recovered from the river after the WWTP outlet, respectively. This species was predominant also in the activated sludge of the waste water treatment plant representing the 51% of the *Aeromonas* species (total strains isolated: 41). The principal species collected from the alpine lake was *A. salmonicida* (88% among 49 strains).

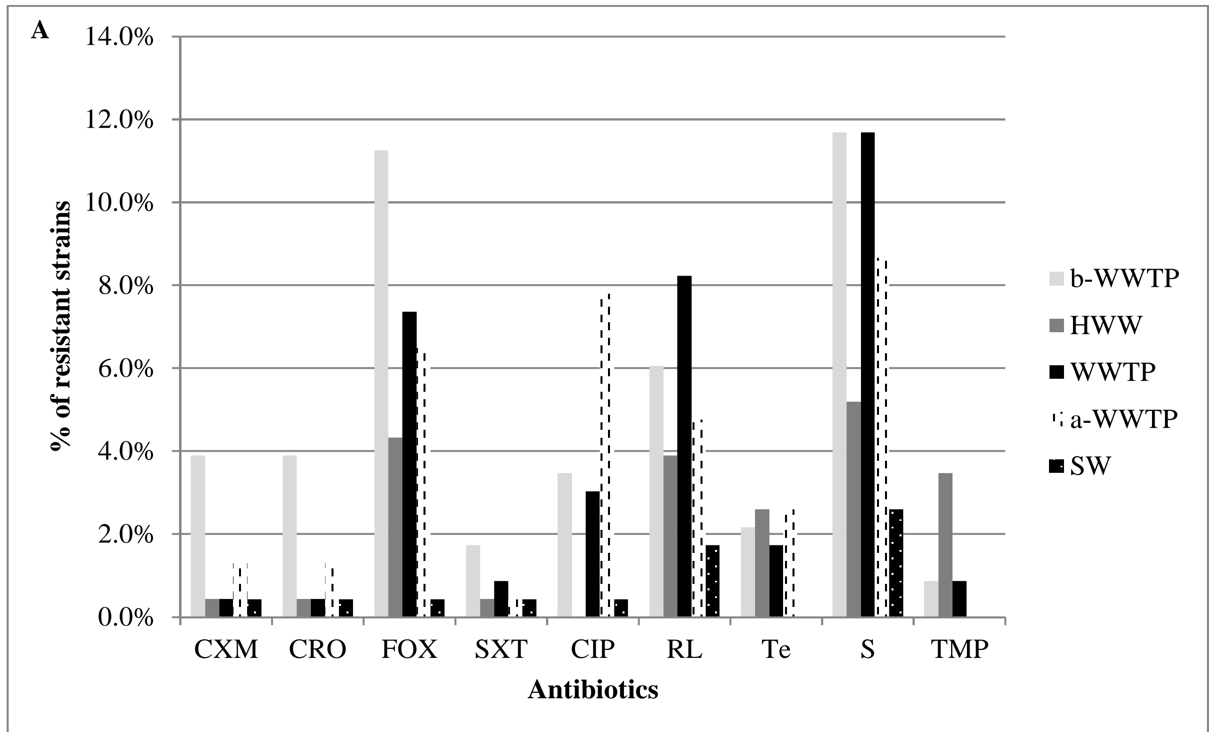
The most representative species belonging to the Faecal Coliforms, for which 50 colonies were obtained from each sample, was *Escherichia coli*. The recovery of *Klebsiella pneumoniae* and *Enterobacter cloacae* was also significant in the waste water treatment plant (34% and 20% respectively). *K. pneumoniae* represented the 22% of the species isolated from the river after the treatment plant.

4.3.2.Antibiotic susceptibility

The majority of the strains, regardless of the species and the sampling areas, were resistant to cefoxitin (43%), streptomycin (32%) and sulfamethoxazole (25%). Among *Aeromonas spp*. resistance to ciprofloxacin was also frequent (15%). As *Aeromonas* are generally resistant to ampicillin, this antibiotic was tested only for Faecal Coliforms, the 32% of which tested resistant (78 out of 250). Faecal Coliforms showed low resistance levels in the river before and after the waste

water treatment plant and in the alpine lake (Figure 1 B). The percentage of resistant strains increased in hospital waste water and in the activated sludge. In *Aeromonas* strains the resistance

percentages in the river were higher than those in the hospital sewage; the level of resistance increased in the waste water treatment plant (Figure 1 A).



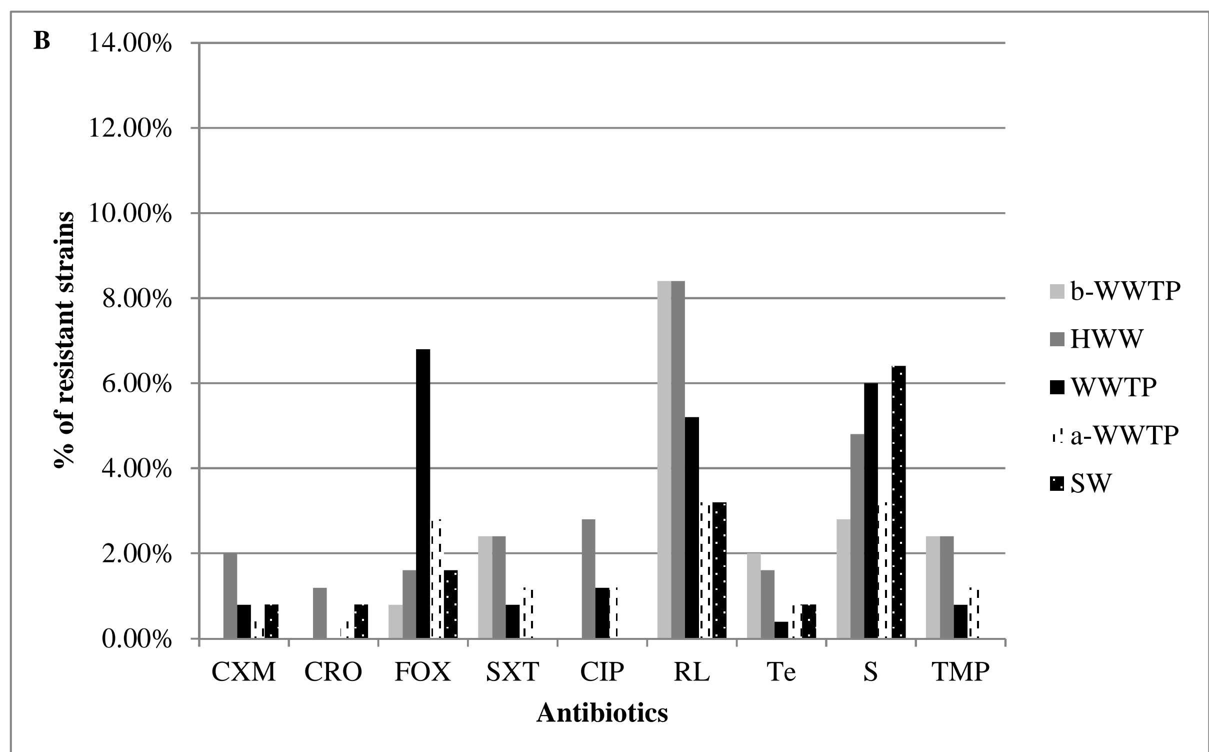


Figure 1. (A) Distribution of Aeromonas resistances in the five different sampling points. **(B)** Distribution of Fecal Coliforms resistances in the five different sampling points. b-WWTP river before waste water treatment plant; HWW hospital waste water; WWTP activated sludge of waste water treatment plant; a-WWTP river after waste water treatment plant; SW superficial water alpine lake. CXM cefuroxime, CRO ceftriaxone, FOX cefoxitin, SXT bactrim, CIP ciprofloxacin, NA nalidixic acid, RL sulfamethoxazole, Te tetracycline, S streptomycin, TMP trimethoprim. The antibiotics shown in figure are the most representative

Strains carrying plasmids (Table 1) and showing three or more antibiotic resistances (Table 2) were particularly abundant in the wastewater treatment plant (88% for *Aeromonas spp*. and 40% for Faecal Coliforms). Despite the high percentage of strains carrying plasmids in the alpine lake, only the 2% and the 18% of the *Aeromonas spp.* and the Faecal Coliforms, respectively, showed a multi-resistant profile.

	***Aeromonas* spp.**	**Fecal Coliforms**
b-WWTP	81.63%	96.00%
HWW	82.61%	82.00%
WWTP	90.24%	84.00%
a-WWTP	91.30%	80.00%
SW	97.96%	92.00%

Table 1. Percentage of *Aeromonas* and Fecal Coliforms carrying plasmids.

	***Aeromonas* spp**	**Fecal Coliforms**
b-WWTP	69.39%	22.00%
HWW	60.87%	28.00%
WWTP	87.80%	40.00%
a-WWTP	73.91%	22.00%
SW	2.04%	18.00%

Table 2. Strains with three or more resistances.

4.3.3.Plasmids, MOB typing and conjugation experiments

More than 80% of *Aeromonas* and Faecal Coliforms isolates carried plasmids (Table 1).

MOB_{P13} and MOB_{P14} subfamilies, comprising mobilizable and potentially transferable plasmids, were identified in approx. the 32% of the *Aeromonas* plasmid extracts whereas mobilizable and potentially transferable plasmids belonging to the MOB_{F12}, MOB_{P12}, and MOB_{H11} were highlighted in nearly the 69% of the Faecal Coliforms plasmid extracts.

MOB_{P13} was observed in the 17% of the *Aeromonas* isolated from the wastewater treatment plant while plasmids of the MOB_{P14} were present in the 46% of the same isolates. The MOB_{F12} was detected in the 72% of the Faecal Coliforms isolated from the alpine lake, the MOB_{P12} was identified in the 28% of those isolated from the river before the wastewater treatment plant, and the MOB_{H11} was distributed in the 8% of the strains of the river after the treatment plant and in of those from the hospital sewage This MOB subfamily was present also in the 14% of the Faecal Coliforms isolated from the activated sludge.

Southern blot analysis using MOB probes, revealed the plasmids which were found to harbor *tnpA* and/or *IntI1* genes. In 36% (4/11) of the *Aeromonas spp*. and 75% (12/16) of Faecal Coliforms tested, one or more MOB subfamilies probes annealed on the same plasmid of *IntI1* or *tnpA* (Table 3).

Conjugations were performed using as donor 33 *Aeromonas spp.* carrying one or more resistance genetic determinants (integrons and/or transposons) located on plasmids or on chromosome. Recipients were *Aeromonas spp.* and Faecal Coliforms without plasmids and resistances, and the strain *E. coli* J53. None of the *Aeromonas* tested was able to transfer resistance genetic elements through conjugation.

4.3.4.Distribution and putative location of the IntI1 gene, and characterization of the associated gene cassettes.

16 *Aeromonas* strains (7%) tested positive by Dot Blot for the *IntI1* specific probe. There was no particular distribution pattern of positive strains in the different environments, excepted that the isolates from the sampling of the alpine lake were all negative. Class 1 integrons had a plasmid location in the 75% (12 out of 16) of the *Aeromonas* spp. (Table 3). *Aeromonas* strains showing

plasmids carrying integrons belonged to the species *A. media* (5 strains), *A. hydrophila* (3 strains), *A. sobria* (2 strains), and *A. caviae* (2 strains).

22 (9%) Faecal Coliforms were positive for the presence of an *intI1* gene. An elevated frequency of positive strains was highlighted in the isolates from the hospital wastewater (26%) and in a lesser extent in those from the river before the treatment plant outlet (16%). 15 (68%) of these strains carried integrons on plasmids and, with the exception of two *Kl. pneumoniae*, all belonged to the species *E. coli*.

The gene cassettes characterization took place on the strains carrying integrons on plasmids (Table 3).

The most common gene cassettes within the class 1 integrons found in *Aeromonas* strains were *aadA1* (62.5%), *catB8* (37.5%), and *catB3* (25%); in Faecal Coliforms the most common gene cassettes were *aadA1* (63.6%), *catB2* (36.4%) and *dfrA14* (22.7%). The *aadA1* gene, codifying for the resistance to streptomycin and spectinomycin, was therefore the most frequent resistant gene found in class 1 integrons located on plasmids, followed by the *catB*, group of genes responsible for the resistance to chloramphenicol. The *dfr* group of genes, codifying for the resistance to trimethoprim and sulfamethoxazole, was particularly present on plasmids of the Faecal Coliform population. The genes highlighted in the integrons conferred the corresponding resistance phenotype to the strains, except for those conferring resistance to chloramphenicol (*catB* genes) in the *Aeromonas* spp. strains.

4.3.5.Distribution and putative location of the tnpA gene

The *tnpA* probe hybridized to approximately 16.5% (38/231) of the isolated Aeromonad and to the 10% (25/250) of the Faecal Coliforms (Table 3).

This gene had a putative plasmid location in 11 *Aeromonas* strains (approx. 29% of the *tnpA* positive strains) and in 4 Faecal Coliforms (approx. 16% of the *tnpA* positive isolates). The plasmid location was not observed in any of the *Aeromonas* spp. or Faecal Coliforms isolated from the hospital waste water. In strains isolated from the other sampling sites, plasmid location of the *tnpA* gene was

highlighted in *A. media* (from the river before and after the waste water treatment plant outlet and from the activated sludge), in *A. caviae* (from activated sludge and from the river after the waste water treatment plant outlet) and in *A. hydrophila* (from the activated sludge). As far as Faecal Coliforms are concerned, plasmid location of *tnpA* was evidenced only in the *E. coli* isolated from the river before and after the waste water treatment plant outlet.

Strains carrying plasmids, integrons, transposons and mobility genes							
Source	**Species**	**Resistances**	***IntI1* gene**	**Gene cassettes**	**Integron putative location / plasmid's MOB gene**	***TnpA* gene**	**Trasposon putative location / plasmid's MOB gene**
b-WWTP	*A.media*	CZ,CXM,FOX,NA,CIP(	-			+	**P**
b-WWTP	*A.media*	CZ,CXM,CRO,FOX,NA,CIP(I)	-			+	**P**
b-WWTP	*A.sobria*	CZ,SXT,NA,S,RL	+	dfrB1-aadA1b-CatB2	**P**	+	C
b-WWTP	*A.media*	CZ,NA,S(I),RL	+	OXA10-aadA1	**P**	+	**P / P14**
b-WWTP	*A.sobria*	CZ,NA,S(I),RL	+	empty	**P**	+	C
b-WWTP	*A.media*	CZ,FOX,SXT,NA,S(I),TMP,RL	+	dfr22	**P**	+	**P**
b-WWTP	*A.media*	CZ,FOX,CIP,C,NA,S(I),RL,NN	+	CatB3- aadA1	**P**	+	C
b-WWTP	*E.coli*	SXZ,CZ(I),Te,TMP,RL	+	dfrA14-aadA1-CatB2	**P / F12**	+	**P / F12**
b-WWTP	*E.coli*	SXT,Te,TMP,RL	+	dfrA14-aadA1-CatB2	**P / F12**	+	**P / F12**
b-WWTP	*E.coli*	SXT,AM,CZ(I),TMP,RL	+	dfrA17-aadA5	C	-	
b-WWTP	*K.pneumoniae*	FOX,AM,S,RL	+	aadA4a-hypotetical protein	C	+	C
b-WWTP	*E.coli*	SXT,CZ(I),Te,TMP,RL	+	dhfrA14-aadA1-catB2	**P / F12**	+	**P / F12**
b-WWTP	*E.coli*	SXT,Te,TMP,RL	+	dfrA14-aadA1-CatB2	**P / F12**	+	C
b-WWTP	*E.coli*	SXT,Te,TMP,RL	+	dfrA14-aadA1-CatB2	**P / F12**	+	C
b-WWTP	*K.pneumoniae*	AM,S(I),RL	+	aadA2	**P**	-	
HWW	*A.hydrophila*	CZ,FOX,NA,RL	+	CatB8-aadA1	C	-	
HWW	*A.hydrophila*	CZ,FOX,NA,S,RL,NN	+	CatB8-aadA1	**P**	-	
HWW	*A.hydrophila*	CZ,CXM,CRO,GM,CIP,NA,S,RL,ATM,NN,NET	+	aacA4cr-Oxa1-CatB3-aar3	**P / H121**	+	C
HWW	*A.hydrophila*	CZ,NA,S(I),ATM(I),RL	+	CatB8-aadA1	C	-	
HWW	*E.coli*	CZ,CXM,CRO,SXT,CIP,C,NA,AM,Te,S,TMP,RL,ATM,NN(I),K	+	aacA4-aadA1-CatB2	**P / F12; H121**	+	C
HWW	*E.coli*	CZ,CXM,CRO,FOX,CIP,NA,AM,S(I),RL,ATM	+	aacA4-aadA1-CatB2	**P / F12; H121**	+	C
HWW	*E.coli*	CZ,SXT,AM,S,TMP,RL	+	dhfrA1-aadA1	C	-	
HWW	*E.coli*	CZ,SXT,AM,S,TMP,RL	+	dfrA1-aadA1	**P**	-	
HWW	*E.coli*	CZ,C,AM,Te,S,RL	+	EstX putative esterase/hydrolase	**P / F12**	+	C
HWW	*E.coli*	CZ,CXM,FOX,GM,SXT,CIP,C,NA,AM,Te,S,TMP,RL,NN,NET	+	dfrA17-aadA5	**P**	+	C
HWW	*E.coli*	SXT,C,AM,Te,S,TMP,RL	+	dfrA12-OrfF	C	+	C
HWW	*E.coli*	CZ,CXM,CRO,AM,CIP(I),NA(I),S(I),RL,ATM	+	aacA4-aadA1-CatB2	**P / F12**	-	
HWW	*E.coli*	CZ,SXT,AM,S,TMP,RL	+	dhfrA1-aadA1	**P / F12; P12**	-	
WWTP	*A.caviae*	CZ,FOX,NA,S(I),RL	-		C	+	**P**
WWTP	*A.caviae*	NA,CIP(I),S,RL	+	CatB8-Transposase	**P / P13; P14**	+	C
WWTP	*A.caviae*	CZ,FOX	-			+	**P**
WWTP	*A.media*	CZ,NA,S(I),RL(I)	-			+	**P / H121**
WWTP	*A.media*	CZ,(FOX),SXT,NA,Te(I),S,TMP,RL	+	drf-aadA1	**P**	-	
WWTP	*A.media*	CZ,FOX,GM(I),S(I),RL	+	aadA2	C	+	C
WWTP	*A.hydrophila*	CZ,(FOX),CIP,NA,SXT(I)	+	CatB3-aadA1	**P**	-	
WWTP	*A.hydrophila*	CZ,NA,S	-			+	**P**
WWTP	*A.media*	CZ,NA,S(I),TMP,RL(I),ATM(I)	-			+	**P**
WWTP	*E.coli*	SXT,AM,CZ(I),S(I),TMP,RL	+	dhfrA1-aadA1	C	-	
a-WWTP	*A.caviae*	CZ,NA,Te(I)	-			+	**P**
a-WWTP	*A.caviae*	CZ,FOX,NA,SXT(I),S(I),RL	+	CatB8-aadA1	**P**	+	C
a-WWTP	*A.media*	CZ,NA,CIP(I),S(I),RL	+	CatB8-aadA2	**P**	+	C
a-WWTP	*A.media*	CZ,FOX,NA,Te(I),RL	+	aacA3-Blaoxa21-CatB3-aadA16	C	-	
a-WWTP	*A.caviae*	CZ,CIP,NA	-			+	**P**
a-WWTP	*A.caviae*	CZ,CIP,NA,S(I)	-			+	**P**
a-WWTP	*A.punctata*	CZ,CIP,NA,S(I)	-			+	**P**
a-WWTP	*A.media*	CZ,NA,S(I)	-			+	**P**
a-WWTP	*K.pneumoniae*	AM,S	+	empty	**P**	-	
a-WWTP	*E.coli*	CIP,NA,AM,CZ(I),RL	-			+	**P / F12**
a-WWTP	*E.coli*	CZ,CXM,CRO,SXT,CIP,NA,AM,Te,S,TMP,RL,ATM	+	dfrA17-aadA5	**P / F12**	-	
a-WWTP	*E.coli*	CZ.SXT,CIP,C,NA,AM,TE,TMP,RL	+	dfrB4	C	+	C
SW	*E.coli*	CZ,CXM,CRO,FOX,AM,Te,S,RL,ATM	+	aadA1	C	+	C

Table 3 *Aeromonas spp*. and fecal coliforms strains carrying plasmids, integrons, transposons and mobility genes. CZ cefazoline, CXM cefuroxime, CRO ceftriaxone, FOX cefoxitin, SXT bactrim, GM gentamicin, CIP ciprofloxacin, PB polymyxin, C chloramphenicol, NA nalidixic acid, AM ampicillin, RL sulfamethoxazole, Te tetracyclin, S streptomacyn, TMP trimetroprim, ATM aztreonam, NN tobramycin, NET netilmycin. P plasmidic; C chromosomal.

4.4. Discussion and Conclusions

In the present study, the prevalence and the characterization of genetic resistance determinants, namely plasmids, integrons and transposons, were investigated in two bacterial populations: *Aeromonas* species, representing natural environmental bacteria living in particular in aquatic environments and Faecal Coliforms, bacteria contaminating these environments from animal and human guts.

The resistance patterns evidenced phenotypically for Faecal Coliforms correlated with the presumptive concentrations of antibiotic compounds in water. The number of resistant strains, and in particular of those with three or more resistances, was higher in hospital waste water and in activated sludge of the waste water treatment plant, the most antibiotic polluted environments that are considered hot spots for the spreading of antibiotic resistance genes[18], than in the river and the alpine lake. The few multi-resistant Faecal Coliforms isolated from the alpine lake could be due to the runoff of sewage derived from cattle grazing on its banks. As far as *Aeromonas* are concerned, the role of the waste water treatment plant in increasing resistance to antibiotics commonly used in clinical settings[19] was evident. In fact, the majority of multi-resistant *Aeromonas* species was found in this sampling and in the river receiving its depurated waters. These results demonstrate the use of this bacterium as a model in studying the diffusion of antibiotic resistances among environmental bacteria.

The phenotypic resistance profiles that we observed in our strains was in some case due to mechanisms not necessary linked to integrons and/or transposons. Streptomycin resistance was due in some strains to the presence of a single gene on the chromosome (data not shown), and resistance to ciprofloxacin, frequent in *Aeromonas* strains, was caused by mutations in the active site of the

gyrase A[20]. Likewise, the high percentage of resistance to cefoxitin (FOX) was due to the chromosomally encoded *ampC* gene both in *Aeromonas* strains and in *Enterobacteriaceae*[21].

More than 80% of both *Aeromonas* and Faecal Coliforms carried plasmids regardless of their origin. The high number of *Aeromonas* strains carrying plasmids, evidenced in the water of the alpine lake, can be explained with the prevalence in this sampling of the species *Aeromonas salmonicida*, which typically harbour three small cryptic plasmids[22].

The characterization of the plasmids through MOBs probes evidenced that many of them were transmissible either through conjugation or mobilization. The 25% of the plasmids evidenced in *Aeromonas* strains belonged to the MOB_P subfamily, in particular MOB_{P13} and MOB_{P14}, which is frequently found in environmental bacteria[13]. MOB_{P13} and MOB_{P14} bring together the incompatibility groups IncL/M, and IncQ2 and IncP-6, respectively. IncL/M plasmids are large, broad host range, and self-transmissible replicons most frequently found in bacteria of the family *Enterobacteriaceae*. These plasmids are reported to be able to persist in diverse environments even without an antibiotic selective pressure. The IncQ2 family consists mostly of small, broad host range, promiscuous plasmids that can be mobilized. These plasmids are spread worldwide in many bacterial species including *Aeromonas sp*. where they are associated with quinolone and tetracycline resistance[23]. Finally, IncP-6 plasmids have been classed as broad-host-range due to their ability to replicate in both *Escherichia coli* (where they are designated IncG) and *Pseudomonas* species[24].

In Faecal Coliforms the majority of plasmids belonged to the MOB_{F12}, MOB_{P12}, MOB_{H11}, and MOB_{H121}. The 32% showed MOB_F relaxases which are found in clinically relevant plasmids[17]. MOB_{F12} contains antibiotic resistant and virulence plasmids of the IncF complex, which are largely found in *Enterobacteriaceae*, and have also been identified in *A. salmonicida* [17]. Plasmids of the IncI1 complex, IncK and Inc9 are members of the MOB_{P12}. IncI1 plasmids carrying *bla*$_{CMY-2}$ have been reported to spread extensively among clinical *E. coli*[25]; an IncK plasmid containing *bla*$_{KPC-2}$ has been characterized in carbapenem-resistant *Serratia marcescens* isolates[26]; Inc9 plasmids are found in Gram positive bacteria[27]. The MOB_{H11} family groups plasmids of the incompatibility group IncHI1, self-transmissible plasmids which can confer a multidrug resistance phenotype to their hosts.

They have been detected in pathogenic isolates of *Salmonella enterica* and *Escherichia coli*[28]. IncHI2, plasmids are frequently encountered in clinical enterobacterial strains associated with the dissemination of relevant antimicrobial resistance genes[17], and IncP7, large conjugative plasmids found in *Pseudomonas* which often carry genes encoding enzymes involved in the degradation of man-made and natural contaminants found in polluted environments[29]. Finally, the MOB_{H121} family covers the incompatibility group IncA/C. IncA/C plasmids were first identified among multidrug resistant *Aeromonas hydrophila* and *Vibrio* spp. over 40 years ago. Todays, these plasmids commonly circulate among Gram negative pathogens bringing with them the ability to encode resistances to a large number of antibiotics[30].

Although the predominating MOB families in the two bacterial populations were different, they group promiscuous plasmids frequently associated to *Enterobacteriaceae*, Pseudomonads or Aeromonads, that are reported to often carry antibiotic resistance and virulence genes.

Several studies demonstrate that Class 1 integrons play an important role in the dissemination of resistance genes, particularly among Gram negative [9, 31]. Moreover, their abundance in microorganisms can rapidly change in response to environmental pressures so that they can be used as indicators for pollutions originating in human activity[32]. Strains carrying the Integrase I gene were found in all kind of waters analysed. The majority of *intI1*-carrying *Aeromonas* were collected from the activated sludge of the wastewater treatment plant (ca. 12% of the strains) whereas *intI1*-carrying Faecal Coliforms from the hospital wastewater (26%). The mean frequency of 8% of strains carrying Class 1 integrons was less elevated in respect to that found in a slaughterhouse wastewater treatment plant, where Moura et al.[33] reported in *Aeromonas spp.* and *Enterobacteriaceae* a percentage of 31%, whereas it was more similar to the 12% found by Mokracka et al. 2012[34] in *Enterobacteriaceae* isolated at all stages of wastewater treatment. No *Aeromonas* strains and only the 2% of Faecal Coliforms isolated from the alpine lake carried an *intI1* gene. These results support that the distribution of this genetic structure is strongly linked to the selective pressure imposed by the contamination of the environment.

Integrons are genetic elements that allow efficient capture and expression of exogenous genes, but they are not "per se" mobile elements. When combined with transposons and/or transferable plasmids, they can play a major role in the spread of resistance to antibiotics among bacteria[35]. The majority of our strains, i.g. 12 out of 16 *Aeromonas*, and 15 out of 22 Faecal Coliforms contained a plasmid-borne *intI1* gene. In these strains, the presence and the characterization of gene cassettes was carried out. As reported in many studies[33,36,37] the *aadA1* gene cassette, codifying for the resistance to streptomycin and spectinomycin, was the most frequent resistance gene identified in these genetic structures followed by the *dfr* genes, involved in trimethoprim resistance. Henriques et al.[36] highlighted that these genes confer resistance to older antibiotics, such as early aminoglycosides and trimethoprim, and speculated that this is probably the result of a selective pressure due to antibiotics different from those now used in clinical settings. Chloramphenicol, a broad-spectrum antibiotic compound, is produced by *Streptomyces*. After years of limited use as therapy due to a number of adverse side-effects in humans, there is a renewed interest in its use due to the lack of new antibiotic compounds necessary to treat infections with emerging multi-resistant strains [38,39]. The oldest and still the most frequent mechanism of resistance to chloramphenicol is the enzymatic inactivation by different chloramphenicol acetyltransferases (CAT). The type B *cat* genes, found frequently in our strains, are widely distributed among Gram-negative and Gram-positive bacteria[40]. Several of these genes are part of cassettes and are therefore transcribed from a promoter located in the *intI1* gene. As a rule, the gene in the cassette closer to the promoter is that strongly expressed[39]. However, gene cassettes may be differently expressed depending on the promoter variant. In a population of *E. coli* clinical strains, the weakest variants were prevalent[41]. In 50% of integrons detected in our *Aeromonas* strains, *catB3* or *catB8* were the first genes of the cassette, but did not confer resistance to chloramphenicol to the strains. This could be due to a weak promoter or to a metabolic condition as that reported in an *E. coli* strain in which, despite the expression of the chloramphenicol acetyltransferase gene, decreased levels of acetyl coenzyme A in the presence of chloramphenicol caused the bacterium to be sensitive to this antibiotic[42]. It has been demonstrated that there is an inverse correlation between the strength of the promoter and the integration efficiency

of exogenous gene cassettes[43, 44]. A better aptitude to excise cassettes could in theory improve the capacity of the integron to adapt to antibiotic pressure and therefore represent a survival advantage[41].

The Tn*21* transposons are considered the transposable elements principally responsible for the problem of multiple resistances to antibiotics in Gram negative bacteria[45]. They have often been associated with multi drug resistance plasmids both in *Aeromonas*[46] and in *E. coli*[47]. These structures were evidenced in the 14% of our strains, a frequency far less important than that of more than 60% reported in Gram-negative bacteria isolated from soil[48] but higher than found in bacterial communities of marine environments[49], where the Tn*21 tnpA* was evidenced once per 10^3 or 10^4 bacteria. Our result is comparable to a previous report[45] describing a total of 19% of unselected Gram negative clinical isolates carrying part of the *tnpA tnpR* or *intI1* gene. In a more recent study[50] the prevalence of Tn*21* in *E. coli* was settled to be of 22%.

Similarly to the distribution of Class 1 integrons, for *Aeromonas* strains the *tnpA* gene was mostly found in isolates from the activated sludge and from the river waters. Even in the Faecal Coliforms that we analysed, the distribution of these transposons was homogeneous in the different sampling sites. Thus, the diffusion of the Tn*21*-like transposons in our sampling seemed submitted to the influence of the environment and of the normal human activities rather than to that of clinical setting. The genetic linkage between Class 1 integrons and mobile genetic elements increases the potential of the former to be disseminated. It has to be noted that a Class 1 integron, called In2, which includes the *aadA1* cassette[51], the most frequent cassette that we found in our strains, is associated to the transposons Tn*21*. Moreover, 11 *Aeromonas* strains and 4 *E. coli* carried the *tnpA* gene on plasmids.

A physical linkage among the different mobile elements investigated in this study was evidenced in many strains. Class 1 integrons, and to a lesser extent Tn21-like transposons, were often associated to plasmids which were identified as transmissible by MOB identification and that are known to circulate extensively among *Enterobacteriaceae*. Even if integrons and transposons were located in our *Aeromonas* strains on plasmids of unknown incompatibility groups or of different MOBs than those evidenced in Faecal Coliforms, the possibility of a lateral exchange of the resistant plasmids found cannot be excluded, as some of them have already been isolated from both types of bacteria.

Our investigations pointed out that both *Aeromonas* and Faecal Coliforms populations are influenced by the potential antibiotic contamination of the environment, and that they reacted to this pollution following different rules. *Aeromonas* species are ubiquitous in aquatic environments and are a model for environmental bacteria. Multi-drug resistance, *intI1* and *tnpA* genes, and transferable plasmids that are frequently identified in the environment were mostly found in *Aeromonas* sp. isolated from the wastewater treatment plant and from the river receiving its depurated waters. On the other hand, Faecal Coliforms are allochthonous in natural hydric environments, having their normal habitat in the gut of animals; the genetic traits involved in antibiotic resistance were evidenced mainly in strains isolated from the hospital waste water and from the activated sludge of the treatment plant. Our results did not allow highlighting common genetic determinants in the two populations, and we were unable to transfer antibiotic resistances through conjugation. However, the genetic elements involved in resistance that we investigated have already been evidenced in both *Aeromonas* and Faecal Coliforms. Moreover, we cannot exclude a lateral transfer implicating others microbial partners, since the aquatic environment is particular rich in microorganisms whereof approx. 99% of them are uncultivable[52].

In conclusion we confirm that *Aeromonas* spp. represents a reservoir of antimicrobial resistance determinants in the aquatic environment, facilitated by numerous plasmids and integrons which help the bacterium to acquire genetic resistance elements. The possibility that these traits are transferred by transformation or transduction is under study.

4.5. Acknowledgements

We thank Joachim Frey Prof. Dr. of the Institute of Veterinary Bacteriology, University of Berne and Paola Barbieri Prof. of the Department of Theoretical and Applied Science, University of Insubria of Varese for the review of the manuscript; and AP Caminada for her technical assistance.

4.6. References

1. Martinez JL. Environmental Pollution by Antibiotics and by Antibiotic Resistance Determinants. *Environmental Pollution Barking, Essex : 1987* 2009; **157 (11):** 2893–902. doi:10.1016/j.envpol.2009.05.051.
2. Gillings, Michael R. Evolutionary Consequences of Antibiotic Use for the Resistome, Mobilome and Microbial Pangenome. *Frontiers in Microbiology* doi:10.3389/fmicb.2013.00004.
3. Carnahan AM, Altwegg M. Taxonomy. In: Austin B., et al., ed. *The genus Aeromonas.* Chichester: John Wiley & Sons Ltd, 1996; 1-38.
4. Janda JM, Abbott SL Evolving concepts regarding the genus *Aeromonas*: an expanding Panorama of species, disease presentations, and unanswered questions. *Clin Infect Dis* 1998; **27:** 332–344.
5. Figuera MJ and Beaz-Hidalgo R. *Aeromonas* infections in humans. In: Graf G, ed. *Aeromonas*, Caister Academic Press, 2015; 4: 65-108.
6. Doyle MP, Erickson MC Closing the door on the Faecal coliform assay. *Microbe* 2006; **1:** 162-163
7. Girones R, Ferrùs MA *et al.* Molecular detection of pathogens in water – The pros and cons of molecular techniques. *Water Research* 2010; **44 (15):** 4325-4339.
8. Rowe-Magnus DA, Mazel D. Integrons: natural tools for bacterial genome evolution. *Current Opinion in Microbiology* 2001; **4 (5):** 565-569.
9. Stalder T, Barraud O, Casellas M, Dagot C, Ploy M-C. Integron involvement in environmental spread of antibiotic resistance. *Front Microbiol* 2012; **3**: 119. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3321497&tool=pmcentrez&rendertype=abstract. Accessed March 26, 2014.
10. Liebert CA, Hall RM *et al.* Transposon Tn21, Flagship of the Floating Genome. *Microbiology and Molecular Biology Reviews : MMBR* 1999; **63 (3)**: 507–22.
11. Nardelli M., Scalzo PM *et al*. Class 1 integrons in environments with different degrees of urbanization. *Plos One* 2012; **7 (6):** 1-13.

12. Garcillàn-Barcia MP, Francia MV *et al.* The diversity of conjugative relaxase and its application in plasmid classification. *FEMS Microbiology Review* 2009; **33 (3):** 657-687.

13. Smillie C, Garcillàn-Barcia MP *et al.* Mobility plasmids. *Microbiology and Molecular Biology Reviews* 2010 **74 (3):** 434-452

14. Benagli C, Demarta A *et al*. A rapid MALDI-TOF MS identification database at genospecies level for clinical and environmental Aeromonas strains. *Plos one* 2012; **7** (**10**): 1-6.

15. Lanz R, Kuhnert P *et al.* Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. *Veterinary Microbiology* 2003; **91** (**1**)**:** 73-84

16. Dahlberg C and Hermansson M. 1995. Abundance of Tn3, Tn21, and Tn501 Transposase (tnpA) Sequences in Bacterial Community DNA from Marine Environments. *Applied and Environmental Microbiology* **61 (8):** 3051–3056.

17. Alvarado A, Garcillàn-Barcia MP *et al*. A Degenerate Primer MOB Typing (DPMT) method to classify gamma-proteobacterial plasmids in clinical and environmental settings. *Plos One* 2012; **7 (7):** 1-15.

18. Baquero F, José-Luis M *et al.* Antibiotics and Antibiotic Resistance in Water Environments. *Current Opinion in Biotechnology* 2008; **19** (**3**): 260–5.

19. Marti E, Juan J *et al*. Prevalence of Antibiotic Resistance Genes and Bacterial Community Composition in a River Influenced by a Wastewater Treatment Plant. *PloS One* 2013 **8** (**10**): e78906. doi:10.1371/journal.pone.0078906.

20. Carnelli, A. Could Aeromonas spp. be considered a vector of resistance genes from the environment to human intestinal flora? (Doctoral Thesis, Università degli Studi dell'Insubria, 2013). http://hdl.handle.net/10277/570

21. George J. AmpC Beta-Lactamases. *Clinical Microbiology Reviews* 2009 **22** (**1**): 161–82

22. Boyd J, Williams J, *et al*. Three Small, Cryptic Plasmids from Aeromonas Salmonicida Subsp. Salmonicida A449. *Plasmid* 2003 **50 (2):** 131–144.

23. 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. Available at: http://jb.asm.org/cgi/doi/10.1128/JB.00864-09.

24. Haines AS, Jones K, Cheung M, Thomas CM. The IncP-6 plasmid Rms149 consists of a small mobilizable backbone with multiple large insertions. *J Bacteriol* 2005; **187**: 4728–38. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1169491&tool=pmcentrez&rendertype=abstract.

25. Sidjabat HE, Seah KY, Coleman L, *et al.* Expansive spread of IncI1 plasmids carrying blaCMY-2 amongst Escherichia coli. *Int J Antimicrob Agents* 2014; **44**: 203–8. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0924857914001617.

26. Costa Guimarães AC, Almeida ACS, Nicoletti AG, Vilela MA, Gales AC, de Morais MMC. Clonal spread of carbapenem-resistant Serratia marcescens isolates sharing an IncK plasmid containing blaKPC-2. *Int J Antimicrob Agents* 2013; **42**: 369–70. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0924857913002082.

27. Shintani M, Sanchez ZK, Kimbara K. Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. *Front Microbiol* 2015; **6**: 1–16. Available at: http://www.frontiersin.org/Evolutionary_and_Genomic_Microbiology/10.3389/fmicb.2015.00242/abstract.

28. Wiesner M, Calva E, Fernández-Mora M, *et al.* Salmonella Typhimurium ST213 is associated with two types of IncA/C plasmids carrying multiple resistance determinants. *BMC Microbiol* 2011; **11**: 9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3025833&tool=pmcentrez&rendertype=abstract. Accessed March 26, 2014.

29. GarcillÃ¡n-Barcia MP, Francia MV, de la Cruz F. The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiol Rev* 2009; **33**: 657–87. Available at: http://doi.wiley.com/10.1111/j.1574-6976.2009.00168.x. Accessed March 21, 2014.

30. Timothy J. Johnson* and Kevin S. Lang. IncA/C plasmids An emerging threat to human and animal health? *Mob Genet Elements* 2012: 193–5.
31. Hall RM, Collis CM. Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resist Updat* 1998; **1**: 109–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16904397.
32. Gillings M, Paulsen I, Tetu S. Ecology and Evolution of the Human Microbiota: Fire, Farming and Antibiotics. *Genes (Basel)* 2015; **6**: 841–57. Available at: http://www.mdpi.com/2073-4425/6/3/841/.
33. Moura A, Isabel H *et al.* Prevalence and Characterization of Integrons from Bacteria Isolated from a Slaughterhouse Wastewater Treatment Plant. *The Journal of Antimicrobial Chemotherapy* 2007; **60 (6):** 1243–50.
34. Mokracka J, Koczura R, Kaznowski A. Multiresistant Enterobacteriaceae with class 1 and class 2 integrons in a municipal wastewater treatment plant. *Water Res* 2012; **46**: 3353–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22507248. Accessed March 26, 2014.
35. Gillings MR. Integrons: past, present, and future. *Microbiol Mol Biol Rev* 2014; **78**: 257–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4054258&tool=pmcentrez&rendertype=abstract.
36. Henriques IS, Fonseca F, Alves A, Saavedra MJ, Correia A. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res Microbiol* 2006; **157**: 938–47. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0923250806002129.
37. Ndi OL, Barton MD. Incidence of class 1 integron and other antibiotic resistance determinants in Aeromonas spp. from rainbow trout farms in Australia. *J Fish Dis* 2011; **34**: 589–99. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21762170. Accessed March 26, 2014.
38. Fernandez M, Conde S, de la Torre J, Molina-Santiago C, Ramos J-L, Duque E. Mechanisms of Resistance to Chloramphenicol in Pseudomonas putida KT2440. *Antimicrob Agents Chemother* 2012; **56**: 1001–9. Available at: http://aac.asm.org/cgi/doi/10.1128/AAC.05398-11.

39. Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev* 2004; **28**: 519–42. Available at: http://femsre.oxfordjournals.org/lookup/doi/10.1016/j.femsre.2004.04.001.

40. Carattoli A. Importance of integrons in the diffusion of resistance. In: *Veterinary Research*. Vol 32., 2001; 243–59.

41. Marchant M, Vinué L, Torres C, Moreno M a. Change of integrons over time in Escherichia coli isolates recovered from healthy pigs and chickens. *Vet Microbiol* 2013; **163**: 124–32.

42. Potrykus J. Chloramphenicol-Sensitive Escherichia coli Strain Expressing the Chloramphenicol Acetyltransferase (cat) Gene. *ant* 2001; **45**: 3610–2.

43. Wei Q, Jiang X, Li M, *et al.* Transcription of integron-harboured gene cassette impacts integration efficiency in class 1 integron. *Mol Microbiol* 2011; **80**: 1326–36. Available at: http://doi.wiley.com/10.1111/j.1365-2958.2011.07648.x.

44. Jové T, Da Re S, Denis F, Mazel D, Ploy M-C. Inverse Correlation between Promoter Strength and Excision Activity in Class 1 Integrons. *PLoS Genet* 2010; **6**: e1000793. Available at: http://dx.plos.org/10.1371/journal.pgen.1000793.

45. Zühlsdorf MT, Wiedemann B. Tn21-specific structures in gram-negative bacteria from clinical isolates. *Antimicrob Agents Chemother* 1992; **36**: 1915–21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=192209&tool=pmcentrez&rendertype=abstract.

46. Del Castillo C, Jun-Ichi Hikima S *et al*. Comparative Sequence Analysis of a Multidrug-Resistant Plasmid from *Aeromonas Hydrophila*. *Antimicrobial Agents and Chemotherapy* 2013; **57 (1):** 120–9.

47. Wibberg D, Rafael S *et al*. The IncF Plasmid pRSB225 Isolated from a Municipal Wastewater Treatment Plant's on-Site Preflooder Combining Antibiotic Resistance and Putative Virulence Functions Is Highly Related to Virulence Plasmids Identified in Pathogenic E. Coli Isolates. *Plasmid* 2013 ; **69 (2):** 127–37.

48. Pearson J, Bruce KD *et al.* Distribution of Class II Transposase and Resolvase Genes in Soil Bacteria and Their Association with Mer Genes. *Applied and Environmental Microbiology* 1996; **62 (8):** 2961–5.

49. Cecilia D, Hermansson M *et al.* Abundance of Tn3, Tn21, and Tn501 Transposase (tnpA) Sequences in Bacterial Community DNA from Marine Environments. *Applied and Environmental Microbiology* 1995; **61 (8):** 3051–3056.

50. Kiiru J, Butaye P, Goddeeris BM, Kariuki S. Analysis for prevalence and physical linkages amongst integrons, ISEcp1, ISCR1, Tn21 and Tn7 encountered in Escherichia coli strains from hospitalized and non-hospitalized patients in Kenya during a 19-year period (1992–2011). *BMC Microbiol* 2013; **13**: 109. Available at: http://www.biomedcentral.com/1471-2180/13/109.

51. Liebert C a, Hall RM, Summers a O. Transposon Tn21, flagship of the floating genome. *Microbiol Mol Biol Rev* 1999; **63**: 507–22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=103744&tool=pmcentrez&rendertype=abstract.

52. D'Costa VM, Griffiths E, Wright GD. Expanding the soil antibiotic resistome: exploring environmental diversity. *Curr Opin Microbiol* 2007; **10**: 481–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17951101. Accessed March 26, 2014.

5. Second manuscript submitted to the *Applied and Environmental Microbiology*

The role of natural transformation in spreading resistance to antibiotics in *Aeromonas* strains of environmental origin

Federica Mauri and Antonella Demarta*

SUPSI Laboratory of applied microbiology, via Mirasole 22A, 6501 Bellinzona, Switzerland

*Corresponding author: antonella.demarta@supsi.ch

5.1. Abstract

Aeromonas sp. is a gram negative bacillus that is found ubiquitously in the aquatic environment. Because of its large diffusion in a variety of habitats, such as polluted or unpolluted environments, chlorinated and potable waters, oligotrophic or rich in nutrients aquatic environments, it is considered a good model for monitoring the impact of antibiotics or heavy metal pollutions on aquatic microorganisms. As observed for pathogenic bacteria, the number of *Aeromonas* strains resistant to antibiotics is increasing. We have speculated that transformation might be a mechanism of primary importance in the horizontal gene transfer of resistant genes that spread among *Aeromonas* and the different populations of bacteria present in the environment.

In order to test our hypothesis, we performed intra- and interspecies transformation assays using *Aeromonas* strains isolated from different aquatic environments. The intergeneric transformation ability was tested using the naturally competent *Acinetobacter baylyi* BD413.

Contrary to other published findings, our strains were difficult to transform and showed frequently only phenotypic changes of their antibiotic resistance profiles.

In the light of our results, lateral gene transfer trough transformation do not seems the preferential way of spreading of resistance determinants among bacteria belonging to the genus *Aeromonas.*

5.2. Introduction

Aeromonas spp. are Gram negative, oxidase positive and facultative anaerobic bacilli, primarily associated to fish diseases (furonculosis) (1) that may also be a human pathogen causing gastroenteritis, wound infections, septicaemias and a variety of others infections (2). *Aeromonas* may be isolated from different aquatic environments, polluted or unpolluted, oligotrophic or hypereutrophic such as groundwater, superficial and marine waters, waste water treatment plants, hospital and household sewages (3, 4). In the last years environmental and human *Aeromonas* have become more resistant to antibiotics used in human and veterinary therapy (5, 6, 7). Many studies have highlighted that these bacteria can carry a large variety of antibiotic resistance determinants which are located on mobile elements such as plasmids, transposons or insertion sequences as well as on the chromosome (8, 9, 10, Carnelli et al. submitted). *Aeromonas* are thus considered a reservoir of resistance genes (11, Carnelli et al. submitted).

Conjugation, transduction, and transformation are distinct mechanisms that allow the horizontal transfer of genetic elements. Conjugation is generally considered to play an important role in horizontal gene transfer (12, 13) but it requires the contact between a donor and a recipient organism. Bacteria must therefore be close to one another in order to exchange genetic material. Actually, conjugation occurs at higher frequencies between members of biofilm communities rather than when they are in a planktonic state (14). Most of the plasmids detected in *Aeromonas* are broad-host-range, capable of conjugative transfer or capable of mobilization. The large majority of these replicons carry antibiotic and metal resistance genes or virulence factors, and there are evidences that they are transferred by conjugation in natural environments (15). Except Carnelli et al. (submitted) in vitro conjugation experiments between *Aeromonas* strains have been mostly carried out in order to demonstrate the transferability of conjugative plasmids (16, 17 and 18).

In nature, antibiotic resistance genes could also spread via transformation, which refers to the ability of bacteria to bind and internalize foreign naked DNA, without the need of contact between a donor and a recipient. Bacteria that are able to take up exogenous DNA from the environment are in a particular physiological state called competence (19). Generally, competence is induced by high cell

density and/or nutritional limitation. The capacity for natural transformation appears to occur in many prokaryotic species (20). Huddleston et al. (21) have demonstrated that *Aeromonas* are capable of natural transformation even if at a very low frequency (1.95×10^{-3} transformants/recipient cell) and under very specific conditions: during starvation, mutants *Aeromonas* strains were able to acquire genes from donors and therefore to survive in stressing conditions (21, 22).

In a recent study, we examined the distribution and we characterize the genetic determinants involved in antibiotic resistance in 250 *Aeromonas* strains isolated from different aquatic environments (Carnelli et al. submitted). Class 1 integrons, transposons of the Tn*21*-like family, and resistant plasmids were frequently identified, mostly in *Aeromonas* strains isolated from heavily polluted waters, but we were not successful in transferring these antibiotic resistance traits through conjugation.

The aims of this work were therefore to evaluate if *Aeromonas* strains may acquire resistance genes through transformation and if they may act as donors of their resistant determinants to a natural competent strain, *Acinetobacter baylyi* BD413 (23), or to other bacteria of the same genus.

5.3. Materials and Methods

5.3.1. Bacterial strains

The *Aeromonas* strains used in this work are listed in Table 1. The strains are part of a collection made up during 2011, and it is composed of isolates from water samples collected in five areas located in Ticino (South part of Switzerland), namely water of a river before (b-WWTP) and after a waste water treatment plant (a-WWTP), activated sludge of a waste water treatment plant (WWTP), hospital wastewater (HWW) and water from an alpine lake (SW) located at 2000 m.a.s in the Swiss Alps. during a previous study (Carnelli et al., submitted), the isolates were tested for antibiotic susceptibility by disk diffusion method according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2014) guidelines; when present, plasmids were characterized by MOB typing (24), and strains were screened for the presence of *intI1* gene, representative of Class I integrons, and *tnpA* gene, representing transposons of the Tn*21*-like family, by dot blot; and

part of the genes cassettes were sequenced. In order to establish the selective conditions to be used in transformations, we set the minimal inhibitory concentration (MIC) for the antibiotics of interest for our bacteria following a method previously described (5), and using an absorbance micro-plate reader (ELx808 Absorbance Reader-BioTek).

Acinetobacter baylyi BD413 (strain ATCC 33305/ BD413/ ADP1) was kindly provided by Prof. Kaare M. Nielsen, University of Tromsø (Norway).

Source	Strains	Species	Resistance phenotype	Gene cassettes	tn21	Recipients
Donors						
b-WWTP	7 FT	*A.sobria*	CZ,SXT,NA,**S,Sp**,RL	*dfrB1*-***aadA1b***-*catB2*	+	AC, 18 FT-Aer, 20 FT-Aer, 21 FT-Aer, 33 FT-Aer, 89 SG-Aer, 177 FDD-Aer 202 LC-Aer, 206 LC-Aer, 248 LC-Aer
b-WWTP	16 FT	*A.media*	CZ,NA,**S**,RL	*bla_{OXA10}*-***aadA1***	+	AC, 206 LC-Aer
b-WWTP	39 FT	*A.media*	CZ,FOX,SXT,NA,S(I),**TMP**,RL	***dfr22***	+	AC, 18 FT-Aer, 20 FT-Aer, 89 SG-Aer, 177 FDD-Aer, 206 LC-Aer, 248 LC-Aer
b-WWTP	42 FT	*A.media*	CZ,FOX,CIP,**C**,NA,**S**,RL,NN	***catB3-aadA1***	+	AC, 18 FT-Aer, 20 FT-Aer, 89 SG-Aer, 177 FDD-Aer, 206 LC-Aer, 248 LC-Aer
HWW	52 SG	*A.hydrophila*	CZ,FOX,NA,RL,**S**	*catB8*-***aadA1***	-	AC
HWW	57 SG	*A.hydrophila*	CZ,FOX,NA,**S,Sp**,RL,NN	*catB8*-***aadA1***	-	AC, 18 FT-Aer, 20 FT-Aer, 21 FT-Aer, 33 FT-Aer, 89 SG-Aer, 177 FDD-Aer 202 LC-Aer, 206 LC-Aer, 248 LC-Aer
HWW	77 SG	*A.hydrophila*	CZ,CXM,CRO,GM,CIP,NA,**S**,RL,ATM,NN,**NET,CTX**	***aacA4cr***-***bla_{OXA1}***-*catB3*-***aar3(***bla_{SHV12}***)***	-	AC, 18 FT-Aer, 20 FT-Aer, 75 SG-Aer, 89 SG-Aer, 177 FDD-Aer, 206 LC-Aer, 248 LC-Aer
HWW	96 SG	*A.hydrophila*	CZ,NA,**S**,ATM(I),RL	*catB8*-***aadA1***	-	AC
WWTP	101 DG	*A.caviae*	CZ,FOX,NA,**S,Sp,RL**	-	+	AC, 18 FT-Aer, 20 FT-Aer, 89 SG-Aer, 177 FDD-Aer, 248 LC-Aer
WWTP	105 DG	*A.caviae*	NA,CIP(I),**S**,RL	*catB8*-***aadA1***	+	AC
WWTP	129 DG	*A.media*	CZ,NA,**S**,RL(I)	-	+	AC
WWTP	130 DG	*A.media*	CZ,(FOX),SXT,NA,Te(I),**S,TMP**,RL	***drf22-aadA1***	-	AC, 18 FT-Aer, 20 FT-Aer, 21 FT-Aer, 33 FT-Aer, 89 SG-Aer, 177 FDD-Aer 202 LC-Aer, 206 LC-Aer, 248 LC-Aer

WWTP	135 DG	*A.media*	CZ,FOX,GM(I), **S**,RL	***aadA2***	+	AC
WWTP	137 DG	*A.hydrophila*	CZ,(FOX),CIP,NA,SXT(I),**S**	*catB3*-***aadA1***	-	AC
WWTP	139 DG	*A.hydrophila*	CZ,NA,**S**	-	+	AC
a-WWTP	161 FDD	*A.caviae*	CZ,FOX,NA,SXT(I),**S**,RL	*catB8*-***aadA1***	+	AC, 18 FT-Aer, 21 FT-Aer, 33 FT-Aer, 202 LC-Aer, 206 LC-Aer
a-WWTP	167 FDD	*A.media*	CZ,NA,CIP(I),S, RL	*catB8*-***aadA2***	+	AC
a-WWTP	171 FDD	*A.media*	CZ,FOX,NA,Te(I),RL,**S, NET,C**	***aacA3***-*Blaoxa21*-***catB3***-***aadA16***	-	AC
a-WWTP	198 FDD	*A.media*	CZ,NA,**S**	-	+	AC
Recipients						
Reference strain	AC	*Acinetobacter baylyi*	R	-	-	
b-WWTP	18 FT	*A. caviae*	CZ,FOX	-	-	
b-WWTP	20 FT	*A. bestiarum*	CZ	-	-	
b-WWTP	21 FT	*A. media*	CZ	-	-	
b-WWTP	33 FT	*A. sobria*	CZ,NA,Te(I),S(I)	-	-	
HWW	75 SG	*A. hydrophila*	CZ,Te(I)	-	-	
HWW	89 SG	*A. hydrophila*	CZ	-	-	
a-WWTP	177 FDD	*A. bestiarum*	CZ	-	-	
SW	202 LC	*A. salmonicida*	CZ	-	-	
SW	206 LC	*A. media*	CZ	-	-	
SW	248 LC	*A. eucrenophila*	CZ	-	-	

Table 1. Source**,** Name, species and antibiotic resistance profiles (phenotype and genotype) of donor and recipient strains used in this work. b-WWTP river before waste water treatment plant; HWW hospital waste water; WWTP activated sludge of waste water treatment plant; a-WWTP river after waste water treatment plant; SW superficial water alpine lake. **In bolt** antibiotic used as selection in transformation experiments and genetic determinants related to phenotype. CZ cefazoline, CXM cefuroxime, CRO ceftriaxone, FOX cefoxitin, SXT bactrim, GM gentamicin, CIP ciprofloxacin, PB polymyxin, C chloramphenicol, NA nalidixic acid, AM ampicillin, RL sulfamethoxazole, Te

tetracyclin, S streptomacyn, TMP trimetroprim, ATM aztreonam, NN tobramycin, NET netilmycin, R rifampicin

5.3.2.DNA and plasmid extraction

Total DNA was extracted with a standard phenol chloroform method slightly modified. Briefly, three bacterial colonies were boiled at 100°C for 5 minutes in 300 µL of distilled water, and centrifuged at 2500 x g for 3 minutes (Eppendorf centrifuge 5427 R, rotor FA-45-24-11). The supernatant was transferred in a tube containing 2 µL of RNase (10 mg/mL) and 4 µL of proteinase K (8.3 mg/mL). The lysate was incubated at 50°C for 30 minutes, twice the volume of phenol/chloroform (1:1) was added, and the mix was spun at 14000 x g for 15 minutes at 4°C. The supernatant was transferred in a clean tube, twice the amount of chloroform was added, and centrifugation was made as in the previous step. Finally, the supernatant was transferred in a last clean tube. Plasmid extractions were made using a midi-prep protocol (Plasmid DNA Purification, NucleoBond® PC100, Machery-Nagel) or a mini-prep procedure (Plasmid DNA Purification NucleoBond® PC20, Machery-Nagel), according to the manufacturer. The DNA concentration was quantified with a spectrophotometer (NanoDrop ND-1000).

5.3.3.PCR detection of specific antibiotic resistance genes

Table 2 summarizes information about primers, size of the amplicon, and reference or accession number of published sequences used to design primers for the amplification of some gene cassettes. PCR mix consisted in 0.75 µL of each primer (10 µM), 12.5 µL Taq PCR Master Mix (Qiagen), 1 to 5 µL of DNA (approx. 10 ng) and water to a final volume of 25 µL. PCR conditions were identical for each pair of primers, apart from *bla* genes, and consisted of an initial step of denaturation (94°C for 15') followed by 30 cycles of denaturation (95°C for 30"), annealing (56°C for 30"), and extension (72°C for 1'). A final extension at 72°C for 7' terminated the amplification of the target genes. For *bla* genes, the PCR mix contained 5x PCR buffer, 0.2 µM of each dNTPs, 10 µM of each primers, 2.5 U/reaction of Taq DNA polymerase, 1 to 5 µL of DNA (approx. 10 ng), and water to a

final volume of 50 µL. PCR conditions were as following: 95°C for 15' (denaturation); 95°C for 30'' (denaturation), 59°C for 1' (annealing), 72°C for 1' (extension) for 30 cycles and 72°C for 10'.

Gene	codified antibiotic resistance	Primer sequence	bp	Refesence or Accession number
dfr22	trimethoprim	for 5'CACCGTGGAAACGGATGAAG 3' rev 5'TAACCCGATTGGCACCCATG 3'	354	AJ628423.2
catB3	chloramphenicol	for 5'CAATATCAAAGTTGGGCGGTACAG 3' rev 5'CAACGATAGCGTAAGGCTCCAC 3'	398	KR338352
sulI	sulfamethoxazol e	for 5'GTGACGGTGTTCGGCATTCT 3' rev 5'TCCGAGAAGGTGATTGCGCT 3'	779	KR338352
aadA	streptomycin, spectinomycin	for 5'ATTTGCTGGTTACGGTGACC 3' rev 5'TCAGCCCGTCATACTTGAAG 3'	533	25
tnpA	transposon tn21	for 5'TACTGCCGCGCATCAAGATC 3' rev 5'AGAAAGTTCGTCCTGGGCTG3'	400	26
bla_{SHV}	ESBL	for 5'ATGCGTTATWTTCGCCTGTGT 3' rev 5'TTAGCGTTGCCAGTGCTCG 3'	800	27
bla_{TEM}	ESBL	for 5'GTATCCGCTCATGAGACAATA 3' for 5'TCTAAAGTATATATGAGTAAACTTGGTC TG 3'	100 0	27
bla_{OXA}	ESBL	for 5'ATATCTCTACTGTTGCATCTCC 3' rev 5'AAACCCTTCAAACCATCC 3'	619	27
bla_{CTX-M}	ESBL	for 5'SCSATGTGCAGYACCAGTAA 3' for 5'CCGCRATATGNTTGGTGGTG 3'	600	28

Table 2. List of primers used for amplification of antibiotic resistance genes. *dfr22*, *catB3*, *sulI* were designed on the basis of published sequences; *aadA* and ESBL primers were previously used by Rahmani et al. (25), Dahlberg et al. (26), Colom et al. (27) and Lartigue et al. (28).

5.3.4.Natural transformation assays

Natural transformation experiments were performed with either the total DNA or the plasmid extracts of 19 *Aeromonas* strains used as donors (Table 1). *Acinetobacter baylyi* BD413, that is spontaneously rifampicin resistant, and 10 *Aeromonas* strains, sensitive to almost all the antibiotics

tested and at least to those employed for selecting transformants, were used as recipients (Table 1). When *Acinetobacter baylyi* BD413 was the recipient strain, assays were performed on nitrocellulose filters as previously described (29). In short, 100 µl of a mixture of naturally competent *Acinetobacter baylyi* BD413 and DNA or plasmid extracts (1- 20 µg per reaction) of a donor was applied to the centre of a 0.2 µm porosity filter (Whatman®) placed on a nutrient agar plate, and incubated at 30°C for 24 h. Filters were then placed in a 50 mL falcon with 4 mL of NaCl 0.9 %. The tube was vortexed to remove all the cells from the filter, and serial dilutions were prepared. 100 µl of the 10^{-7} dilution were spread on LB agar without selection to enumerate the recipient, and 100 µl of the 10^{-1} dilution were spread on LB agar with the appropriate antibiotic selection, to enumerate the transformants. All plates were incubated 24 – 48 h at 30°C. Transformation frequency was calculated as the number of transformants divided by the number of viable cells of recipients.

Transformation experiments with *Aeromonas* strains were made in a transformation buffer following the method previously describe by Huddleston et al. (21). To induce competence, recipient cells were grown in 20% LB broth until when they reached the late stationary phase of growth (OD_{600} 0.45 - 0.5), that was determined after cell growth experiments. The transformation mixture was composed by 100µl of the transformation buffer (53mM Tris pH 7.9, 20mM MgSO4 and 50mM NaCl), 40µl of competent cells and approximatively 7 ng/mL of donor DNA or plasmid extracts. Fresh LB medium was added and the mixture was incubated for 1.5 h at 30°C. Serial dilutions and spread on selective or non-selective agar were performed as described above.

Selection of the transformants was done in LB agar containing the appropriate antibiotic(s), agreeing to the known resistance profile of the donors. A negative control was included in each assay, and consisted in the spread of the recipients cells without addition of the donor DNA. This control was carried out in order to detect and quantify possibly mutation events.

5.3.5.Electroporation

Electrocompetent cells of *E.coli* DH5α were prepared according to standard procedure. Electroporation experiments were performed using 1µg of DNA (total or plasmidic) of some donors

Aeromonas strains. Cells were pulsed at 1.8 kV/cm in a pre-chilled cuvette, using a Gene Pulser electroporator (Bio-rad). The electroporated preparation was mixed with 350 μL of pre-warmed LB and incubated at 37°C with shaking for 1 h. Plasmid pUC19 was used as the positive control of electroporation. Electroporation using *Aeromonas* strains as recipients was performed according to Fengqing et al. (30). Cells were grown in LB broth and in diluted LB broth (20%) and electrocompetence were assessed at early and late stationary phase, determined by growth curves. Cells were pulsed at 12.5 kV/cm with 1µg of DNA of the donor.

5.4. Results and Discussion

Interspecies and intraspecies transformations of ten *Aeromonas* strains belonging to different species were attempted using total DNA and plasmid extract of eight *Aeromonas* strains selected as donor (Table 1). The antibiotics used for selection were chosen among those whose resistance could be due to genes that were present in the donor strains, namely streptomaycin and spectynomycin (*aadA* genes), sulfamethoxazole (*sulI* gene), chloramphenicol (*cat* genes), and cefotaxime (*bla* genes). The concentrations that had to be reached in the agar plates in order to select transformants were determined on the basis of the MIC values obtained for the donors and for the recipients, taking also into account of what reported by other authors (16, 23).

The MIC values for streptomycin and spectinomycin of all the donor *Aeromonas* strains were ascertained to be of 64 mg/L, whereas these of the recipients were comprised between 16 and 32 mg/L, a level that has been reported by other authors (31). In the first trials of transformation, we used a streptomycin concentration of 64 mg/L for selecting transformants. Unfortunately, all the recipient strains could grow. Attempts were therefor done using 128 mg/L, but in this case neither growth nor transformation occurred. Despite the MIC established for the receivers, the streptomycin concentration of 64 mg/L seems to be non-selective. At the successive concentration (128 mg/L), receivers were completely inhibited, demonstrating that the transformation assays failed. The same experiments, which also ended in no transformations, were carried out with the DNA and the plasmid extract of the donors 7FT, 57SG, and 101DG to transform receivers that were selected using spectinomycin. Spectinomycin and streptomycin target ribosomes and either prevent translocation of

tRNAs during translation or cause errors in selecting the right tRNA, thus inhibiting protein synthesis (32). Bacteria can become resistant to these antibiotics trough the production of inactivating enzymes, the adenyltransferases largely diffused in Gram negative and present also in our *Aeromonas* donors, but also trough mutations in the 16S rRNA and/or in some ribosomal proteins, known to be involved in the binding of both streptomycin and spectinomycin (33). A different mechanism might explain our results. Aeromonads are known to have 9-10 rRNA operons (The Ribosomal RNA Database: https://rrndb.umms.med.umich.edu/) per genome, so the high number of receiving cells and the high number of molecules able to bind these aminoglycoside compounds, could have led to the depletion of the antibiotic from the medium and promote growth of the strains. When the concentration of the antibiotics has been increased, the receivers couldn't no more grow or form colonies. On the other hand, it has been observed that pre-treatment of E. coli with sub-lethal doses of kanamycin or streptomycin induced an adaptive response of the cells that increased their antibiotic resistance compared to not exposed cells; this response was related with a significant increase in the production of capsular polysaccharide (34). A similar mechanism could have happened in our cultures.

Sulphamethoxazole was used as an alternative selection in natural transformation when the strain 101 DG acted as the donor. Since the MIC of the receivers was 128 mg/L for this antibiotic, we established to select transformants with a concentration of sulphamethoxazole of 256 mg/L. In this case too, all the receivers tested were capable of growing. Several resistance determinants have been found in Gram negative bacteria that cause resistance to sulfamethoxazole, i.e. spontaneous mutants in the *dhps* genes on chromosome, and the presence of *sul1*, *sul2*, and *sul3* genes on chromosome or associated to integrons and transposons. Other mechanisms can also be involved, such as decreased permeability and efflux pumps (35; Podnecky 2013 http://hdl.handle.net/10217/80967). Although spontaneous *dhps* mutants can easily be isolated in the laboratory, we don't believe that mutations alone can explain our result. Adaptive mechanisms such as those indicated for streptomycin and spectinomycin resistance are probably involved.

Two donors (42 FT *A. media* and 171 FDD *A. media*) were resistant to chloramphenicol (MIC 16 mg/L), and were shown to carry the respective genetic determinant (*catB*) as an integron cassette. The recipients strains were sensitive to this antibiotic having a MIC of 0.025 mg/L, but none of them could be transformed. In natural transformation, the transfer of DNA is initiated by the recipient cells that have to be in a physiological state called competence. It seems that most naturally transformable bacteria regulate expression of the genes involved in competence in response to various cellular and/or environmental signals (20). To achieve an efficient transformation "in vitro", it is essential to fulfil the physical and chemical conditions that influence the competence of the receiver strains. For *Aeromonas* cells, these conditions were studied by Huddleston et al. (21). These authors found that in vitro competence of transformable strains occurred when *Aeromonas* were cultivated in dilute growth medium and was induced during the late stationary phase of growth. Moreover, cells transformation could occur only in the presence of sodium and either magnesium or calcium. Even if all the conditions mentioned above were satisfied, not all aeromonads form transformant colonies, a behaviour that we also experienced. Furthermore, the same authors suggested that in *Aeromonas*, transformation is under an important genetic control, phylogenetically determined, in both the ability to act as DNA donors and the ability to catch DNA. The majority of the *Aeromonas* they studied preferred to accept DNA from close related strains (21). It is possible that the conditions applied in that study to induce competence were not adapted to our strains. On the other hand, the negative results could also be due to the phylogenetic distance between the donors and the receivers we have chosen. Finally, the receiver strains must be able, in addition to take extracellular DNA, to integrate and express it. To test this last hypothesis, DNA and/or plasmid extracts of donors were electroporated in receiving competent *Aeromonas* cells. Again, no transformants were obtained, indicating either the non-competent status of the cells or their inability to integrate and express foreign DNA.

A further transformation assay was attempted exploiting the MIC value of 16 mg/L for cefotaxime of the strain *A. hydrophila* 77SG, that was used as donor. All the receiving strains had MIC values around 0.5 mg/L. The donor strain, isolated from hospital waste water, is multi-resistant (cefazoline, cefotaxime, cefuroxime, ceftriaxone, gentamicin, ciprofloxacin, nalidixic acid, ampicillin,

sulfamethoxazole, streptomacyn, aztreonam, tobramycin, and netilmycin), possess a Class 1 integron with the cassettes aacA4cr-oxa1-catB3-aar3, carried on a plasmid belonging to the MOB family H_{121}, and the *tnpA* gene, indicating the presence of a transposon Tn*21*-like (Table 2). Moreover, the strain possesses a bla_{SHV} gene as established by PCR amplification. After the transformation assays, four recipients became cefotaxime resistant (table 3). This phenotypic change was never observed in negative controls indicating that it was induced by the exposure to the DNA of the strain 77SG, but the genotypic screening (PCR) of the receivers did not highlight the acquisition of any of the known resistant determinants of the donor. Nevertheless, the phenotypic resistance to cefotaxime was maintained for approximately 100 generations in absence of selection. Domingues and colleagues (36) suggested that changes in susceptibility patterns after exposure to bacterial DNA could be due to the transfer of resistance determinants not yet known, neither regarding the mechanisms employed nor their genetic nature. Another possible explanation of this result could be that receivers had undergone some mutational events which can confer low-level resistance. Finally, the receivers cells may have reacted to the exposure to cefotaxime with an adaptive response. Adaptive resistance is the ability to survive in stressing environmental conditions, as those represented by sub-inhibitory levels of antibiotics, thanks to alterations in gene and/or protein expression (37, 38). It has to be underlined that adaptive resistance mechanisms are not stable and the phenotype that they give reverts to the wild-type upon the removal of the inducer (39). Since our strains continued to show cefotaxime resistance for at least 100 generations and the phenotypic change was induced only by exposure to DNA, it is unlike that adaptive resistance had played a major role in our case. On the other hand, in *vivo, Aeromonas* strains are capable to adapt and live in different conditions such as polluted or unpolluted environments, chlorinated and potable waters, oligotrophic or rich in nutrients aquatic environments. These evidences demonstrate that *Aeromonas* are able to survive in many different environments; even when submitted to highly stressing conditions, and therefore that they must have and express some adaptive responses. The receivers we tested were grown in diluted medium until the late stationary phase before exposition to exogenous DNA. Since these conditions have undoubtedly influenced the antibiotic resistance profile of the strains, it could be that our recipients have displayed some unidentified responses to survive the antibiotic pressure.

Antibiotic selection (mg/L)	Donor	Donor resistance phenotype	Recipient	Recipients resistance phenotype	Resistance phenotype of transformant	Acquired generic determinants
RA, C (50, 16)	42 FT	CZ,FOX,CIP,C,NA,S,RL,NN	AC	R	CXM, **FOX**, **CIP**, **C**, TMP, R	na
CTX (10)	77 SG	CZ,CXM,CRO,GM,CIP,NA,S,RL,ATM,NN,NET,CTX	AC	R	**CXM**, FOX, **CIP**, C, TMP, **CTX,** R	na
RA, S (50, 5) or RL (256)	101 DG	CZ,FOX,NA,S,Sp,RL	AC	R	**S**, TMP, **RL,** R	*tnpA*
CTX (8)	77 SG	CZ,CXM,CRO,GM,CIP,NA,S,RL,ATM,NN,NET,CTX	18 FT	CZ, FOX	CZ, FOX, **CTX**	na
			75 SG	CZ,Te(I)	CZ,Te(I), **CTX**	
			206 LC	CZ	CZ, **CTX**	
			248 LC	CZ	CZ, **CTX**	

Table 3. Transformation of *Acinetobacter baylyi* (AC) and of *Aeromonas spp*. In **bold** acquired antibiotic resistances linked to donors profiles. CZ cefazoline, CXM cefuroxime, CRO ceftriaxone, FOX cefoxitin, SXT bactrim, GM gentamicin, CIP ciprofloxacin, PB polymyxin, C chloramphenicol, NA nalidixic acid, AM ampicillin, RL sulfamethoxazole, Te tetracyclin, S streptomacyn, TMP trimetroprim, ATM aztreonam, NN tobramycin, NET netilmycin, R rifampicin. na: not acquired.

Changes in phenotype profile might be due also to epigenetics modifications (e.g. DNA methylation) that regulate gene expression at transcriptional level in response to environmental variations (40), which can be represented among other by the presence of naked DNA. It is believed that the resistance phenotype acquired by adaptive resistance usually disappears after few generations in the absence of antibiotics and that the continuous exposure to these compounds leads to a stable form of resistance (39). The phenotypic diversity in a population without significant genotypic variation can be due to stochastic differences in gene expression patterns. Under selection, these phenotypes might become heritable. The epigenetic inheritance could be due to DNA methylation, chromatin modifications, superhelical domain configuration, or other mechanisms (41). In *Aeromonas hydrophila* it has been demonstrated that the overproduction of the DNA adenine methyltransferase (DAM) alters the virulence properties of the strains acting on gene expression (42). It is conceivable that some DNA methylation mechanisms might also influence the expression of genes involved in antibiotic resistance.

Intrergeneric transformations were carried out using as receiver the natural competent *Acinetobacter baylyi* BD413. Transformers were the 18 *Aeromonas* strains reported in Table 1.

The plasmid extract of *A. caviae* 101 DG allowed the transformation of naturally competent cells of *Acinetobacter baylyi* BD413, that acquired some resistances property of the donor (resistance to streptomycin and sulfamethoxazole) as well as the *tnpA* gene (table 3). The transformation frequency was however low ($4*10^{-8}$ CFU/mL). The *tnpA* gene has a plasmid location in the donor, but only the gene was detected in the transformant. Again, transformation caused further phenotypical changes in the recipient strain without any acquisition of genetic determinants. As in the inter- and intraspecies transformation assays, these phenotypic variations occurred only when donor DNA and/or plasmid extract were added to the transformation mixture. Furthermore, the phenotypic resistance to trimethoprim was a completely new characteristic not linked to the donor, which was sensitive to this antibiotic. Domingues et al. (23, 36) supposed that natural competent organisms, such as *Acinetobacter baylyi*, exposed to heterologous DNA could undergo to genetic rearrangements or acquisition of small fragments of DNA that affect the antimicrobial susceptibility profile. Rearrangements and acquisition of small DNA fragments can create genetic mosaic that is undetectable by sequencing, but that can confer new phenotypic characters.

DNA and plasmid extract of two other strains of *Aeromonas* (42 FT and 77 SG) induced phenotypical resistance changes in *Acinetobacter baylyi* BD413 (Table 3) that, again, was not ascribed to the detectable exchange of genetic determinants.

In conclusion, horizontal gene transfer trough transformation do not seems, in the light of our results, the preferential way of spreading of resistance determinants among bacteria belonging to the genus *Aeromonas*, in disagreement with other authors (21, 22, 43). In fact, Sakai (22) found that a protease-deficient mutant (NTG-1) of *Aeromonas salmonicida* was easily transformed not only by DNA fragments of the parent strain and intraspecific strain but also by DNA fragments of interspecific *A. hydrophila*, and intergeneric *P. fluorescence* and *V. anguillarum*. More recently, Huddleston et al. (21) stated that 73% of the environmental *Aeromonas* strains they tested were able to act as recipients, and 100% were able to act as donors to at least some other aeromonads under optimal

assay conditions of transformation. Despite we adopted the same transformation conditions, to the better of our efforts we were only able to obtain phenotypic transformants showing an increased resistance toward some antibiotics in both intra- and interspecific transformation assays. Possible explanations of our unsuccessful attempts are that we were unable to induce competence in our receives, that receivers were unable to incorporate and to express the foreign DNA and/or the transformers had specific donor DNA preferences that we have not fulfilled.

On the opposite, we could transform the natural competent *Acinetobacter baylyi* BD413. This demonstrated that at least some *Aeromonas* strains are able to act as donors of resistant determinants that could be express in intergeneric strains.

Aeromonas is able to live in complex microbial ecosystems, which can be rich in bacteriophages and free DNA, and where possession of restriction-modification systems might provide important advantages to the host protecting it from genome subversion through any invading foreign DNA. *Aeromonas* are known to code for different restriction endonucleases (44). This characteristic might also be taken into account to explain the lack of efficient transformation that we experienced.

5.5. Acknowledgments

We want to thank Professor Kaare Magne Nielsen, University of Tromsø (Norway), for kindly providing us the strains *Acinetobacter baylyi* BD413; and Paola Barbieri Prof. of the Department of Theoretical and Applied Science, University of Insubria of Varese for the review of the manuscript

5.6. References

1. **Janda JM, Abbott SL**. 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev **23**:35–73.
2. **Figuera MJ and Beaz-Hidalgo R.** 2015 *Aeromonas* infections in humans. In: Graf G, ed. *Aeromonas,* Caister Academic Press. **4:** 65-108.
3. **Janda JM, Abbott SL.** 1998 Evolving concepts regarding the genus *Aeromonas*: an expanding Panorama of species, disease presentations, and unanswered questions. Clin Infect Dis. **27:** 332–344.
4. **Carnahan AM, Altwegg M.** 1996 Taxonomy. In: Austin B., et al., ed. The genus *Aeromonas*. Chichester: John Wiley & Sons Ltd. 1-38.
5. **Huddleston JR, Zak JC, Jeter RM**. 2006. Antimicrobial susceptibilities of *Aeromonas spp*. isolated from environmental sources. Appl Environ Microbiol **72**:7036–42.
6. **Blasco MD, Esteve C, Alcaide E.** 2008. Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. J Appl Microbiol **105**:469–75.
7. **Figueira V, Vaz-Moreira I, Silva M, Manaia CM.** 2011. Diversity and antibiotic resistance of *Aeromonas spp*. in drinking and waste water treatment plants. Water Res **45**:5599–611.
8. **Alcaide E, Blasco M-D, Esteve C**. 2010. Mechanisms of quinolone resistance in *Aeromonas* species isolated from humans, water and eels. Res Microbiol **161**:40–5.
9. **Kadlec K, von Czapiewski E, Kaspar H, Wallmann J, Michael GB, Steinacker U, Schwarz S**. 2011. Molecular basis of sulfonamide and trimethoprim resistance in fish-pathogenic *Aeromonas* isolates. Appl Environ Microbiol **77**:7147–50.
10. **Vincent AT, Trudel M V, Paquet VE, Boyle B, Tanaka KH, Dallaire-Dufresne S, Daher RK, Frenette M, Derome N, Charette SJ.** 2014. 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 **58**:7367–74.
11. **Picão RC, Poirel L, Demarta A, Silva CSF, Corvaglia AR, Petrini O, Nordmann P**. 2008. Plasmid-mediated quinolone resistance in Aeromonas allosaccharophila recovered from a Swiss lake. J Antimicrob Chemother **62**:948–50.

12. **Thomas CM, Nielsen KM**. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol **3**:711–21.

13. **Filutowicz M, Burgess R, Gamelli RL, Heinemann J a, Kurenbach B, Rakowski S a, Shankar R.** 2008. Bacterial conjugation-based antimicrobial agents. Plasmid **60**:38–44.

14. **Madsen JS**, **Burmølle M**, **Hansen LH**, **Sørensen SJ**. 2012. The interconnection between biofilm formation and horizontal gene transfer. FEMS Immunol Med Microbiol **65**:183–195.

15. **Piotrowska M**, **Popowska M**. 2015. Insight into the mobilome of Aeromonas strains. Front Microbiol **6**:1–16.

16. **Moura A, Henriques I, Ribeiro R, Correia A**. 2007. Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. J Antimicrob Chemother **60**:1243–50.

17. **Moura A, Oliveira C, Henriques I, Smalla K, Correia A**. 2012. Broad diversity of conjugative plasmids in integron-carrying bacteria from wastewater environments. FEMS Microbiol Lett **330**:157–64.

18. **Bello-López JM, Vázquez-Ocampo NJ, Fernández-Rendón E, Curiel-Quesada E**. 2012. Inability of some *aeromonas hydrophila* strains to act as recipients of plasmid pRAS1 in conjugal transfer experiments. Curr Microbiol **64**:332–337.

19. **Lorenz MG, Wackernagel W**. 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev **58**:563–602.

20. **Johnsborg O**, **Eldholm V**, **Håvarstein LS**. 2007. Natural genetic transformation: prevalence, mechanisms and function. Res Microbiol **158**:767–78.

21. **Huddleston JR, Brokaw JM, Zak JC, Jeter RM**. 2013. Natural transformation as a mechanism of horizontal gene transfer among environmental *Aeromonas* species. Syst Appl Microbiol **36**:224–34.

22. **Sakai DK**. 1987. Transformation of a nonpathogenic *Aeromonas salmonicida* mutant with intraspecific, interspecific and intergeneric bacterial DNA fragments in a laboratory model of river sediments. Nippon SUISAN GAKKAISHI.

23. **Domingues S, Harms K, Fricke WF, Johnsen PJ, da Silva GJ, Nielsen KM**. a 2012. Natural transformation facilitates transfer of transposons, integrons and gene cassettes between bacterial species. PLoS Pathog **8**: e1002837.

24. **Alvarado A**, **Garcillán-Barcia MP**, **de la Cruz F**. 2012. A degenerate primer MOB typing (DPMT) method to classify gamma-proteobacterial plasmids in clinical and environmental settings. PLoS One **7**:e40438.

25. **Rahmani M, Peighambari SM, Svendsen CA, Cavaco LM, Agersø Y, Hendriksen RS**. 2013. Molecular clonality and antimicrobial resistance in Salmonella enterica serovars Enteritidis and Infantis from broilers in three Northern regions of Iran. BMC Vet Res **9**:66.

26. **Dahlberg C, Hermansson M.** 1995. Abundance of Tn3, Tn21, and Tn501 transposase (tnpA) sequences in bacterial community DNA from marine environments. Appl Environ Microbiol **61**:3051–6.

27. **Colom K, Pérez J, Alonso R, Fernández-Aranguiz A, Lariño E, Cisterna R**. 2003. Simple and reliable multiplex PCR assay for detection of blaTEM, blaSHV and blaOXA-1 genes in Enterobacteriaceae. FEMS Microbiol Lett **223**:147–151.

28. **Lartigue MF, Zinsius C, Wenger A, Bille J, Poirel L, Nordmann P**. 2007. Extended-spectrum β-lactamases of the CTX-M type now in Switzerland. Antimicrob Agents Chemother **51**:2855–2860.

29. **Ray J, Nielsen K**. 2005. Experimental methods for assaying natural transformation and inferring horizontal gene transfer. Methods Enzymol.

30. **Fengqing H, Song Y**. 2005. Electroporation-mediated transformation of *Aeromonas hydrophila.* Plasmid **54**:283–7.

31. **Goñi-Urriza M**, **Pineau L**, **Capdepuy M**, **Roques C**, **Caumette P**, **Quentin C**. 2000. Antimicrobial resistance of mesophilic Aeromonas spp. isolated from two European rivers. J Antimicrob Chemother **46**:297–301.

32. **Criswell D**, **Tobiason VL**, **Lodmell JS**, **Samuels DS**. 2006. Mutations conferring aminoglycoside and spectinomycin resistance in Borrelia burgdorferi. Antimicrob Agents Chemother **50**:445–52.

33. **Kehrenberg C**, **Catry B**, **Haesebrouck F**, **de Kruif A**, **Schwarz S**. 2005. Novel spectinomycin/streptomycin resistance gene, aadA14, from Pasteurella multocida. Antimicrob Agents Chemother **49**:3046–9.
34. **Lu E**, **Trinh T**, **Tsang T**, **Yeung J**. 2008. Effect of growth in sublethal levels of kanamycin and streptomycin on capsular polysaccharide production and antibiotic resistance in Escherichia coli B23. J Exp Microbiol Immunol **12**:21–26.
35. **Sköld O**. 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist Updat **3**:155–160.
36. **Domingues S, Nielsen KM, da Silva GJ**. b 2012. Various pathways leading to the acquisition of antibiotic resistance by natural transformation. Mob Genet Elements **2**:257–260.
37. **Martinez J and Baquero F**. 2006. Mutation Frequencies and Antibiotic resistance. J Paediatr Child Health **15**:1771–1777.
38. **Fernández L**, **Hancock REW**. 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev **25**:661–81.
39. **Motta SS, Cluzel P, Aldana M**. 2015. Adaptive Resistance in Bacteria Requires Epigenetic Inheritance, Genetic Noise, and Cost of Efflux Pumps. PLoS One **10**:e0118464.
40. **Casadesús J**, **Low D**. 2006. Epigenetic gene regulation in the bacterial world. Microbiol Mol Biol Rev **70**:830–56.
41. **Adam M**, **Murali B**, **Glenn NO**, **Potter SS**. 2008. Epigenetic inheritance based evolution of antibiotic resistance in bacteria. BMC Evol Biol **8**:52.
42. **Erova TE**, **Pillai L**, **Fadl AA**, **Sha J**, **Wang S**, **Galindo CL**, **Chopra AK**. 2006. DNA adenine methyltransferase influences the virulence of Aeromonas hydrophila. Infect Immun **74**:410–424
43. **Nguyen HNK**, **Van TTH**, **Nguyen HT**, **Smooker PM**, **Shimeta J**, **Coloe PJ**. 2014. Molecular characterization of antibiotic resistance in Pseudomonas and Aeromonas isolates from catfish of the Mekong Delta, Vietnam. Vet Microbiol **171**:397–405.
44. **Furmanek-Blaszk B**. 2014. Phenotypic and molecular characteristics of an Aeromonas hydrophila strain isolated from the River Nile. Microbiol Res **169**:547–552.

6. Third manuscript submitted to the *Future Microbiology*

First detection of antibiotic resistance genes in lytic and temperate *Aeromonas* bacteriophages

Federica Mauri and Antonella Demarta*

SUPSI Laboratory of applied microbiology, via Mirasole 22A, 6501 Bellinzona, Switzerland

*Corresponding author: antonella.demarta@supsi.ch

6.1. Abstract

The increase of antibiotic resistance in (pathogenic) bacteria is a worldwide problem, and studies on how antibiotic resistant genes can spread among microorganisms are mandatory. Besides conjugation and transformation, it is believed that bacteriophage transduction play an important role in the horizontal gene transfer, particularly in those environments rich in phages and bacteria.

Aeromonas are potentially pathogenic, ubiquitous bacteria that colonize almost all hydric environments. Many studies demonstrated the rise of resistance to antibiotics of environmental and clinical *Aeromonas* strains, and attested that these bacteria are a reservoir of genetic determinants of resistance.

In order to investigate if these resistance traits could be transferred horizontally among *Aeromonas* spp., we examined the presence of resistance genes in lytic and temperate *Aeromonas* bacteriophages and we studied the potential transduction of these genes.

We demonstrated for the first time that lytic and temperate bacteriophages of *Aeromonas* are able to integrate resistance genes from their hosts. Specific bacteriophages can therefore be involved in the horizontal gene transfer of resistant determinants among this widespread genus of aquatic bacteria.

6.2. Introduction

Antibiotic resistance of pathogenic bacteria is a worldwide problem and the increasing number and spread of super-bugs resistant to almost all antibiotics is frightening, particularly in clinical settings.

Antibiotic resistant bacteria are more and more detected also in the environment, especially water, even if at percentages lower than those found in human or veterinary medicine [1]. Resistant (pathogenic) bacteria, resistant determinants, and active antibiotic compounds reach the aquatic environment through waste water treatment plants, hospital waste waters, agricultural runoff, and other human and animal sources, and interact with the resident bacterial populations. Due to the strong selective pressure they generate, aquatic ecosystems are considered "hot spots" in the spreading of antibiotic resistances [2, 3].

Resistance can arise by acquisition of mutations and by exchange of genetic determinants trough horizontal gene transfer that is considered the main contributor to the rapid diffusion of antibiotic resistances [4]. In fact, resistance genes are often located on mobile genetic elements such as integrons, transposons and plasmids, and can be laterally transferred among even distant bacterial taxa by conjugation, by natural transformation or by transduction [5]. Transduction is mediated by bacteriophages and consists in the transfer of genetic material from a donor, who has been infected by a phage, to a recipient, which afterword can express the acquired genetic trait.

Bacteriophages are divided in virulent phages (or lytics), that lyse their host to release mature virions, and temperate phages (or lysogens), that integrate their DNA (prophage) in the host genome, replicating it in synchrony with the host cell. Temperate phages may revert to the lytic cycle through the action of natural (for example UV radiations) or of human origin (pollutants as some antibiotics) inducers [6]. During the infection stage, phages can acquire genetic material from their host, because the packaging of bacteriophage DNA is a low fidelity mechanism. These bacterial DNA portions can afterwards be transferred to another cell. The huge number of phages distributed in many ecosystems and their ability to survive for a long time in the environment [7] should render phage transduction an important mode of lateral transfer of DNA, comprising antibiotic resistance genes [8]. Indeed,

many studies demonstrate the presence of antibiotic resistance genes in bacteriophages [9, 10, 11, 12].

Aeromonas are good examples of ubiquitous bacteria that can colonize almost all hydric environments. The potential of these bacteria to develop resistance to many classes of antibiotics has been largely demonstrated [13, 14]. Recently, we characterized the resistance determinants of a collection of 231 *Aeromonas* strains isolated from different aquatic sources. Furthermore, we evaluated the potentiality of these strains as vectors of resistance genes disseminated trough conjugation and transformation [Carnelli et al. submitted, Mauri et al. submitted]. The aim of the present work was to evaluate the role played by bacteriophages in the horizontal transfer of resistance genes in this widespread genus of aquatic bacteria.

6.3. Material and methods

6.3.1. Bacterial strains

Aeromonas strains were isolated and characterized in a previous work [Carnelli et al. submitted].

6.3.2. Lytic bacteriophages

The lytic bacteriophages used in this study are part of a collection used in a phage typing scheme [15]. Phages were isolated from sewage effluents collected in our region (Ticino, Switzerland) following the enrichment protocol of Adams [16] with some modification. Briefly, a few colonies of an overnight culture of an *Aeromonas* isolate were inoculated in 10 mL of the collected water containing 100µL of $MgSO_4$ 1M and 1mL of Luria Bertani (LB) broth 10X. After incubation at 30°C for 24h, the suspension was centrifuged at 10.000 x g for 10 minutes and filtered on a 0.22µm membrane filter (Millipore). 100 µL of phosphate buffer (pH 7.1), 10 µL of $MgSO_4$ 1M, 100 µL of supernatant and a few colonies of a fresh culture of the same *Aeromonas* strain were mixed together in a glass tube. 3ml of soft agar (LB broth with 0.7% of agarose) were added to the tube, vortexed and poured onto a LB agar plate. The presence of visible plaques on the agar surface after over-night incubation at 30°C indicated the presence of lytic bacteriophages that were further propagated and enumerated (PFU/mL, plates forming units).

In order to purify and enrich the bacteriophage suspensions, a single plaque was removed from the soft agar layer with a sterile loop, put in 10 mL of LB and incubated at 30°C for 24 h. The suspension was then centrifuged, filtered and tested for plaques formation as described above. This step was repeated as many times as necessary to obtain a pure phage suspension. The determination of the concentration of phage suspensions was performed by the double agar overlay plaque assay as described by Kropinski et al. [17]. Briefly, phages suspension were diluted (10^{-1} – 10^{-7}) in LB broth, mixed with soft agar and poured off on agar plates. After incubation the titer of the original phage preparation was determined by counting the number of plaques formed, taking into consideration the dilutions.

6.3.3.Induction of lysogenic bacteriophages using mitomycin C

An overnight culture of *Aeromonas spp.* was diluted 1:100 in LB broth and incubated for 4 h at 30°C. Mitomycin C (0.5 µg/mL) was added to the growing cultures, that were further incubated for approx. 6 h until the lysis of the cells, detected by a decrease of turbidity. The lysates were centrifuged at 3000 x g for 12 minutes at 4°C and filtered on a 0.22µm membrane filter [18].

6.3.4.Bacteriophage host range

The ability of bacteriophages to lyse different *Aeromonas* strains was tested by lysotyping (phage typing) as following. 1 mL of an overnight culture of *Aeromonas sp.* was transferred in 2 mL of phosphate buffer pH 7.1 with 200 µL of $MgSO_4$ 1M, the suspension was poured onto an agar plate, excess was eliminated, and plates were let dry at room temperature. One drop of each bacteriophage suspension, previously diluted according to its RDT (routine dilution test), was deposited on the plate surface. After the drops dried, the plates were incubated (24 h at 30°C), and the lytic reactions of each phage were recorded as positive (lysis of the strain) or negative (absence of lysis).

6.3.5.Phage concentration by PEG/NaCl

To eliminate bacteria and to concentrate the phage suspensions, a PEG/NaCl solution was used following the method of Sayers and Eckstein [19] slightly modified. 10 mL of 2% PEG/NaCl (PEG

6000 2% w/w and NaCl 2.5M) were added to 50mL of a phage filtrate and the suspension was centrifuged 15000 x g for 10 minutes at 4°C. 10 mL of 20% PEG/NaCl (PEG 6000 20% w/w and NaCl 2.5M) were added to the supernatant and incubated at 4°C overnight. The phage suspensions were then centrifuged 15000 x g for 10 minutes at 4°C. The pellet was resuspended in phage buffer (Tris-HCl 10mM pH7.6, NaCl 100mM, $MgSO_4$ 1mM and $CaCl_2$ 1mM), in PBS 1 x or in NaCl 0.9%, depending on the experiments that followed.

6.3.6.DNA extraction from bacteria

Total DNA was extracted with a standard phenol chloroform method slightly modified. Briefly, three bacterial colonies were boiled at 100°C for 5 minutes in 300 µL of distilled water, and centrifuged at 2500 x g for 3 minutes. The supernatant was transferred in a tube containing 2 µL of RNase (10 mg/mL) and 4 µL of proteinase K (8.3 mg/mL). The lysate was incubated at 50°C for 30 minutes, twice the volume of phenol/chloroform (1:1) was added, and the mix was spun at 14000 x g for 15 minutes at 4°C. The supernatant was transferred in a clean tube, twice the amount of chloroform was added, and centrifugation was made as in the previous step. Finally, the supernatant was stocked in a clean tube.

6.3.7.DNA extraction from bacteriophages

DNA from bacteriophages was extracted with the phenol-chloroform method described by Muniesa and Jofre [20]. Briefly, 2.5 µL of RNAse and 0.5 µL of DNAse were added to 500 µL of phage suspension and incubated for 2 hour at 37°C. Proteinase K (50 µL) and SDS 10% were added and the mix was incubated 1h at 56°C. One volume of phenol, one volume of phenol- chloroform (1:1) and one volume of chloroform were added sequentially in the supernatant, the solution was mixed by inversion and centrifuged at 10000 x g for 5 minutes each time. Two volumes of cold absolute ethanol were added and the solution was incubated for 10 min at -70°C. The mixtures were centrifuged at 13500 x g at 4°C for 20 min, supernatants were discarded and the dried pellets were resuspended in water.

6.3.8. Screening of antibiotic resistance genes by PCR

The presence of resistance genes in bacteria and in bacteriophages was screened by PCR. Table 1 summarizes information about primers, product sizes, and references or accession numbers of published sequences used to design primers. PCR mix consisted in 0.75 µL of each primer (10 µM), 12.5 µL Taq PCR Master Mix (Qiagen), 1 to 5 µL of DNA (approx. 10 ng) and water to a final volume of 25 µL. PCR conditions were identical for each pair of primers and consisted of an initial step of denaturation (94°C for 15') followed by 30 cycles of denaturation (95°C for 30"),annealing (56°C for 30"), and extension (72°C for 1'). A final extension at 72°C for 7' terminated the amplification of the target genes.

Table 1. List of primers used for amplification of antibiotic resistance genes. *dfr22*, *catB3*, *sulI* were designed on the basis of published sequences; *aadA* and *tnpA* primers were previously used by Rahmani et al. [20] and Dahlberg et al. [21] respectively. ESBL primers were designed by Colom et al. [22].

Gene	codified antibiotic resistance	Primer sequence	Bp	Reference or Accession number
dfr22	trimethoprim	for 5'CACCGTGGAAACGGATGAAG 3' rev 5'TAACCCGATTGGCACCCATG 3'	354	AJ628423.2
catB3	chloramphenicol	for 5'CAATATCAAAGTTGGGCGGTACAG 3' rev 5'CAACGATAGCGTAAGGCTCCAC 3'	398	KR338352
sulI	sulfamethoxazole	for 5'GTGACGGTGTTCGGCATTCT 3' rev 5'TCCGAGAAGGTGATTGCGCT 3'	779	KR338352
aadA	streptomycin, spectinomycin	for 5'ATTTGCTGGTTACGGTGACC 3' rev 5'TCAGCCCGTCATACTTGAAG 3'	533	20
tnpA	transposon tn21	for 5'TACTGCCGCGCATCAAGATC 3' rev 5'AGAAAGTTCGTCCTGGGCTG3'	400	21
bla_{SHV}	ESBL	for 5'ATGCGTTATWTTCGCCTGTGT 3' rev 5'TTAGCGTTGCCAGTGCTCG 3'	800	22
bla_{OXA}	ESBL	for 5'ATATCTCTACTGTTGCATCTCC 3' rev 5’AAACCCTTCAAACCATCC 3’	619	22

6.4. Results

6.4.1.Host range of lytic bacteriophages

In order to determine their host range, 21 lytic bacteriophages, prevalently isolated on *Aeromonas caviae, A.hydrophila* and *A.media* as propagator strains, were used at a RDT of approx. 10^3 in the phage typing of 231 *Aeromonas* strains of environmental origin. Eleven phages were able to infect almost one of the strains tested, aside from the original host. Overall, 16% (36/231) of the *Aeromonas* sp. could be lysed by at least one phage, and almost all phages were able to infect more than one *Aeromonas* species (table 2).

Table 2. Species and number of *Aeromonas* spp. strains used for phage amplification and phage typing results of the eleven lytic bacteriophages those were able to form plaques on our strains (number and species).

Lytic Phages	*Aeromonas* strains used for phage amplification (*species*)	*Aeromonas* strains that support plaques formation (*species*)
1	6.84 (*A.hydrophila*)	156-FDD (*A.hydrophila)*
3	6.84 (*A.hydrophila*)	156-FDD (*A.hydrophila)*
		160-FDD (*A.caviae)*
5	11.88 *(A.hydrophila*)	5-FT, 77-SG, 147-DG, 157 FDD, 164 FDD (*A.hydrophila)*
		32-FT, 177-FDD (*A.bestiarum)*
		105-DG, 109-DG, 123-DG, 178-FDD, 192-FDD (*A.caviae)*
		116-DG, 166-FDD, 169-FDD, 171-FDD, 206-LC (*A.media)*
		205-LC, 229-LC (*A.salmonicida)*
18	18 *(A.media*)	30-FT (*A.bestiarum)*

		34-FT, 43-FT, 115-DG, 166-FDD, 169-FDD (*A.media)*
		164-FDD (*A.hydrophila)*
42	42 *(A.media*)	43-FT, 169-FDD (*A.media)*
43	43 *(A.caviae*)	30-FT, 32-FT (*A.bestiarum)*
		164-FDD (*A.hydrophila)*
		34-FT, 115-DG, 169-FDD, 176-FDD (*A.media)*
45-a	45 *(A.caviae*)	18-FT, 24-FT, 26-FT, 105-DG, 123-DG (*A.caviae)*
		30-FT, 32-FT (*A.bestiarum*)
		34-FT, 43-FT, 166-FDD, 169-FDD, 176-FDD (*A.media)*
		164-FDD (*A.hydrophila)*
		229-LC (*A.salmonicida)*
50-a	50 (*A.caviae*)	137-DG (*A.hydrophila)*
		154-FDD, 167-FDD, 181-FDD (*A.media)*
94 Ca	94 (*A.bestiarum*)	64-SG, 73-SG, 81-SG (*A.salmonicida)*
		114-DG (*A.hydrophila)*
		135-DG (*A.media*)
94 pla	94 (*A.bestiarum*)	64-SG, 73-SG, 81-SG (*A.salmonicida)*

6.4.2.Antibiotic resistance determinants in the DNA of lytic bacteriophages

We analyzed by PCR the DNA extracted from phages to detect the presence of the resistant determinants (genes, transposons or integrons) carried by the *Aeromonas* strains in which the phage had a lytic cycle.

Five of the 21 bacteriophages acquired at least an antibiotic resistance determinant from their hosts (Table 3). We were unable to find acquired genetic determinants by the phage 50-a. Phage 5 acquired *catB3* and *sulI* from the strain *A. media* 171-FDD-Aer but it was unable of taking one of the many determinants present in strain *A. hydrophila* 77-SG-Aer. The more frequently antibiotic resistance determinants detected in our phages were *sulI* and *tnpA*.

Table 3. Genetic determinants acquired by lytic bacteriophages.

***Aeromonas* spp. host**	**Species**	**Genetic determinants of hosts**	**Lytic bacteriophages**	**Genetic determinants acquired by bacteriophages**
105-DG	*A.caviae*	*aadA, sulI, tnpA*	**45-a**	***aadA, sulI, tnpA***
171-FDD	*A.media*	*catB3, sulI*	**5**	***catB3, sulI***
34-FT	*A.media*	*tnpA*	**18, 43, 45-a**	***tnpA***
123-DG	*A.caviae*	*tnpA*	**45-a**	***tnpA***
154-FDD	*A.media*	*tnpA*	**50-a**	na
167-FDD	*A.media*	*aadA, sulI, tnpA*	**50-a**	na
77-SG	*A.hydrophila*	*catB3,sulI, tnpA,* bla_{SHV}, bla_{OXA}	**5**	na

na not acquired

6.4.3.Host range of lysogenic bacteriophages

All the 231 *Aeromonas* strains were induced with mitomycin C in order to detect lysogenic bacteriophages. On the basis of the turbidity decrease observed in the *Aeromonas* cultures after the addition of mitomycin C, prophages were successfully induced in 17 of the 231 *Aeromonas* strains. Lysogenic strains belonged mostly to the species *A.caviae, A.media, A.bestiarum,* and *A.hydrophila*. Phage typing was performed using only the temperate phages whose host possessed at least one known determinant of resistance. Bacteriophage 19-L, which was induced in *A. caviae*, had a lytic

cycle in 12 *Aeromonas* strains belonging to the species *A.caviae, A.salmonicida*, *A.hydrophila,* and *A.eucrenophila*. For phage 39-L, induced from an *A. media*, only one propagation host belonging to the species *A.hydrophila* was found, whereas phage 34-L, obtained from an *A. media*, infected 30 different strains of *Aeromonas*, particularly of the species *A.salmonicida*, but also of species *A.media, A.hydrophila,* and *A.eucrenophila.*

6.4.4.Antibiotic resistance determinants in the DNA of lysogenic bacteriophages

Three lysogenic strains, 19FT-Aer (*A. caviae*), 34FT-Aer (*A. media*), and 39FT-Aer (*A.media*), carried at least one of the following genetic determinants: the *tnpA* gene, suggesting the presence in the cells of a transposon of the Tn21-like family, and the *dfr22* gene, encoding a trimethoprim-resistant dihydrofolate reductase. As demonstrated by PCR, these genes were acquired by the respective temperate bacteriophages (table 4).

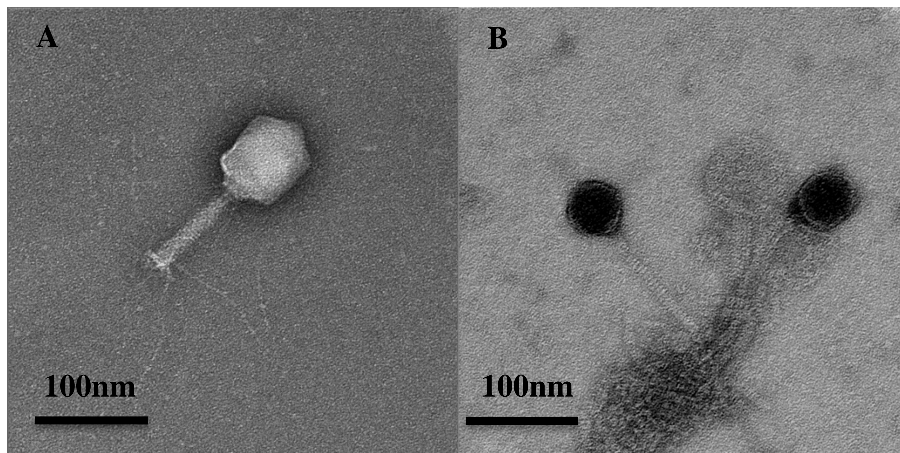
Table 4. Genetic determinants acquired by lysogenic bacteriophages

Lysogenic *Aeromonas* strains (*species*)	**Genetic determinants of resistance on the lysogenic strain**	**Lysogenic bacteriophages**	**Genetic determinants acquired by temperate bacteriophages**
19FT-Aer *(A.caviae)*	*tnpA*	19-L	*tnpA*
34FT-Aer *(A.media)*	*tnpA*	34-L	*tnpA*
39FT-Aer *(A.media)*	*tnpA, dfr22*	39-L	*tnpA, dfr22*

6.4.5.Morphology

Transmission electron microscopy (TEM) images of suspensions of phages 45-a and 39-L were taken at the Geneva University (Barja F.) allowing an initial characterization of these two bacteriophages (Figure 1 A and B). Both bacteriophages belong to the order *Caudovirales*, which groups phages with icosahedral heads and tails. Phage 45-a, that shows a strong resemblance with phage T4, is most probably a member of the *Myoviridae* family that comprises phages characterized by an icosahedral head, a contractile tail, and a base plate with tail fibres.

Fig. 1 Electron micrograph of phages particles 45-a (A) and 39 L (B). Scale bar (100nm).



6.5. Discussion and conclusions

To contribute to the evaluation of the role played by bacteriophages in the transfer of resistance genes among bacteria in aquatic environments, we studied a collection of lytic and temperate specific phages of *Aeromonas* strains. *Aeromonas* spp. were considered a model of natural aquatic bacterial populations owing to their large diffusion in all kind of waters, polluted or unpolluted, oligotrophic or hypereutrophic. Due to their ecology, Aeromonads are submitted to the selection that arise in aquatic environments, and have become more and more resistant to antibiotics [1, 23, 24]. *Aeromonas* bacteriophages are known since many years, and a number of them have been identified and characterized [25]. The lytic bacteriophages that we used in the present work came from a collection of *Aeromonas* phages made in the late eighties for being used to type clinical and environmental *Aeromonas* strains [15]. The abundance of bacteriophages being connected to the abundance and activity of their bacterial hosts [26], our bacteriophages, as well as those described by other authors [27 28, 29], were isolated from aquatic environments charged in *Aeromonas* strains such as fish farm water and sediments, sewage, waste water treatment plants, and rivers.

Temperate bacteriophages were induced by mitomycin C in 271 *Aeromonas* strains. Despite lysogeny appears to be widely spread in nature and some bacterial strains can release up to five different type of temperate phages [16], our results revealed that only 7% of the *Aeromonas* sp. were lysogenic. This percentage was slightly more elevated than that found by Beilstein and

Dreiseikelmann [30], who were able to induce by UV radiation the 2% of 74 *Aeromonas* isolated from surface waters.

The electron microscopy imagines of some of our phages showed the typical structure of the tailed bacteriophages, indicating that they belong to the order *Caudovirales* that groups phages with icosahedral heads and tails. The length and contractile abilities of the tail differentiate among the families *Myoviridae*, with long and contractile tails, *Podoviridae*, short non contractile tails, or *Siphoviridae*, the larger family composed by virus with long tails that are not contractile. We did not investigate further the phages particles nor their genome but the majority of the *Aeromonas* specific phages reported in literature, belongs to the family *Myoviridae* and resembles to the T4-like phages, which infect several species of Enterobacteriaceae as well as distant bacterial genera such as *Aeromonas*, *Vibrio* and cyanobacteria (29). The phage 45-a is highly similar to the T4 phages and could therefore belong to the *Myoviridae.*

Although our lytic bacteriophages were active on a little fraction of the *Aeromonas* sp. tested, infecting only the 16% of the strains, the majority showed a wide host range. In fact, nine phages were polyvalent, being able to display a lytic cycle on different *Aeromonas* species. A similar observation can be made for the temperate bacteriophages we could induce from the 231 *Aeromonas* strains. Polyvalent bacteriophages act better in spreading horizontally genetic determinants, due to their ability to transduce among different strains, species and even genera [31, 32]. On the contrary, in phage therapy it is preferable to select the bacteriophages for the pathogenic bacterial species or even for the single strain in order to not interfere on the equilibrium among the many bacterial populations of the system treated.

Recent studies have emphasized the role of bacteriophages in the emergence and spread of antibiotic resistance genes, highlighting the presence in the genomes of bacteriophages isolated from activated sludge, rivers, urban sewages, and animal faecal waste of genetic determinants for resistance to antibiotics such as chloramphenicol, tetracycline, kanamycin, gentamycin, methicillin, beta-lactams and others [9, 10, 11, 12]. To our knowledge this is the first time that bacteriophages specific for *Aeromonas* sp. have been screened for the presence of antibiotic resistance genes. Resistance genes

or resistance markers were found in all of the temperate bacteriophages, probably reflecting their ability to integrate into the bacterial genome, and in some lytic phages. All the antibiotic resistance genes present in the donor strains could be detected in our phages, namely *aadA* (genes conferring resistance to streptomycin and spectinomycin), *catB* (chloramphenicol resistance), *sulI* (sulfamethoxazole resistance) and *dfr* (trimethoprim resistance). The *tnpA* genes, genetic markers of the transposons Tn21-like that are involved in the spread of antibiotic resistances [33], were also acquired by our phages. Conversely, we were unable to find resistance determinants in phages propagated on some donors. In particular, phage 5, that was able to capture *catB3* and *sulI* from the strain *A. media* 34FT-Aer, was found incapable of transduce the many resistance determinants of the strain *A. hydrophila* 77SG-Aer.

To our knowledge, this is the first finding of antibiotic resistance genes and genetic elements involved in the spread of resistance to antibiotics in lytic and temperate bacteriophages specific for *Aeromonas* spp. This result reinforces the role that bacteriophages might play in the aquatic environment in the horizontal gene transfer of resistance genes.

6.6. Acknowledgments

We want to thank Dr Graciela Castro Escarpulli of the National polytechnic institute (Mexico). We are indebted to Dr François Barja of the University of Geneva, Department of Botany and Plant Biology, for TEM samples preparation and analyses of bacteriophages. And we want to thank Paola Barbieri Prof. of the Department of Theoretical and Applied Science, University of Insubria of Varese for the review of the manuscript

6.7 References

1. Martinez JL. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ Pollut [Internet]. Elsevier Ltd; 2009 Nov [cited 2014 Jul 11];157(11):2893–902. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19560847
2. Baquero F, Martínez J-L, Cantón R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol [Internet]. 2008 Jun [cited 2014 Mar 26]; 19(3):260–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/
3. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol [Internet]. Nature Publishing Group; 2014; 12(7):465–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24861036
4. Courvalin P. Predictable and unpredictable evolution of antibiotic resistance. J Intern Med [Internet]. 2008;264(1):4–16. Available from: http://doi.wiley.com/10.1111/j.1365-2796.2008.01940.x
5. Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol [Internet]. 2005 Sep [cited 2014 Mar 20]; 3(9):711–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16138099
6. Orlova EV, (iInstitute for Structural and Molecular Biology Department of Biological Sciences BC. Bacteriophages and Their Structural Organisation. Bacteriophages. 2012; 3–30.
7. Muniesa M, Colomer-Lluch M, Jofre J. Could bacteriophages transfer antibiotic resistance genes from environmental bacteria to human-body associated bacterial populations? Mob Genet Elements [Internet]. 2013 Jul 1; 3(4):e25847. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3812792&tool=pmcentrez&rendertype=abstract
8. Balcazar JL. Bacteriophages as vehicles for antibiotic resistance genes in the environment. PLoS Pathog [Internet]. 2014 Jul [cited 2014 Aug 2]; 10(7):e1004219. Available from: http://www.ncbi.nlm.nih.gov/pubmed/
9. Colomer-Lluch M, Jofre J, Muniesa M. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PLoS One [Internet]. 2011 Jan [cited 2014 Mar 21];

6(3):e17549. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3048399&tool=pmcentrez&rendertype=abstract.

10. Colomer-Lluch M, Imamovic L, Jofre J, Muniesa M. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. Antimicrob Agents Chemother [Internet]. 2011 Oct [cited 2014 Mar 21]; 55(10):4908–11. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3187014&tool=pmcentrez&rendertype=abstract
11. Muniesa M, Imamovic L, Jofre J. Bacteriophages and genetic mobilization in sewage and faecally polluted environments. Microb Biotechnol [Internet]. 2011 Nov [cited 2014 Mar 26];4(6):725–34. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3815409&tool=pmcentrez&rendertype=abstract
12. Mazaheri Nezhad Fard R, Barton MD, Heuzenroeder MW. Bacteriophage-mediated transduction of antibiotic resistance in enterococci. Lett Appl Microbiol [Internet]. 2011 Jun [cited 2014 Mar 26];52(6):559–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21395627.
13. Arias A, Seral C, Navarro F, Miró E, Coll P, Castillo FJ. Plasmid-mediated QnrS2 determinant in an Aeromonas caviae isolate recovered from a patient with diarrhoea. Clin Microbiol Infect [Internet]. Elsevier; 2010 Jul 7 [cited 2015 Nov 10];16(7):1005–7. Available from: http://www.clinicalmicrobiologyandinfection.com/article/S1198743X14617651/fulltext
14. Figueira V, Vaz-Moreira I, Silva M, Manaia CM. Diversity and antibiotic resistance of Aeromonas spp. in drinking and waste water treatment plants. Water Res [Internet]. 2011 Nov 1 [cited 2014 Mar 26]; 45(17):5599–611. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21907383
15. Altwegg M, Altwegg-Bissig R, Demarta A, Peduzzi R, Reeves MW, Swaminathan B. Comparison of four typing methods for Aeromonas species. J Diarrhoeal Dis Res [Internet]. 1988 Jun [cited 2015 Nov 10];6(2):88–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2908357

16. Adams M. Methods of study of bacterial viruses. In: Bacteriophages, 1959. Interscience Publishers, INC. [Internet] Available from: http://www.cabdirect.org/abstracts/19602204111.html
17. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Humana Press; 2009. Chapter 7, pp. 69-76.
18. Raya RR and Hébert ME. Isolation of Phage via Induction of Lysogens. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Chapter 3, p 27.
19. Sayers JR and Eckstein F. Properties of Overexpressed Phage T5 D15 Exonuclease. J Biol Chem. 1990; 265(30):18311–7.
20. Rahmani M, Peighambari SM, Svendsen CA, Cavaco LM, Agersø Y, Hendriksen RS. Molecular clonality and antimicrobial resistance in Salmonella enterica serovars Enteritidis and Infantis from broilers in three Northern regions of Iran. BMC Vet Res [Internet]. 2013 Jan [cited 2014 Mar 20];9:66. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3623788&tool=pmcentrez&rendertype=abstract.
21. Dahlberg C, Hermansson M. Abundance of Tn3, Tn21, and Tn501 transposase (tnpA) sequences in bacterial community DNA from marine environments. Appl Environ Microbiol [Internet]. 1995;61(8):3051–6. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=167581&tool=pmcentrez&rendertype=abstract
22. Colom K, Pérez J, Alonso R, Fernández-Aranguiz A, Lariño E, Cisterna R. Simple and reliable multiplex PCR assay for detection of blaTEM, blaSHV and blaOXA-1 genes in Enterobacteriaceae. FEMS Microbiol Lett. 2003;223(2):147–51.

23. Gillings, Michael R. 2013 Evolutionary Consequences of Antibiotic Use for the Resistome, Mobilome and Microbial Pangenome. Frontiers in Microbiology doi:10.3389/fmicb.2013.00004.

24. Carnahan AM, Altwegg M. Taxonomy. 1996 In: Austin B., et al., ed. The genus *Aeromonas*. Chichester: John Wiley & Sons Ltd; 1-38.

25. Kim JH, Son JS, Choi YJ, Choresca CH, Shin SP, Han JE, et al. Isolation and Characterization of a Lytic Myoviridae Bacteriophage PAS-1 with Broad Infectivity in Aeromonas salmonicida. Curr Microbiol [Internet]. 2012 May [cited 2014 Mar 26];64(5):418–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22314935

26. Muniesa M, Colomer-Lluch M, Jofre J. Potential impact of environmental bacteriophages in spreading antibiotic resistance genes. Future Microbiol [Internet]. 2013 [cited 2014 Apr 1];739–51. Available from: http://www.futuremedicine.com/doi/abs/10.2217/fmb.13.32

27. Kim JH, Son JS, Choi YJ, Choresca CH, Shin SP, Han JE, et al. Complete genome sequence and characterization of a broad-host range T4-like bacteriophage phiAS5 infecting Aeromonas salmonicida subsp. salmonicida. Vet Microbiol [Internet]. Elsevier B.V.; 2012;157(1-2):164–71. Available from: http://dx.doi.org/10.1016/j.vetmic.2011.12.016

28. Shen C, Liu Y, Lu C. Complete Genome Sequence of Aeromonas hydrophila Phage CC2. J Virol [Internet]. 2012 Oct [cited 2015 Oct 15];86(19):10900. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3457290&tool=pmcentrez&rendertype=abstract

29. Haq IU, Chaudhry WN, Andleeb S, Qadri I. Isolation and Partial Characterization of a Virulent Bacteriophage IHQ1 Specific for Aeromonas punctata from Stream Water. Microb Ecol [Internet]. 2012 May [cited 2014 Mar 26];63(4):954–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21947462

30. Beilstein F, Dreiseikelmann B. Temperate bacteriophage PhiO18P from an Aeromonas media isolate: characterization and complete genome sequence. Virology [Internet]. 2008 Mar 30 [cited 2015 Oct 15];373(1):25–9. Available from: http://www.sciencedirect.com/science/article/pii/S0042682207007702

31. Jensen EC, Schrader HS, Rieland B, Thompson TL, Lee KW, Nickerson KW, et al. Prevalence of broad-host-range lytic bacteriophages of Sphaerotilus natans, Escherichia coli, and Pseudomonas aeruginosa. Appl Environ Microbiol [Internet]. 1998 Feb;64(2):575–80. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=106085&tool=pmcentrez&rendertype=abstract

32. Khan MA, Satoh H, Katayama H, Kurisu F, Mino T. Bacteriophages isolated from activated sludge processes and their polyvalency. Water Res. 2002;36(13):3364–70.

33. Zühlsdorf MT, Wiedemann B. Tn21-specific structures in gram-negative bacteria from clinical isolates. Antimicrob Agents Chemother [Internet]. 1992 Sep;36(9):1915–21. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=192209&tool=pmcentrez&rendertype=abstract

7. Transduction

Transduction is the horizontal gene transfer mechanism by which genetic material from a donor cell is transfer to a recipient by means of a bacteriophage. Two principal mechanisms are known: generalized transduction, which consists in transfer of any fragment of the genome of the donor, and specialized transduction, in which only specific genes, which are located adjacent to the prophage attachment site, are transferred [1]. Both lytic and lysogenic bacteriophages can transfer any piece of DNA (general transduction); on the contrary only temperate phages can do specialized transduction [2].

It is reported that transducing particles represent generally 1% of the total phages particles and that transduction is a rare event, occurring approximately only once every 107–109 phage infections. Moreover, abortive transduction, in which the DNA of the transducing particle is injected into the cell but it is not integrated and it is lost, is also reported to be frequent and to occur in the 90% of the recipient cells [3, 4].

To overcome these known difficulties in the experimental evaluation of phage transduction, some critical points have to be considered in planning of in vitro investigations.

In the population of bacteriophages to be used, transducing particles have to be present and the more they are the more the transducing efficiency is high. It has been showed that some chelating agents, such as EDTA and sodium citrate, can increase the number of gene copies of the bacterial donor in the DNA of phages, thus resulting in an enrichment of transducing particles [5].

Transduction depends on different conditions such as the physical interactions between phage and host, an adequate physiological condition of the host and the characteristics of the environment in which interactions occurred [6]. The presence and the concentrations of certain ions (Ca++ and Mg++) are particularly important due to their capacity to facilitate the adsorption of phage particles to the host cells. On the other hand, their concentration has to be controlled (by means of chelating agents) in order to prevent subsequent reinfection [7].

The multiplicity of infection (m.o.i.) is defined as the ratio of infectious virions to cells in a culture. In other terms, m.o.i. is roughly the average number of phages per bacterium, assuming that all phages are infectious, that all cells are susceptible and that the phages infecting each bacterium has a random distribution. In most virus infections, however, there are uninfected cells and bacteria that are infected with multiple virus particles [8]. As the m.o.i. increases, the percentages of cells infected with at least one phage particle also increases: for a m.o.i of 1.0, 63.2% of the cells are infected with at least one phage, whereas with a m.o.i of 5 this percentage increases to 99.3%.

The m.o.i is therefore an important parameter that influences the effectiveness of transduction; the m.o.i. should be comprised between 0.01 and 1 in order to infect a recipient bacterium with no more than one phage particle [9]. In fact, if the infecting particle is a transducing phage, one has to avoid the lysis of the transductants.

To allow the spread of genetic determinants into a microbial niche, the transduced genetic material have to be stable retained and expressed into the recipient host. The genes acquired by bacteriophages could be check for their integrity (by PCR and sequencing) and for their ability to be expressed in the recipient (inserting the phage DNA by electroporation into bacterial competent cells).

The choice of the recipient bacterial strain is another critical point in transduction experiments. The recipients must be able to evidence that the transformation has occurred successfully. Transducing particles behave like a bacteriophage, thus adsorbing on the cell surface of their specific host and injecting their DNA into the cell cytoplasm. The outcome of the adsorption and injection steps is dependent of the bacterial host. If transductants are not able to integrate the DNA, this last will be lost causing an abortive transduction [3, 4]. Since transducing particles are defective, they are unable to undergone both the lytic and the lysogenic cycle and they cannot promote plaque formation. Therefore, it should be considered that a “silent transfer”, that is the transduction of DNA to bacterial strains that the phage cannot parasitize, could occur [10]. To investigate this kind of transduction, that can represent an important way for spreading resistant determinants, many different strains, of the same as well of different species and even genera, has to be checked [11].

7.1. Preliminary attempts for the demonstration of phage transduction between Aeromonas strains

7.1.1 Material and methods

Transduction was tested accordingly to the following three methods using both lytic and lysogenic phages lysate. Phages lysate were prepared as previously described [12, 13] using a m.o.i. value of 10 for the infection of donor strains. i) One milliliter of a high titer phage suspension (10^{10} PFU/mL) was added to 9 mL of an overnight recipient culture in LB broth (10^{9} CFU/mL) and incubated 1h at 30°C (m.o.i.: 1). Bacterial cells were then washed twice with LB broth, resuspended in 1mL of LB broth with sodium citrate 20 mM and incubated 2 h at 30°C. 100 µL of the suspension were plated on selective LB agar plates, containing the antibiotic for which the transfer of the corresponding resistance gene was checked [14]. ii) Two or three colonies of the recipient *Aeromonas* were resuspended in 200 µL of phage buffer with 200 µL of a phage suspension (m.o.i.: 1). The mix was incubated at 30°C for 30 minutes; 100 µL were then plated on a selective LB agar plate (Corvaglia AR, personal communication). iii) Lytic phages particles were further enriched as following: 10 µL of an overnight culture of the *Aeromonas* strain that was a possible donor of the resistant gene were added to 1mL of LB broth containing 0.2% of glucose and 5mM of $CaCl_2$. The culture was incubated until it reached the early exponential phase (about 2 h). 10 µL of the specific lytic phage suspension (about 10^{8} PFU/mL) were added to the culture and the mix was incubated again for 3 h at 30°C. 100 µL of chloroform were added, the suspension vortexed and centrifuged at 13000 rpm for 1 minute at 4°C; supernatant was transferred in a clean tube and stored at 4°C. For transduction, few colonies of a recipient culture were inoculated in 1mL of LB broth and incubated O/N at 30°C. Cells were centrifuged at 13000 rpm for 30 seconds and the pellet was resuspended in 500 µL of CaMg (5nM $CaCl_2$ + 10mM $MgSO_4$). Different amounts of the phage preparation were added to 100 µL of the recipient cells and leaved at room temperature for 30 minutes, whereupon 1mL of LB was added and each tube was incubated for 1 h at 30°C. (m.o.i. 0.01-1). The mix was centrifuged as described above and pellet was resuspended in 1mL of LB with 20mM sodium citrate. Finally 100 µL were plated on selective agar and incubated at 30°C for 24-48 h [7].

7.1.2 Preliminary Results.

Transduction experiments were carried out with phages 45-a, 5, 39-L, 34-L, and 19-L. Each phage suspension was tested to confirm the presence of the genetic resistance element of the propagator or lysogenic original host. Antibiotics used for the selection of transductants were streptomycin, spectinomycin, trimethoprim, sulfamethoxazole or chloramphenicol according to the genetic determinants detected in the bacteriophage suspensions and the original host. Recipient strains were sensitive to these antibiotics and were propagators for the infecting phage (Table 1). None of the 15 recipients were able to express phenotypically the resistance trait of the original host and detected in the phage DNA by PCR.

Table 1. Donor and recipient bacterial strains, phages and antibiotic selection used in transduction experiments

Propagator or lysogenic host (species)	Genetic determinants of the original host present on the phage DNA	Phage used as transducer	Recipient host (species)	Antibiotic selection (mg/L)
19-FT	*tnpA*	19-L	218-LC, 244-LC	S, Sp (128)
34-FT	*tnpA*	34-L	218-LC, 244-LC	S, Sp (128)
39-FT	*dfr22*	39-L	110-DG	TMP (2), RL (256)
105-DG	*aadA1*	45-a	18-FT, 24-FT, 26-FT, 32-FT, 123-DG, 164-FDD, 176-FDD 180-FDD	S, Sp (128)
		5	5-FT, 32-FT, 123-DG	S, Sp (128)
171-FDD	*catB3*	5	109-DG, 147-FDD, 206-LC	C (2)

S streptomycin; Sp spectinomycin; TMP trimethoprim; RL sulfamethoxazole; C chloramphenicol

7.1.3. Outlooks for the future.

Future transduction experiments will take into consideration the critical experimental points highlighted above. First of all, in order to ensure a transfer of genetic material, transducing particles will be enriched. Much attention will be devoted to the choice of the recipients strains and the antibiotic selection, since previous data (Mauri *et. al.*, submitted) have demonstrated that

streptomycin and sulfamethoxazole, two of the antibiotics used for selecting transductants, induce adaptability in our *Aeromonas* strains, namely the exposure of a sensitive strain to the minimal inhibitory concentration of these antibiotics did not inhibit its growth.

7.2 Acknowledgments

We want to thank Paola Barbieri Prof. of the Department of Theoretical and Applied Science, University of Insubria of Varese for the review of the manuscript, and Anna-Rita Corvaglia, Ph.D of Genomic Research Lab Service of Infectious Diseases of the Geneva University Hospital for her methodological advices.

7.3 References

1. Thierauf A., Perez G. and Maloy S. 2009. Generalized transduction. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Chapter 23:267-268.
2. Balcazar J.L. 2014. "Bacteriophages as Vehicles for Antibiotic Resistance Genes in the Environment." *PLoS Pathogens* 10 (7) (July): e1004219.
3. Thierauf A., Perez G. and Maloy S. 2009. Generalized transduction. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Chapter 23: 274-275.
4. Kenzaka T, Tani K, Sakotani A, Yamaguchi N, Nasu M. High-Frequency Phage-Mediated Gene Transfer among Escherichia coli Cells, Determined at the Single-Cell Level. Appl Environ Microbiol [Internet]. 2007 Mar 23 [cited 2015 Oct 19];73(10):3291–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1907122&tool=pmcentrez&rendertype=abstract
5. Colomer-Lluch, M., Jofre, J., & Muniesa, M. (2014). Quinolone resistance genes (qnrA and qnrS) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes. *Journal of Antimicrobial Chemotherapy*, *69*(5), 1265–1274. http://doi.org/10.1093/jac/dkt528
6. Muniesa M, Imamovic L, Jofre J. Bacteriophages and genetic mobilization in sewage and faecally polluted environments. Microb Biotechnol [Internet]. 2011 Nov [cited 2014 Mar 26];4(6):725–34. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3815409&tool=pmcentrez&rendertype=abstract

7. Thierauf A., Perez G. and Maloy S. 2009. Generalized transduction. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Chapter 23:280-282.
8. Shabram, P. (2000). Multiplicity of Infection/Multiplicity of Confusion. Molecular Therapy, 2(5), 420–421. http://doi.org/10.1006/mthe.2000.0212
9. Zhang, B., Metharom, P., Jullie, H., Ellem, K. A., Cleghorn, G., West, M. J., & Wei, M. Q. (2004). The significance of controlled conditions in lentiviral vector titration and in the use of multiplicity of infection (MOI) for predicting gene transfer events. Genetic Vaccines and Therapy, 2(1), 6. http://doi.org/10.1186/1479-0556-2-6
10. Penadés JR, Chen J, Quiles-Puchalt N, Carpena N, Novick RP. Bacteriophage-mediated spread of bacterial virulence genes. Curr Opin Microbiol [Internet]. 2014;23C:171–8. Available from: http://www.sciencedirect.com/science/article/pii/S1369527414001866
11. Thierauf A., Perez G. and Maloy S. 2009. Generalized transduction. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Chapter 23: 271-274.
12. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Humana Press; 2009. Chapter 7, pp. 69-76.
13. Raya RR and Hébert ME. Isolation of Phage via Induction of Lysogens. In: Clokie MRJ and Kropinski AM, editors. Bacteriophages methods and protocols, volume 1: Isolation, characterization, and Interactions, vol. 501, 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-60327-164-6_3. Chapter 3, p 27.

14. Mazaheri Nezhad Fard R, Barton MD, Heuzenroeder MW. Bacteriophage-mediated transduction of antibiotic resistance in enterococci. Lett Appl Microbiol [Internet]. 2011 Jun [cited 2014 Mar 26];52(6):559–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21395627.

8. Conclusions

Since the end of the last century, antibiotic resistant bacteria and antibiotic resistance genes are considered as worldwide emerging environmental contaminants; microorganisms are developing resistance not only to single antibiotic families but also to several of them (multiresistance). Most resistant organism and their associated genes are dispersed in the aquatic environment that is considered a vast reservoir of antibiotic resistance. Little is known about the influence of environmental antibiotic resistance on natural microbial populations and about its clinical impact. In the environment, antibiotic resistance determinants may become part of the environmental gene pool, may spread horizontally, and may move back to human and animal bacteria via food and drinking water. Our investigations have confirmed that bacteria, also human related pathogens, isolated from different aquatic environment with different degrees of pollution, are affected by selective pressure of antibiotics. *Aeromonas* species that are ubiquitous in aquatic environments and Faecal Coliforms that are allochthonous in natural hydric environments, having their normal habitat in the gut of animals are differently influenced by potential antibiotic contamination of the environment. Although, the genetic elements involved in resistance that we investigated have already been evidenced in both *Aeromonas* and Faecal Coliforms, our results did not allow highlighting common genetic determinants in the two populations and resistant strains are not distributed in the same aquatic environments. Multi-drug resistance profile and plasmids were mostly found in *Aeromonas* sp. isolated from the wastewater treatment plant and from the river receiving its depurated waters; on the other hand the genetic traits involved in antibiotic resistance were mainly evidenced in Faecal Coliforms strains isolated from the hospital waste water and from the activated sludge of the water treatment plant. In this study we used *Aeromonas* spp. multi-resistant strains as donors in mating experiments and sensitive Fecal Coliforms and *Aeromonas* as recipients but we were unable to transfer antibiotic resistances through conjugation.

We decided to study other two mechanisms of horizontal gene transfer (HGT): natural transformation and transduction, in order to explain the high number of antibiotic resistant *Aeromonas* strains found in the environment and their possible role as vectors of antibiotic resistance

genes. Our study on natural competence has pointed out that *Aeromonas* spp. is able to adapt and survive under antibiotics selective pressure especially during starvation of nutrients and when exogenous DNA was put in contact with recipient strains. These results gave to us several insights for future outlooks especially on epigenetics that could affect resistance profile by genes modifications at transcriptional level. Our results suggest that horizontal gene transfer trough transformation do not seems the preferential way of spreading of resistance determinants among bacteria belonging to the genus *Aeromonas.* Possible explanations of our unsuccessful attempts are that we were unable to induce competence in strains, that recipients were unable to incorporate and express the foreign DNA and/or that the transformers had specific donor DNA preferences that we have not fulfilled. On contrary natural transformation (intergenic transformation) could be the mechanism by which *Aeromonas* spp. spreads resistance genes among competent bacteria such as *Acinetobacter.* Finally our preliminary results suggest the possible role of *Aeromonas* bacteriophages in spread of antibiotic resistance because of their ability to acquired antibiotic resistance genes from hosts.

Since the spread of antibiotic resistance is a worldwide problem, monitoring the presence and diffusion of resistance determinants is crucial. *Aeromonas* spp. are considered a reservoir of antibiotic resistance genes and, in our opinion, represent a good model for monitoring antibiotic influences on bacterial populations of aquatic environments.

9. Ringraziamenti

Grazie

Ad Antonella per avermi seguito e sostenuto lungo questo percorso;

Alla Professoressa Paola Barbieri per i suoi preziosi consigli;

A Mauro per avermi dato l’opportunità di far parte di questo meraviglioso gruppo;

Ad AnnaPaola per aver messo a disposizione del mio lavoro la sua esperienza in laboratorio;

A Cristina per la revisione di alcune parti della tesi e i preziosi suggerimenti;

Ad Alison per la sua preziosa manodopera;

A tutto il gruppo del LMA: Flavia, Valeria, Nicola, Francesco, Damiana, Sophie, Samuel, Franziska.

Grazie

Ai miei genitori, Patrizia e Fiorenzo, per aver sempre creduto nelle mie capacità;

A mio marito Alessandro per aver condiviso con me ogni istante anche dal punto di vista scientifico;

A Maria Angela e Maurizio per il loro sostegno;

A Noris per l’amicizia che ci lega e per il conforto nei momenti difficili;

A Erika e Massi per la loro ospitalità e per quella fantastica prima visita a Londra che è stata una tappa importante del lavoro;

A Francesco perché sarebbe stato felice del mio traguardo e perché è sempre nel mio cuore.