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TITLE OF THE DOCTORAL THESIS:

Genetic elements carrying virulence and antibiotic-resistant genes: incidence and exchange mechanisms among microorganisms of the marine community also including allochthonous bacterial species of medical interest.

S.S.D. Med/07

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Rationale & Objective of the thesis

It is known that bacteria can acquire antimicrobial resistance (AR) and virulence genes by horizontal transfer of genetic elements and that antibiotic use selects for existing resistance mechanisms and for novel resistance mutations (Smith *et al*., 2002). Recently, some *Vibrio cholerae* clinical strains containing antibiotic resistance genetic elements previously detected in marine bacteria have been isolated (Pande *et al*., 2012). During previous studies conducted in our lab (Labella *et al*., 2013; Gennari *et al*., 2012; Caburlotto *et al*., 2010), a significant number of environmental bacteria carrying virulence genes and antibiotic resistant and multi-resistant marine bacterial strains were isolated from the coastal area of the Venetian lagoon and from a number of Italian fish farms. Data obtained in these studies support the view that the autochthonous marine micro-flora might constitute a reservoir of virulence and AR genes. Considering that most of those AR and virulence determinants are located in mobile genetic elements (MGE), they can be transferred to other bacteria by horizontal gene transfer (HGT). In particular, it is important to determine if HGT can occur in the marine environment not only among autochthonous bacteria, such as *Vibrio*, *Aeromonas*, *Photobacterium*, but also between members of the marine bacterial population and human pathogenic bacteria of medical interest transitorily present in seawaters.

The first part of the PhD project has focused on the development of a DNA microarray to detect over 200 genes of medical and veterinarian interest in the marine environment. The device would represent an alternative method to PCRs, offering the advantage of interrogating a unique sample for thousands of loci. It would be useful for the screening of collections of marine bacterial strains or for monitoring changes in the bacterial community over time and areas.

The aim of the second part of the study, made in collaboration with the PGBA team at the INRA-Tours Institute, has been to evaluate the possibility for marine bacteria to transfer MGEs to allochtonous bacteria present in the marine environment. In particular, conjugations were set up in different environmental conditions to verify the transfer of antibiotic resistance determinants from environmental *Vibrio* donor strains to members of medical interest of the *Enterobacteriaceae* family, and to better understand the horizontal gene transfer mechanism. An additional objective of the project has been to test the possibility for specific MGEs of mobilizing genetic elements involved in virulence and AR and lacking transfer autonomy.

PART 1:

DEVELOPMENT OF A DNA MICROARRAY FOR THE DETECTION OF ANTIMICROBIAL RESISTANCE, VIRULENCE AND SPECIES-SPECIFIC GENES

Introduction

Reports of diseases associated to marine waters are on rise, both because of climate changes, in particular long-term warming trend, and because of anthropic activities. Human activities have enhanced global transport of marine bacteria species including pathogens and human-facilitated epidemics are common in aquaculture sites. This phenomenon has an economic relevance and can also represent a public health problem. Most of the marine waters-associated diseases occur by host shift and not by the emergence of new microorganisms (Chang *et al*., 2012).

Many of the bacterial fish pathogens such as *Clostridium, Legionella, Pseudomonas* and *Vibrio* are naturally present in estuarine waters and oceans, and can even persist in a dormant but viable state. *Aeromonas hydrophila* is an etiological agent of fish diseases, resulting in hemorrhagic septicemia. *Edwardsiella tarda* causes in channel fish, eels, and flounders the serious systemic septicemia commonly known as edwardsiellosis. *Photobacterium damselae* ì infects a wide range of marine species, leading to pasteurellosis. Outbreaks of disease caused by *Vibrio anguillarum* represent one of the most commonly occurring examples of fish vibriosis; this pathogen usually determines hemorrhagic septicemia and it is worldwide distributed, affecting a wide range of fish and shellfish. These pathogens altogether cause massive fish mortality and large economic losses in fish farming every year (Chang *et al*., 2012).

Bacteria can also infect humans through recreational exposure and/or consumption of contaminated fish and shellfish. Cholera is a good example of how human health threats from the ocean are affected by climate; annual epidemics of *V. cholerae* occurring in Bangladesh have been associated with high sea surface temperatures. Using 26 years of historical data for North India, the main parameters influencing epidemics in the marine environment have been determined, in particular a combination of warm air temperature, followed by heavy rainfall, and appropriate transmission mechanisms, have been showed to trigger for cholera epidemics (Jutla *et al*., 2013).

Moreover because nutrients enter riverine and coastal systems during heavy rain-fall, El Nino Southern Oscillation-related events play a deep role in cholera outbreaks. Those events also promote microbial and toxic contaminant inputs from terrestrial sources thus influencing the emergence of new infections among marine and human organisms (Harvell *et al*., 1999).

It is known that bacteria can acquire antimicrobial resistance (AR) by horizontal transfer of genetic elements and that antibiotic use selects for existing resistance mechanisms and for novel resistance mutations (Smith *et al.*, 2002; Stokes, 2011). It has been

hypothesized that, under the selective pressure of the antibiotics used in fish farming, AR marine bacteria could persist and constitute an environmental reservoir of genes of medical interest (Seyfried *et al.*, 2010). However, the real impact of aquaculture activities in the generation and spread of AR bacteria in the marine environment has not been accurately evaluated in large scale studies.

Most of those epidemics are caused by agents that are difficult to identify; and for this reason there is an urgent need for development of better molecular tools for the identification not only of the bacterial species but also of the genetic pool present in aquatic environments.

The study here presented is part of a project that aimed to estimate the incidence of antibiotic resistance genes in bacteria isolated from four fish farms in northern Italy along the Italian Adriatic Coast. The incidence of antibiotic-resistant strains in fish farms was compared to that found in coastal areas where aquaculture centers are not present, in order to have a baseline rate of AR incidence occurring naturally. In the first part of this study, we found that the AR bacteria global incidence is similar in both marine microenvironments, but a significant higher incidence of antibiotic multi-resistant strains (resistance towards 3-4 of the tested antibiotics) in samples obtained from the studied fish farms if compared with samples from control sea water. In particular, there are significant differences regarding the incidence of multi-resistant bacteria which is higher when fish farms are considered globally (4% versus 10%) and much higher (4% versus 18–28%) when the incidence in coastal environment was compared with some of the aquaculture centers (see Table 1) (Labella *et al*., 2013).

	strains tested	TET (8 µg/ml)	TIM (32 µg/ml)	TIM+SULF (32+60 µg/ml)	FLU (2 µg/ml)	Resistance to 3 or 4 antibiotics	Total (%)
Total Veneto I	*410*	*33 (8%)*	*11 (3%)*	*33 (8%)*	*1 (0.2%)*	*19 (5%)*	*97 24% (avg)*
Total Veneto II	*299*	*63 (21%)*	*1(0.3%)*	*17 (6%)*	*1(0.3%)*	*53 (18%)*	*135 45% (avg)*
Gargano I (July)	119	28 (23%)	5 (4%)	8 (7%)	0	5 (4%)	46 (39%)
Gargano II (July)	44	23 (50%)	0	0	0	12 (28%)	35 (79%)
TOTAL aquaculture STRAINS (%)	**872**	**147 (17%)**	**17 (2%)**	**58 (7%)**	**2 (0.2%)**	**89 (10%)**	**313 (36%)**
Coastal area	**402**	**78 (19%)**	**7 (2%)**	**18 (4%)**	**2 (0.5%)**	**16 (4%)**	**121 (30%)**

Table 1: Incidence of antibiotic-resistant bacterial strains in four fish farms and in a control coastal area (Labella et al., 2013).

From these and other data we deduced that flumequine and oxytetracycline are the most frequently used antibiotics within the Italian context and have been found in the sediments within the fish farms and in the surrounding environments. The flumequine-resistant strains were also resistant to ciprofloxacin, an antibiotic used in clinical settings; cross-resistance between older quinolones and fluoroquinolones has been already reported in veterinary isolates. A high attention should be given to these strains, considering that flumequine has a high environmental impact because it remains active in sediments for prolonged periods of time (half-life up to 60 days) (Labella *et al*., 2013). Differences in AR frequency have been seen to depend also on the sampling periods, with the highest peaks of AR in association with highest temperatures. These results indicate a relationship with optimal temperature and environmental conditions that favour the selection of AR strains by accelerating their growth rate.

On the basis of those results, we concluded that surveillance and frequent controls of both the bacterial species present in these environments, and the virulence/AR genes that they harbour should be planned. In fact the AR bacterial strains isolated constitute an environmental reservoir involved in the seafood chain and they might represent both a risk for human health and a public health concern.

Nevertheless, the analysis of natural samples are not easy to be conducted; traditional methods for the identification of pathogenic bacteria include culture, microscopy, serology and biochemical assays. However they are laborious, especially considering that the cited bacteria are phylogenetically diverse. PCR and molecular biology techniques are better options for bacterial detection in environmental samples, enabling rapid identification of bacteria in many different matrices with a high specificity. Their limiting factor is that they are time-consuming and expensive especially if a large amount of genes is under analysis.

A multiplex PCR approach that can simultaneously identify several pathogens on the basis of the PCR amplicon size using gel electrophoresis has successfully been applied by Chang and colleagues in 2009 to detect fish and shellfish pathogens. However, there are practical limits to this technique: it is not easy to incorporate more than six primer sets because of the cross-reactions, and there are challenges inherent in size discrimination among PCR products by conventional electrophoresis. In addition, sequencing is needed to confirm product identity, and it is a relatively costly and laborious process, also due to the expensive equipment needed.

Microarrays are systems that can manage simultaneous detection of multiple analytes in a single experiment, permitting relatively rapid samples interrogation for the presence of hundreds to thousands of gene targets. They are frequently used to interrogate a mixture

of nucleic acids that is produced from a PCR reaction to increase their sensitivity (Kim *et al.*, 2010). Another hybridization strategy consists in extracting the nucleic acid and applying it to the device. RNA is the preferred target for direct hybridization because of the higher number copy, although it is less stable than DNA (Call, 2005).

Considering the large number of genes that should be analysed and wanting to extend the analysis both geographically and temporally, we have designed and validated an oligonucleotide-based microarray, to detect bacterial genes of medical and veterinarian interest in the marine environment.

Materials & Methods

Bacterial strains and sample preparation: the 43 different strains used to validate the microarray are listed in Table 2. They included a number of bacterial species of fishery and clinical interest (*Vibrio, Escherichia, Enterococcus, Aeromonas* and *Photobacterium)* whose genes are present on the device, and strains of *Pseudomonas, Candida* and *Staphylococcus* used as negative controls; these latter were introduced in the study to validate the specificity of the array's probes. The results obtained with the microarray were compared with those obtained by multiplex PCRs that were set up as a validation tool.

Target bacterial species used to test the microarray
Vibrio cholerae N16961
Vibrio cholerae M010 0139
Vibrio cholerae N16961 O1 El tor
Vibrio parahaemolyticus Vpe17
Vibrio parahaemolyticus QM97097
Vibrio harveyi CECT 525
Vibrio mimicus
Vibrio vulnificus
Vibrio alginolyticus NPV2
Photobacterium damselae subsp. *damselae*
Photobacterium damselae subsp. *piscicida* ATCC 17911
Aeromonas salmonicida subsp. salmonicida
Aeromonas hydrophila
Aeromonas veronii
Escherichia coli PE9i 12
Escherichia coli PE9i 17
Escherichia coli PE9i 19
Escherichia coli FE5E 8
Escherichia coli FE5E 11
Escherichia coli FE7E 1
Escherichia coli PE11i 1
Escherichia coli PE11i 7
Escherichia coli PE9i 27
Escherichia coli ATCC 35150
Escherichia coli CAG18439 SXTMO10
Enterococcus faecalis 25
Enterococcus faecalis A.2.2.16
Enterococcus faecium A.2.2.14
Enterococcus faecium SeH2 tet
Enterococcus faecium SeH2 ery
Enterococcus faecium SeH4
Enterococcus faecium SeH13
Enterococcus casseliflavus A1.1.24
Enterococcus gallinarum S3.1.40
Edwarsiella tarda
Listonella anguillarum
Yersinia ruckeri
Streptococcus pyogenes 7008
Proteus
Salmonella enteritidis CQ8335

other bacterial species used as negative control
Pseudomonas aeruginosa
Candida albicans
Staphylococcus aureus MU 50

Table 2: Bacterial strains used to validate the microarray device.

To validate the microarray with natural environmental samples, water and sediment aliquots were obtained from the Adriatic Sea and added with different bacteria

concentrations, in order to determine the sensitivity of the device. Natural samples were then filtered with 0,22 µm filters, and filters were resuspended in physiological solution (0.98% NaCl); three consecutive washing-centrifugation steps were done, and the DNA extraction procedure was finally applied following the phenol-chloroform protocol (described in the other Materials & Methods section).

Design of the device and probes: For the microarray, 164 target genes were selected (see Table 3), including genes for AR towards many antibiotics (such as quinolones, tetracycline, trimethoprim, eritromycin), mobile genetic elements carrying AR and virulence genes (class I integron, conjugative plasmids, pathogenicity islands), and species-specific 16S rDNA to identify a number of *Vibrio, Aeromonas* and *Photobacterium* species and the faecal contamination indicators *Escherichia coli* and *Enterococcus faecalis.*

GENE FUNCTION	TARGET GENE	Conferred or predicted protein function	ASSOCIATED PROBES	PROBE SEQUENCE
ANTIBIOTIC RESISTANCE (Tetracycline)	*tetA*	tetracycline efflux protein, class A Gram-	tetA P1	CGCCTTTCCTTTGGGTTCTCTATATCGGGCGGATC
			tetA P2	AATTTCCTGACGGGCTGTTTCCTTTTGCCGGAGTC
			tetA P3	CATTCTGCATTCACTCGCCCAGGCAATGATCACCG
	tetB	tetracycline efflux protein, class B Gram-	tetB P1	GCGTTAATGCAGGTTATCTTTGCTCCTTGGCTTGG
			tetB P2	TTAATAGGCGCATCGCTGGATTACTTATTGCTGGC
			tetB P3	GGTGGTTTTGCAGGAGAGATTTCACCGCATAGTCCC
	tetC	tetracycline efflux protein, class C Gram-	tetC P1	CTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACC
			tetC P2	GCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGG
			tetC P3	ATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTT
	tetD	tetracycline efflux protein, class D Gram-	tetD P1	AAGCGGATGTGGCAAACCATTACGGCATTCTGCTG
			tetD P2	TGCTGTGGATGTTGTATCTCGGGCGGATTATCTCC
			tetD P3	CGGCAATACTGAATGCCTGCACCTTTCTGATGGTC
	tetE	tetracycline efflux protein, class E Gram-	tetE P1	CGTTATTACGGGAGTTTGTTGGAAAGGCTAATGTTG
			tetE P2	ACTACGGTGTTTTATTGGCGCTGTATGCAATGATG
			tetE P3	TCGCATAGGTCGTCGCCCTGTATTGTTACTTTCAC
	tetG	tetracycline efflux protein, class G Gram-	tetG P1	CATTATTCAACTGATCGGCCAAGTGCCTGCAGCCC
			tetG P2	AGGCAATGCTCTCAAACAATGTCAGCAGTAACAAG
			tetG P3	TATCGCAGGACCGCTTGGCTTCACAGCACTCTATT
	tetM	Ribosomal protection protein, class M	tetM P1	TTTCGGATGCTTGCTCCTATTGTATTGGAACAAGT
			tetM P2	AGTATGGCTTATACTATAGCCCTGTTAGTACCCCA
	tetK	tetracycline efflux protein, class K Gram+	tetK P1	GCTGTCTTGGTTCATTGATTGCTTTTATTGGTCACA
	tetL	tetracycline efflux protein, class L Gram+	tetL P1	TGGTTTTGAACGTCTCATTACCTGATATTGCAAATG
	tetO	Ribosomal protection protein, class O	tetO P1	AGCCATATCTCCACTTTGAAATTTATGCACCGCAGG
			tetO P2	TCGGCTGCTTTCCCCTATCGTATTGGAGCAGGCTTT
			tetO P3	GGGCTGTATGGATGGAAAGTGACAGACTGTAAAATCTGT
	tetS	tetracycline resistance protein class S	tetS P1	AAAGGAGGAAAACGAAAGAATTCAAAGTTGCTCCT

ANTIBIOTIC RESISTANCE (Fluoroquinolone)	*aac6-lbcr*	class I integron aminoglycoside 6'-N-acetyl transferase type Ib-cr	aac6-lbcr P1	GTGTACATGGTTCAAACACGCCAGGCATTCGAGCG
			aac6-lbcr P2	TTGAGAGGCAAGGTACCGTAACCACCCCATATGGT
			aac6-lbcr P3	GAGCAACTTGCGAGCGATCCGATGCTACGAGAAAG
	gyrA	DNA gyrase subunit A (gyrA) gene	gyrA P1	ACTTCGTTACATGTTGGTCGATGGCCAAGGTAACT
			gyrA P2	TAGGTGACGTAATCGGTAAATATCACCCTCATGGT
			gyrA P3	TGAATGTGCTGGGCAACGACTGGAACAAAGCCTAT
	parC	Type II Topoisomerase IV, subunit A	parC P1	GCGGCGATGCGTTATACCGAAGCGAAACTGTCAAA
			parC P2	CAACGACGTATTATTTACGCGATGTCGGAGCTTGG
			parC P3	ACCGTGCATTGCCTTATATTGGCGATGGTTTAAAA
	parC V. cholerae	Type II Topoisomerase IV, subunit A, *V. cholerae*	parC V. cholerae P1	TCGCCCTTGGTTAGTAATCGCTAAAATCTCATCGT
			parC V. cholerae P2	TTGCTCAGTTGCGGCAGATCCTTAATCGGGAACAG
			parC V. cholerae P3	CATCAAGTTGGAGAGCACTTCCTCGCGGCTGTTGG
	qnrA	pentapeptide repeat proteins that blocks the action of ciprofloxacin	qnrA P1	TGCTGGAGCAGATAGGTCTGGTGGTGTTTCCTTGA
			qnrA P2	CCTGGACGGCGTGCAGATCAATGAAGAGCAGCAAC
			qnrA P3	GATCTGAGTGGCTCCGAGTTTGGCCAGATAGACTG
	qnrA1	pentapeptide repeat proteins that blocks the action of ciprofloxacin	qnrA1 P1	GAGTATTACCGCCGGAATGCCGTTTCTCTGCTGCC
			qnrA1 P2	AAATATCACTTGAGTTCCGCGCTGCAACAAGGCAG
			qnrA1 P3	AATAGTGCTGCCGGATTAGCTCGAATGCAAACACA
	qnrB	pentapeptide repeat proteins that blocks the action of ciprofloxacin	qnrB P1	AGCAAACTTCACACATTGCGATCTGACCAATTCGG
			qnrB P2	GATCTCTCCGGTGGCGAGTTTTCGACTTTCGACTG
			qnrB P3	TGGAAAAGTGTGAGCTATGGGAAAACCGCTGGATG
	qnrD	pentapeptide repeat proteins that blocks the action of ciprofloxacin	qnrD P1	ATTAAAATCACACCCTTCCAAGCTATTCCTGTCGT
			qnrD P2	CTAAACACTCACTAATTTCAAGACCTAAGGCGCTAATG

			qnrD P3	CATATTCATAAAATTAGCCCCTCGAAAATCAGCTCCTT
	qnrS	pentapeptide repeat proteins that blocks the action of ciprofloxacin	qnrS P1	AATCTTTCCTATGCCAATATGGAGAGGGTTTGTTTAG
			qnrS P2	TGAGTAATCGTATGTACTTTTGCTCAGCATTTATTTCTGG
			qnrS P3	AAAAGGTGCCAACTTTTCCCGAACAAACTTTGCCCATCAA
	qnrS2	pentapeptide repeat proteins that blocks the action of ciprofloxacin	qnrS2 P1	ACAAGAACAGCTTCTCGAAGCGTTGGGTATTGTTG
			qnrS2 P2	TCGAAAAGTCGATACATCAGGTATCAAAATTGCCAG
			qnrS2 P3	GGTGCTAATTTATGTCACGCCGAACTCGACGGTTTA
	qnrVC3	pentapeptide repeat proteins that blocks the action of ciprofloxacin	qnrVC3 P1	GGCGCTTCATTTAAAGAATCAGATTTAAGCCGTGGTG
			qnrVC3 P2	ACTGAGAGATTGTGATCTTAAAGGAGCGAATTTTAGTCAA
			qnrVC3 P3	TTAAGCGCTCAAACCTCCGAGATACACAGTTCATTAACTG
	qepA	quinolone efflux pump	qepA P1	CAACTGCTTGAGCCCGTAGATCGTCAGCAGCACCG
ANTIBIOTIC RESISTANCE (Trimethoprim)	*dfrA1*	dihydrofolate reductase (DHFRIa)	dfrA1 P1	TACACTACATATATCTACAATAGACATCGAGCCGGAAGGT
			dfrA1 P2	ACCTAAAGAAAATAACGGATCATGTCATTGTTTCAGGTGG
			dfrA1 P3	ACGTTCAAGTTTTACATCTGACAATGAGAACGTATTGATC
	dfrA5	dihydrofolate reductase (DHFRV)	dfrA5 P1	AGCACATAATTGCTCACAGCCAAACTATCAGGTAA
			dfrA5 P2	CCTCTACGCTCCATATATCGACGATTGATATTGAGCCG
			dfrA5 P3	GTCTGGTGGCGGGGAGATTTACAGAGAAACATTGCCCA
	dfr18	dihydrofolate reductase type VIII	dfr18 P1	TAACGTCCAGAGGCCACATTATCGAAAACGGCAGT
			dfr18 P2	GAGGGCGTATCCTGCAGCGGATACTCACGTAGACG
			dfr18 P3	CTGAAATCTATAATCTGCACAAAGACGTCATTACGAAGG
	dfrA12	dihydrofolate reductase (DHFRXII)	dfrA12 P1	TTCCGTACACCCACTCCGTTTATGCGCGTCGAAAC
			dfrA12 P2	AGAATTCGAGCTTGTCTCAACCGAAACCATTCAAG
			dfrA12 P3	AAACCTTCGAGGGTGACGCCTTCTTCCCAATGCTC
	dfrA15	dihydrofolate reductase	dfrA15 P1	TATTTCAACAATCGACATTGAGCCAGAAGGTGATGTCTA
			dfrA15 P2	ATCTGAAGACGATAACGGATCATGTGATTGTGTCTGGTG
			dfrA15 P3	AGAATGTATTGGTATTTCCATCTATCGATGAAGCGCTAAA

ANTIBIOTIC RESISTANCE (Sulfadiazine)	*sulI*	dihydropteroate synthase	sulI P1	GCGACGCCAGAGACCGAGGGTTAGATCATGCCTAG
			sulI P2	CGCAATCACCTTCTCGGAAACCCTCGCGAAATTTC
			sulI P3	TATTGGTCTCGGTGTCGCGGAAATCCTTCTTGGGC
	sulII	dihydropteroate synthase type II	sulII P1	TTGACGATGCCGAAAATGATGAGCGATTTATTCAT
			sulII P2	GATACGCGCGATTTCTGTGTCGGACGAAACAGGCG
			sulII P3	TAACTGTCGAGCGAGACGGGAATGCCATCTGCCTT
ANTIBIOTIC RESISTANCE (MEP)	*vmrA*	vmrA gene for Na+-coupled multidrug efflux pump	vmrA P1	GTGACGGTCTATTACCTGATTGCGGAAGGTATTGCCAA
			vmrA P2	TCGCTATTGGTACATCAAGCTTCTTTATGTACGCTTA
			vmrA P3	TATGCAGCCTTTGGTGAGCTACTACCATGGTGCTCGAA
	MEP *V. cholerae*	MDR efflux pump (Na(+)/drug antiporter),	MEP V. cholerae P1	GTGATGACGTACAGTTACACCTTGCAGCAAAATAA
			MEP V. cholerae P2	GATTTATCATCGGACTCTCGGCGGCCGCACTGATG
			MEP V. cholerae P3	TAGGGATGACCAACTGGCTGACGGAGCAACCCTTA
ANTIBIOTIC RESISTANCE (Erythromycin)	*ermA*	erythromycin resistance protein class A	ermA P1	TGAACGACATCAACCATTGATTTCAAAGAAGGACTAC
			ermA P2	CTATCTTATCGTTGAGAAGGGATTTGCGAAAAGATTGCAA
			ermA P3	CGGATATTGTCAAAAGAATTACCTTTGAAAGTCAGGCTAA
	ermB	erythromycin resistance protein class B	ermB P1	CGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCT
			ermB P2	CGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATT
	ermC	erythromycin resistance protein class C	ermC P1	AGGTGTAATTTCGTAACTGCCATTGAAATAGACCAT
	mefA/E	macrolide-efflux protein	mefA/E P1	CAGCACTCAATGCGGTTACACCACTTTTAGTACCA
			mefA/E P2	TGATAGTATTGTTTATCCGTAGCATTGGAACAGCTTTTC
			mefA/E P3	GGTGCCGATTTAATTATCGCAGCAGCTGGTGCAGTGC
	msrA/B 1	macrolide-efflux protein (msrA) gene	msrA/B P1	GTGCAAATGGCATACTATCGTCAACTTGCTTATGA
			msrA/B P2	TGAAGCTATTTACCACCAAATAGAGGGAATTGATTGTTC
	msrA/B 2	macrolide-efflux protein (msrA) gene	msr A/B 2 P1	ACTGGGACTTACTATACTGACAAAGCAGATTTAGCTATT
			msr A/B 2 P2	GTCTTAATAAGCAAGGAAATGATCGCGGTAGTCAATACC

			msr A/B 2 P3	TGATGCGAACCGTATTAGCTTGAAAGAATTATTACTGCA
	msrC	macrolide resistance-like protein	msrC P1	AGGAAACTCTGACGAAACCGTTGTGTACGTTAAGT
ANTIBIOTIC RESISTANCE (Ampicillin)	*blaZ*	beta-lactamase	blaZ P1	AGTGAAACCGCCAAGAGTGTAATGAAGGAATTTTAA
			blaZ P2	ATCCTAAGGGCCAATCTGAACCTATTGTTTTAGTCA
ANTIBIOTIC RESISTANCE (Vancomycin)	*van A*	VanA ligase (vanA) gene	vanA P1	TGATAGGCCGGTGGCAGCTACGTTTACCTATCCTG
			vanA P2	GCTGGGATAGCTACTCCCGCCTTTTGGGTTATTAA
			vanA P3	ATGTAGCATTTTCAGCTTTGCATGGCAAGTCAGGT
	vanB	VanA ligase (vanB) gene	vanB P1	GGTGCTTGGATGCAGAGGGCTTGCTCGTGTTGATC
			vanB P2	CGGTATCTTCCGCATCCATCAGGAAAACGAGCCGG
			vanB P3	GCTTGCATGGACAAATCACTGGCCTACATTCTTAC
	vanC1	vancomycin resistance protein C1 (vanC1)	vanC1 P1	ATTGGTGCTTGTGATGCGATTTCTCTTGTCGACGG
			vanC1 P2	GACAAAACAGCGCTCCAATCTGCATTAACGACTGC
			vanC1 P3	AAATGGCTCTTGCATCAACTTGCTGATACCATGGG
	vanC2/3	vancomycin resistance protein C2 (vanC2/3)	vanC2/3 P1	AGAAGCCTTTACTTATTGTTCCGCAGTGCTCCTAC
			vanC2/3 P2	ACTAAAGTCACCTGCGTTGAAGAAATCGCTTCTGC
ANTIBIOTIC RESISTANCE (Lincomycin)	*linB*	lincosamide nucleotidyl transferase enzyme	linB P1	ACCACTGCTCGATTAGATAAGGTAGAATTATTTGAAGCC
			linB P3	GTGGTGCAAGACCAAATAGACTTACTGAAGAAAATGCTAA
			linB P2	ATCCCCTCGTTTAAAGATTCAGGTTATATTCCTGATACGA
ANTIBIOTIC RESISTANCE (Synercid)	*vgb*	virginiamycin B hydrolase	vgb P1	GGTGCTGAATCTGGGTTAGGCAATGTATATTCCTTAATAA
			vgb P2	GTAAACCATATATCTCCATTTGGTCCTTCTGTAATACCGT
			vgb P3	CGTCGTCCGTAATACGTCCAATACGGTTGCCATTCATTTC
	vatD(satA)	streptogramin A acetyltransferase	vatD(satA) P1	TGTATGGATAGGAAAAGATGTTGTAATTATGCCAGGAGT
			vatD(satA) P2	GGATGGGAGAAACATATGCCAAAATTAGATCAACTACCTA
			vatD(satA) P3	AATGGAGCAAATCATAGAATGGATGGCTCAACATATCCAT
	vatE(satG)	streptogramin A acetyltransferase	vatE(satG) P1	GTCGGTGGCAATCCAATTCAACTCATCGGACCAAG

			vatE(satG) P2	TCGGAAATGACGTGTGGTTTGGGCAAAATGTGACC
			vatE(satG) P3	TCCTGAACTGACTGATTTGCCGTTGAAAGGTGATA
ANTIBIOTIC RESISTANCE (Streptomycin)	*strA*	aminoglycoside phosphotransferase	strA P1	ACTTCTTACCGGACGAGGACAAGAGTACGCCGCAG
			strA P2	CTATCGGTTGATCAATGTCCGTTTGAGCGCAGGCT
			strA P3	CAGGAGGGTGCATGCTTGGTGATAACGGCAATTCC
	strB	aminoglycoside phosphotransferase	strB P1	CGCCAATATGTTCTACGATCCGGCTGACAGAGACG
			strB P2	GTGATAGATCCCGTCGGTCTGGTCGGTGAAGTGGG
			strB P3	GATGAGCAATGCCTCGGAACTGCGTGGGCTACATG
ANTIBIOTIC RESISTANCE (Chloramphenicol)	*cat*	chloramphenicol acetyl transferase	cat P1	TTCATCATGCCGTTTGTGATGGCTTCCATGTCGGC
			cat P2	TTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGC
			cat P3	TGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGT
	cmlA	chloramphenicol transporter	cmlA P1	TGTGTTACGCCGCTGGAATGGGTAGCTTCTTCGTC
			cmlA P2	GCTACTCCCCGTTAAGTGCCTGAACTTCTGGTTGT
			cmlA P3	CGTTTCTAGGTTTGGGCATGATCGCTGCATCTGCA
MOBILE GENETIC ELEMENTS (Class I Integron)	*qacEdelta1*	quaternary ammonium compound-resistance protein	qacEdelta1 P1	CCTTCCGCCGTTGTCATAATCGGTTATGGCATCGC
			qacEdelta1 P2	AATCGCAACATCCGCATTAAAATCTAGCGAGGGCT
	sulI	dihydropteroate synthase	sulI P1	GCGACGCCAGAGACCGAGGGTTAGATCATGCCTAG
			sulI P2	CGCAATCACCTTCTCGGAAACCCTCGCGAAATTTC
			sulI P3	TATTGGTCTCGGTGTCGCGGAAATCCTTCTTGGGC
	aadA2	aminoglycoside adenyltransferase	aadA2 P1	TGTCTAACAATTCGTTCAAGCCGACCGCGCTACGC
			aadA2 P2	GCTTTGTGAAAGGCGAGATCATCAAGTCAGTTGGT
			aadA2 P3	TCACTTGGCCTCACGCGCAGATCACTTGGAAGAAT
MOBILE GENETIC ELEMENTS (ICE/SXT-R391)	oriTsxt	origin of transfer	oriTsxt P1	TTTCGATCCAAAAGCCAAACGGATAGTGGTTTTGG
			oriTsxt P2	TCGAGACGCCAAACGATCGTTTGCATTCTGGGTTT
	intSXT *V. cholerae*	transposon SXT integrase gene	intSXT V. cholerae P1	CACTATCCTAACAATGTGAAAATCTGGCTCGCAGA

			intSXT V. cholerae P2	AACAAGGTGAGAAAATTGGAGCGCCGATCATGCGG
			intSXT V. cholerae P3	TAAAACTGCTTCTCTTATTGGGAGGGCGCAAGGGC
	transposon sxt like	transposon	tpn sxt like P1	GATTTTCTCAATGAAATCAATAAGCGAAGGACCTTTGC
			tpn sxt like P2	GAGTACTTCCAAAAGTGTGTACAAATCCGTGTACAAAC
			tpn sxt like P3	CGATTAACACGACGGATTTGACAAGCGAAGAACTGAAA
	traI V. cholerae	conjugative relaxase	traI V. cholerae P1	CAATGAGCATGGTATTCCAGCCGGACAGCTGCTTC
			traI V. cholerae P2	GATGGTATTCGCGTCACGATTCAAACCTTACGCCA
			traI V. cholerae P3	TACTGACCGATGGCGATTACCCAGACGTGGATCAT
	traI V. harveyi	conjugative relaxase	traI V. harveyi P1	CTTTGCTACCTCGAAGCAGGTGCAAACTTTTCTCG
			traI V. harveyi P2	ATCTTGCAAAACATCGATGATGGCAAAGGCAAACA
			traI V. harveyi P3	AATACAGTGATGGAACACGCTGGACGACACAAGCG
	traI V. vulnificus	conjugative relaxase	traI V. vulnificus P1	ACTTCAATGGACGAGACATTCAATTCATGTCAGATGATAA
			traI V. vulnificus P2	TCACGACTTAAACCCAAATAACATTGAGAAAATCATCGAG
			traI V. vulnificus P3	GAACTGAGACAGAACATCATTATTGTACTAAACCAAGGCA
	prfC	peptide chain release factor 3	prfC P1	AAGATACTTATCGCACTCTGACCGCAGTTGACTCT
			prfC P2	GGTTAATCTTTTGGATACTCCGGGACACGAAGACT
			prfC P3	GTGACCACCTCTGTTATGCAATTCCCGTACGGCGA
	xis V. cholerae	recombination directionality factor Xis	xis V. cholerae P1	AAGATTTGGAGGAGTATGAGAAGAAAGAGGACTGA
			xis V. cholerae P2	TCGTATCAACCGAAATACACGAAACAAGCTGTGGC
			xis V. cholerae P3	GGCTCGTTACCACATTAGCTATACGACGCTCTGGC
MOBILE GENETIC ELEMENTS (R-Plasmids)	*repA* pMRV150	conjugative plasmid	repA pMRV150 P1	AGGCCCTCATCAAACAAGAGGTCATAAGCGACTAC
			repA pMRV150 P2	CTGGACTCAAGCATCACCAGATTCGCCGTACACGC

			repA pMRV150 P3	TCCCCTCTTGCACGGTACATCTACAAGCGAATGAG
	repA pAb5s9	conjugative plasmid	repA pAb5s9 P1	GGCTTCTTCCGCCCGGTCGAATAGTGATAGCTGTT
			repA pAb5s9 P2	GTGGGCATTTCAATCAAGCGGTGTGATGACTGCCG
			repA pAb5s9 P3	ATAAATTCGGCTTGTGTTAGCCAGGTGTCACGGCT
CLINICAL SIGNIFICANCE PATHOGENS (*Enterococcus* spp)	*ddl E. faecalis*	cytoplasmic enzyme D-alanyl-D-alanine ligase	ddl E. faecalis P1	GGAACAATTCAAGGATTCATGGAAACCATTAATATGCCTT
			ddl E. faecalis P2	AAGTACAGTTAGTCTTTATTAGTAAAGACGGTCAATGGGT
			ddl E. faecalis P3	GTGTCTGTTTTGTCTGCATATTCCGTTTTAAATGCAATCT
	ddl E. faecium	cytoplasmic enzyme D-alanyl-D-alanine ligase	ddl E. faecium P1	CGCGAAATCGAAGTTGCTGTATTAGGAAATGAAGA
	esp	biofilm formation gene	esp P1	TTCTGGTTTATCAAAACCTGGAGAAACGATTTGGA
			esp P2	TATCAACCGCTTTTGGTGATTCCTTAATAACGGTT
	esp2	biofilm formation gene	esp2 P1	CCGTGGTAGTTGGATTTAAACCAGATGCTAAAGAATC
	gelE	biofilm formation gene	gelE P1	ACTGCCGGTTTAGAATATTTAGGACAATCAGGTGC
	sprE	serine protease gene	sprE P1	CCAGGCTATATTTCTTTAGGAACAGGCTTTGTTGTTG
			sprE P2	CTTTTGCGTCAATCGGAAGAATCATTTCCCCTGCCAG
	aceVF	adhesin to collagen	aceVF P1	TTTCACTTGCCGAGTTTGAGCAACAAGGTTATGGC
			aceVF P2	GGATCGACAAGGAAGTGGTCAACAATTAAATAAAGAGAG
CLINICAL SIGNIFICANCE PATHOGENS (*Escherichia coli*)	*stx1B*	shiga-like toxin 1 subunit B	stx1B P1	GGGATGACTGTAACCATTAAAACTAATGCCTGTCAT
			stx1B P2	TGATGACGATACCTTTACAGTTAAAGTGGGTGATAAAGA
			stx1B P3	GCCTGATTGTGTAACTGGAAAGGTGGAGTATACAAAATAT
	stx2B	shiga-like toxin 2 subunit B	stx2B P1	CCGGATTTGCTGAAGTGCAGTTTAATAATGACTGA
			stx2B P2	AATGACTGTCACAATCAAATCCAGTACCTGTGAAT
	hlyA	cytotoxic haemolysin	hlyA P1	TACCGTTGATTCATTTTCTGAGCAGCTTAACCAGC
	sfa	S fimbrial adhesins	sfa P1	GCTGTCTTTTAAGACCAGAAGAGGTTAACAGTGAAT
			sfa P2	GGCTTATAACATTTCTTACCTGACCAGGGTACCGG
	afa	proteins involved in adhesion to epithelial cells	afa P1	GCGCTGAGGGATTATCTGGTAATGGGATATAACCG

			afa P2	TGGCTGAGATATCGCCGGTACACAGTGAGAAGGTT
			afa P3	GAGCGATATCTGTATCTTGCTGACACCCCTCAGGG
	papG1	chaperon protein	papG1 P1	CGATGTGCCTGCAAATATTCGTTTTATGCTGTTAAG
			papG1 P2	GCTAATAATCATTATGCGGCTCAGACTCTTTCTGTGT
			papG1 P3	ACAGTCTCTGGAAATAAAGCATGGTGATCTGTCTATTAAT
	papG2	chaperon protein	papG2 P1	CTTTCTGTGTCTTGCGATGTGCCTACAAATATTCG
			papG2 P2	TGTCGATTAATAGCGCTAATAATCATTATGCGGCTCAG
			papG2 P3	CCGTCCTTCTGCACAGTCTCTGGAAATAAATCATGGTGA
	eaeA	outer membrane protein called intimin	eaeA P1	TACGAATAAAATGACTAAAATGTCCCCGGACGCGA
			eaeA P2	TACAGTTCCGAAAGCGAAATGATGAAGGCTGGACCT
			eaeA P3	ACAGGGTATCAGTTTATCGGTAATTTGGTCACTGAATAA
	ibeA	virulence factor	ibeA P1	CGCCGCGTACTACACATTACCGCCGTTGATGTTAT
			ibeA P2	TCAAACTGGTTGCGGATGCAATGCTTGAACAGGCA
			ibeA P3	CTGGCACGCGAAATAGAAGAAACGGCAAAATCAAT
	fyuA	yersiniabactin receptor	fyuA P1	CCGTTTGGCGACCAGGGTAAGAGCAATGACGATCA
			fyuA P2	TGATAAATCCAGTACACAATATCACGGCAGCATGC
			fyuA P3	TAAGCAGTACCGGCTATACCACCGCTGAAACGCTG
	iutA1	ferric aerobactin receptor	iutA1 P1	TACACCACCGGGCGCTATCCGTCGTATGACATCAC
			iutA1 P2	CATGAAACTGACTCTGAACAGCAAACCGATGGACG
			iutA1 P3	GTCGGTCAGGTTTACTACCGCGATGAGTCGTTGCG
	iutA2	ferric aerobactin receptor	iutA2 P1	TATCGACCATATTGAAGTGATCTCCGGCGCGACGG
			iutA2 P2	CTTCACGTTCCGACAGCCGACAACTGGACTCTGTC
	iucA	aerobactin iron transport system	iucA P1	GCGGTGATTCGCTGCTGGTTTCGGCGGTAAAACGC
	traT	outer membrane protein	traT P1	GGGCTGCAGGGCAAAATTGCTGATGCTGTGAAAGC
			traT P2	ATGAGCACAGCAATCAAGAAGCGTAACCTTGAGGT
			traT P3	GTTGCACTGGTCAGTTCCACTCTGGCCCTTTCAGG
	orfI tn1721		orfI Tn1721	GTCAGGATTAACGTACGTGATATGGCTTTGAGGGG
			orfI Tn1721	GGCTGGCCTAATAGTTCTTCCTCAGTGAAACCACTG

			orfI Tn1721	ATGCAGCAGGCGGCATATCTGGGTGTCTTACGATG
	chuA	outer membrane heme/hemoglobin receptor	chuA P1	GATTGGTCGCTTCTATACCAACTATTGGGTGCCAAA
			chuA P2	ACTTACGTCCGGAAACTAACGAAACTCAGGAGTACG
			chuA P3	GGGCTGCGTTTTGATGACCTGATGTTGTCCAATGAT
	yjaA	stress-induced protein	yjaA P1	GTTCTGCAACTCCACCCACGTTCAACCTGGCATTC
			yjaA P2	CCAGCGCCTGTTAATCGCCAATTTCTTTGTTGCAG
	tspE4.C2	phylogenetic marker	tspE4.C2 P1	CCGACGATAATTTATTACTACGGTGGCTGTTTTGTT
			tspE4.C2 P2	ATGACCTTACGAATAGTGTCGCCGCTGAATGCCCC
			tspE4.C2 P3	CAGAAACGCGGGTAGATATTCAGACTATCGAACTT
CLINICAL SIGNIFICANCE PATHOGENS (*Vibrio cholerae*)	16-23Sintergenicseq	intergenic species-specific sequence	16-23S Vc P1	AAAATCTGTCTCGCACTCATGTAAATTAAACGCGA
			16-23S Vc P2	ACATTCAAGTGTGCTTGGTATCGAATAAGACTTCGGTCT
	ctxB_V. cholerae	cholera toxin	ctxB Vc P1	TCGAAAAGTTATGTGTATGGAATAATAAAACGCCTCATGC
			ctxB Vc P2	GAAGGATACCCTGAGGATTGCATATCTTACTGAAGCTAAA
			ctxB Vc P3	TGCAACTTTTCAAGTAGAAGTACCAGGTAGTCAACATATA
	gbpa_V.cholerae	colonization factor	gbpa Vc P1	ATGGCGAGCTGAAATCCATCACGCTAGAGCTGAGC
			gbpa Vc P2	CAAATTGAAGCGCCACAGCCTGAGTATTCACTGAC
			gbpa Vc P3	TCCGTTCTGTGTCGTTGAAGGAAATATGGTGCAGC
	wbfO_V.cholerae	required for the biosynthesis of the somatic O139 antigen	wbfO Vc P1	TGAAAGTAGCCAATTTGATTCTTCTGCTATAGAAAGGCTT
			wbfO Vc P2	GCGTTATAGGTATCATCAAGAGAGTATATCACGTGTAGCT
			wbfO Vc P3	TAGGTTATAAAGATGCCGAAGACTATAAATTTTGGGTCGA
	ace_V.cholerae	accessory cholera enterotoxin	ace Vc P1	TGATTGATATGTTTACCATCTATCCGCTTATCCAACAGG
			ace Vc P2	TTGTGATCAAGCTCGGTATTATGTGGATTGAGAGCAAGA
			ace Vc P3	GGACACCCTTTATGACTGGCTAATTGATGGCTTTACGTG
	ctxA_V.cholerae	cholera toxin	ctxA Vc P1	AGATGGTTATGGATTGGCAGGTTTCCCTCCGGAGC
			ctxA Vc P2	TGATGAACAATTACATCGTAATAGGGGCTACAGAGATAGA
			ctxA Vc P3	CACTTAGTGGGTCAAACTATATTGTCTGGTCATTCTACTT
	hap_V.cholerae	Haemagglutinin Protease	hap Vc P1	GATGGCTATACCCGTTTCTATCCTTTGGTGGATAT

			hap Vc P2	ATAACCAACAGGCTCAGTTAGTGTACTTGGTCGAC
			hap Vc P3	ATCTCCCAACCGTGACCCCTGACATTGAAAGCCAG
	ompW_V.cholerae	outer-membrane protein W	ompW Vc P1	TCCTCAACGCTTCTGTGTGGTATGCCAATATTGAA
			ompW Vc P2	CCACCTACCTTTATGGTCCAATACTACTTTGGTGAAG
	ompU_V.cholerae	outer-membrane protein U	ompU Vc P1	GCCAAGTACTCTGACAACGGTGAAGATGGTTACTCTC
			ompU Vc P2	CTGTTGACGCAATGGGTAATGTTGTAACTGAAACAAA
	flrA_V.cholerae	activator of downstream flagellar gene expression	flrA Vc P1	CAGGAGTTTGTCCCTGATATAGATGCGCCGCAAGC
			flrA Vc P2	TTAAACGTGTTCCCGATTGAAATGCCCGCTCTGCG
			flrA Vc P3	AGATGATTGACGGACAGAAATTTCGTGAAGATCTCTAC
	rstR_V.cholerae	gene of the filamentous bacteriophage CTXphi	rstR Vc P1	GCGGAAGATGGGCCACAGATGAAAATCAAGAGCTTATGCT
			rstR Vc P2	ACAAATGGCTGATGAAATTGGAATTAGTCTAACATCGTAC
			rstR Vc P3	AGGCTAGCCAACCAAAGAAAGGCAATTAATAAGACTCAGG
	tcpI_V.cholerae	toxin-coregulated-pilus	tcpI Vc P1	GTGTCGCTTTAGATATTGCAGGTGATCAGATTGCCTCACC
			tcpI Vc P2	CAACACGTTCTCACTATTGCCACCCCTGTTTATGTAGGCA
			tcpI Vc P3	CGAACTCGGTACTAAACATTACACAGCTTTTGATATCGAT
	zot_V.cholerae	zonula occludens toxin	zot Vc P1	ACATGATAAGAGAGGCGGCGGAGATAGGGTATCGC
			zot Vc P2	GGATATCTGCCTAACCACGCCTAACATTGCCAAAG
	wbfR_V.cholerae	species-specific gene	wbfR Vc P1	GTGGCTTTTACGAGAAGTACTGTATCGTTATGTACCT
			wbfR Vc P2	GGGTTCCCTTGTTAGACCACCGCATTGCTGAGTTTGC
			wbfR Vc P3	ACGGGTTGTCTCCAACTGGGATCTAGATGAGCCTTTGGTT
	epsM_V.cholerae	protein of the general secretion apparatus	epsM Vc P1	CCTGATTGAGTGGTTGATCGCTTGGCGCATCACTG
			epsM Vc P2	GCTCTTGCAAATACGCAATCCATGAGACCAATTGC
			epsM Vc P3	TTCAGTTGCAGACGTTTGACTTCCACAACGCCGTT
	mshA_V.cholerae	mannose-sensitive hemagglutinin	mshA Vc P1	GTCACAGCAGCGCCACGTTTCTTAAACCTGCAAGG
	luxO_V.cholerae	two-component response regulator involved in quorum sensing	luxO Vc P1	TGCTCAATGAAGGTCGTGAAATCACCCTAGATATG
			luxO Vc P2	CAAACTTCTGCGCTTTATTCAGACAGGGACATTCC

	rfbN_V.cholerae	required for the biosynthesis of the somatic O1 antigen	rfbN Vc P1	AGCAAAAGTTTGTTGAACATCTGAATTCACTTGCGAGTGA
			rfbN Vc P2	TTGCGGCGGTAAAAGGTTACCTAAACTTCGCCTCAACATC
			rfbN Vc P3	GAGTTATGCAGGAGGTGTTGGGGCCTAAACGTCGC
	stn-sto_V.cholerae	heat-stable enterotoxins	stn-sto Vc P1	TGCTGCGAAATATGTTGCAATCCAGCTTGCTTTGG
	tcpA_V.cholerae	subunit of the toxin-coregulated pilus	tcpA Vc P1	GGTATGACACTACTCGAAGTGATCATTGTTCTAGGTATT
	toxR_V.cholerae	control of the expression of outer membrane protein	tox R Vc P1	ATTGTTGGGCAAGAGCATGGTTACACAAGCTATGAGCCTA
			tox R Vc P2	TCATCATCCATATGTTGCAAGAATTATCCAAGATTGGCTT
			tox R Vc P3	GATTGGAAACGTTCACCACAATCAAATATAAGGCCAGATA
	hlyA_V.cholerae	cytotoxic haemolysin	hlyA Vc P1	AATGGTGGTAGCTTGAGTTCGGTGGATCTGTCCGG
			hlyA Vc P2	TTGATGCATTAGGGTCCGCCTCCGATTTATTGGTT
			hlyA Vc P3	GTATGCGAGCAGCAACGATGCGGTAAGTTTACGTA
	nanH_V.cholerae	neuraminidase	nanH Vc P1	GAAGGGGTGACATGGAAAGGACCAATTCAACTTGTTAA
			nanH Vc P2	TTTCTTACTCTTTACTAACCCACAAGGAAACCCTGCGG
			nanH Vc P3	CTCCAAAACGGTGATCTACTCCTTACTGCACGCCTTGA
	otnB_V.cholerae	required for the biosynthesis of the somatic O139 antigen	otnB Vc P1	AACCAATGTGGCCGATATTCGCACTGTTTTGTATAAA
			otnB Vc P2	TTCGATGCAAGAAGCGTATAAAGTGTTTAGCAAGC
			otnB Vc P3	ACTTGGGTGCGTGAAGCCAAAGCGCCATTTAAACC
	tcpB_V.cholerae	toxin co-regulated pilus biosynthesis protein B	tcpB Vc P1	TCGATTGGTATCGAGTCCGGTATATTGCCTACTTCAGGT
			tcpB Vc P2	TCAGAGAGTGCTAAGGATTCTCAAGGCACAACCCAAAAG
			tcpB Vc P3	ACCATTAACGCTATTGAATGCCCAACAGGTTTAAATA
	GroEL_V.cholerae	species-specific gene	GroEL Vc P1	CTAACTCAGACTCTAGCGTGGGCAACATCATTGCT
			GroEL Vc P2	TGCCGATACTAAAGCGATTGCCCAAGTAGGTACCA
			GroEL Vc P3	AAAAGTGACCTTAGGTCCTAAAGGCCGTAACGTAGT
	vpsR_V.cholerae	Vibrio polysaccharide regulator	vpsr Vc P1	CGCTTACTGAATAAACACAACTTGATCACCGATGAA
			vpsr Vc P2	TCAAGCGTGTGGTATTAATGTCAGATACTGTGGTG
			vpsr Vc P3	TTCTTACAAGAAGGGACTGTGGAGACGCGCCAAGG

	rtxA_V.cholerae	multifunctional autoprocessing RTX (MARTX) toxin	rtxA P1	GACATGCTGCCATTCTATGCACTTCGTACCGAACG
			rtxA Vc P2	AGCAAGAAAACGTCGCTTCCATAATTAACACCATG
			rtxA Vc P3	ACACGCTTTAGGTGAAACGTCGAATCTAGAGAATA
	otnA_V.cholerae	capsular biosynthesis gene	otnA Vc P1	AGAGGTGCAGGTGCCAGTGACTTATCTGCTCGATA
			otnA Vc P2	TAGGGCGCATGGTGGTACAGCTTAGCCGCATTATG
			otnA Vc P3	GACACCAATCTGAGCTCAGTGATTTCTGATCCAGC
CLINICAL SIGNIFICANCE PATHOGENS (*Vibrio vulnificus*)	*GroEL*	species-specific gene	GroEL Vv P1	TTGCTTCCAGTGTTGGAGTCGGTAGCGAAAGCCTC
			GroEL Vv P2	ATATCTTGTTGGTGGATAAGAAAGTCAGCAGCATT
			GroEL Vv P3	CACCAACCAAGATTCTGGTGCGGTCGAGCTCGATA
	rtxA1	multifunctional autoprocessing RTX (MARTX) toxin	rtxA1 Vv P1	TATTGTTGGCTGCTCTTTGGTGAGTGACGACAAGC
			rtxA1 Vv P2	AATCAAGCGGAAAATGTCAGCAGCAAGCCTGATCA
			rtxA1 Vv P3	CGTTTAAGTGGCTACAGTGCCGATGAGCTGGCAGT
CLINICAL SIGNIFICANCE PATHOGENS (*Vibrio mimicus*)	*gyrB*	gyrase B	gyrB Vm P1	AGAAGTCGCGACCCTGATCACTGCATTAGGCTGTG
			gyrB Vm P2	CGCGCTAGACTTAGCTGGTTTGCCAGGCAAATTGG
			gyrB Vm P3	TACCCACTTGAACCGTAACAAAACGCCGATCCATG
	hlyA	cytotoxic haemolysin	hlyA Vm P1	GTCAGTGGAGATGCCCCGAAAGCTAAACTCGAGGC
			hlyA Vm P2	GGAGTGGGTTTTGCTAATCAATTTATTGTCATCACTGA
	vmhA	hemolysin	vmhA Vm P1	TGACCTGAACTAAAATGTGATTAATGCCCTGATAATTCAG
			vmhA Vm P2	ACGTACCGACAGGAGCCAATATTACCCAAATGAAAGTTTG
			vmhA Vm P3	TCAACAAATGGTCGGTTCGAAAGAGCACATCAGCAATGCT
CLINICAL SIGNIFICANCE PATHOGENS (*Vibrio parahaemolyticus*)	*groEL*	species-specific gene	groEL Vp P1	CTGCTAATCATCGCAGAAGACGTAGAAGGCGAAGC
			groEL Vp P2	TTCTACCAACTCTAGAAGCAGTAGCAAAAGCATCT
			groEL Vp P3	AGCTAGAAAACCCATTCATCCTTCTAGTTGATAAGAAGAT
	tdh	Thermostable direct hemolysin	tdh Vp P1	GCTAACTTTGTTGGTGAAGATGAAGGTTCTATTCCAAGTA

			tdh Vp P2	GTGTTCGTAAAATCAGATCAAGTACAACTTCAACATTCCT
			tdh Vp P3	CAATGGCAGCGGTGTCTGGCTATAAGAGCGGTCATTCTG
	tlh	species specific thermolabile hemolysin	tlh Vp P1	AAAGTAGCAACAACTTAGCCGAGTACCGTTTCTAA
			tlh Vp P2	CACGCACCCAACAACAGCAACTCACCGCTATGTTG
			tlh Vp P3	TGTGCGGCGTCTGGTGCTGAGAAGTTTGTGTTCTG
	trh	thermostable direct hemolysin-related hemolysin	trh Vp P1	GAATGAAGAATCATTGCCAAGTGTAACGTATTTGGATGA
			trh Vp P2	CATTCGCGATTGACCTGCCATCCATACCTTTTCCTTCTC
	gbpA	N-acetyl glucosamine-binding protein A	gbpA Vp P1	GTGAGAGTGCAACACAATTTGAGCCAGCGACAGGC
			gbpA Vp P2	ATGTAAACCATTCCCTTACTCTGGCTACTGCGTAC
			gbpA Vp P3	CACAGCAGGTACAAAAGTGTTAGCAAGCGACGGCG
	orf8	genetic marker for identifying strains	orf8 Vp P1	TGAAGTAACCTCCGATATGTTAGATGGCTATTCTGAAAT
			orf8 Vp P2	ACTCAAACTGTAGATAAACGTTTGAAACAAGAAGCTTACG
			orf8 Vp P3	TTCATATAGAAAACAAAGAGCGCTTCAAAGTGGGGATTAC
	5'VpPAI	5' end of *Vibrio parahaemolyticus* pathogenicity island	5'VpPAI P1	GGGTGAAAACAGGACATTTGCCGCTCGGAAGTTGA
			5'VpPAI P2	CACATGAGTGAGCACTTCCCTTTTGTAGAAGAAGA
			5'VpPAI P3	AGCTAGGTATATACCCAACACATACTTGGTTGCGA
	3'VpPAI	3' end of *Vibrio parahaemolyticus* pathogenicity island	3'VpPAI P1	GGTGAAGAGATGGAAGCGCACGTTAAATATCTTAAGTA
			3'VpPAI P2	AGCAATCGGCTATTCGCTTAATGAATATGCGTAAAGAA
			3'VpPAI P3	ACCGAAGTGGATTCCTGAATCTGATGAAGACAAGGCTC
	toxR	activator of numerous genes involved in virulence	toxR Vp P1	CACTCGTATGAGAACGTGACATTGCGTATTTTCGC
			toxR Vp P2	GGACAAAATAACCAGCTGATTTTGAACTACATTCATGACA
			toxR Vp P3	GCCTTCTATTGAGCAGTGCATTGAACGCTACGTTA
	toxRS	activator of numerous genes involved in virulence	toxRS Vp P1	CTAGATGCTGAACAAAGCCGCCGAATTGACGTGGT
			toxRS Vp P2	TGATAACTATCTGCTTGTTTCTCCTTCTGAGTTCAAAGA

			toxRS Vp P3	TCGACAATTAATATTTCAGAGAAAGGTCGCTGGGAAGTG
	yopP	virulence protein	yopP Vp P1	CGTCCATCAAATCAATATAAGAAGAAAGCTGTTCACGA
			yopP Vp P2	AGCGGTATCTTTAGGTAATTGTTTTCCTTGTCGAATTG
			yopP Vp P3	CGACAAATCTGGCGGTTGTATCCTCTGATGGTATTAAAC
	Mtase	methyltransferase	Mtase Vp P1	GGATTGAAGGTGTAGAAGTCGAAGTTCGCTTTTAA
			Mtase Vp P2	TTTGAAGGTGACGCTGAGTTTTGATGAAGCTCTAG
			Mtase Vp P3	CCTTCTGAACTGCAAAGCCCATTCGAAGGTCAACC
CLINICAL SIGNIFICANCE PATHOGENS (*Vibrio alginolyticus*)	16S	species-specific gene	16S Va P1	ACTCTCCGTAAGTCGTAACACAGGTAGCACGTAGA
			16S Va P2	ATATTTCGTAGCGGGGTCGGGGTAGGTTTACGGCG
	rpoB	RNA polymerase beta subunit	rpoB Va P1	TACCGTCGCGTTGTAGATGGCGTAGTAACAGACGA
			rpoB Va P2	CTCAAGACTTGATCAACGCGAAGCCAATTTCTGCG
			rpoB Va P3	GAAGCTGATCGCGATCCGTAACGGTAAGGGCGAAG
	pyrH	virulence protein	pyrH Va P1	GCGCGCGTTATGTCTGCCATTCCTCTAAAAGGCGTATG
			pyrH Va P2	GGAAGTTCTGGATAAAGAGCTGAAAGTTATGGACCT
			pyrH Va P3	TCTGCTGCTTGCCTACGTGGTATTGAAATCGAAGCGGAT
	asp	alkaline serine protease	asp Va P1	AGAAGTTGACCTTACTTTCTTTGTCTGCGGTAAAATC
			asp Va P2	GCACCATCAACAGTTGGACTCTGACCTTTCAATAAGG
			asp Va P3	ATGGGAACTAAAAGCGGTAGACAGTGCCAGAAGAGAC
CLINICAL SIGNIFICANCE PATHOGENS (*Aeromonas hydrophila* subsp. *hydrophila*)	*gyrB*	gyrase B	gyrB Ahh P1	CGTCGAATACCTGAACCAGAACAAAACCCCGATCC
			gyrB Ahh P2	ATACCTTGTTCCACTACGAGATCCTGGCCAAGCGT
	rpoD	RNA polymerase sigma factor	rpoD Ahh P1	AGATGAAGACGAAGATGAAGACGAAGACGGTGATG
			rpoD Ahh P2	CATTTCCGGCTTCATCGATCCCAACGAGACCGATG
			rpoD Ahh P3	TGAGCAGTACGACAAATACGAAGCGGAACAGCTGC
	TH	virulence protein	TH Ahh P1	GCGAGCGCCTCTATCTGGAGCAGTACCTGGAACAG
CLINICAL SIGNIFICANCE PATHOGENS (*Aeromonas veronii*)	*rpoD*	RNA polymerase sigma factor	rpoD Av P1	AAGCAATTACTTACCTGCTCGAACAGTACGACAAG

	aerA	aerolysin	aerA Av P1	GATATCGAAATCGGTCAGCCCCAGACCCGTTCAGC
			aerA Av P2	GTATGGTTTGTCGACCATGCAGAATAACCTGGGTC
			aerA Av P3	GGTGAAGTGAAGTGGTGGGACTGGAACTGGACCAT
AQUACULTURE SIGNIFICANCE PATHOGENS (*Vibrio harveyi*)	*pyrH*	UMP kinase	pyrH Vh P1	TAACCCATTCTTCACAACAGATTCAGCGGCGTGTCTAC
			pyrH Vh P2	CTTCAAGGCGAAGAAGGTTTCGGTATTGACCCAGC
			pyrH Vh P3	TCTTACGCAGAAGTTCTGGATAAAGAGCTGAAAGTAA
	RTX	toxin	RTX Vh P1	TGCGATCAAGATTATTTGAACGGTTACAAGGGAGAGCGC
			RTX Vh P2	GCGCTATCCTTGTTTACAAACGAAACGCGAGCGGC
			RTX Vh P3	GATAAGCCAAAGCGGAGATGTAATTTTAGTTTCTGCAAAT
AQUACULTURE SIGNIFICANCE PATHOGENS (*Aeromonas salmonicida* subsp. *salmonicida*)	*vapA*	surface protein array known as A-layer	vapA Ass P1	GTGGATATTCAGAATGGTACCCGCGGCACAGCACC
			vapA Ass P2	ATCAGGGTGAAGTAGCTGTTAAGAAATCCAATGCT
			vapA Ass P3	CTGACGTTGGTGCTTCTATCACTGCTGGCCGTCAG
	endopeptidase	proteolytic peptidase	endopeptidase Ass P1	TGACATGAGTGCTCAGGGTCAGTACAGCATCCGCT
			endopeptidase Ass P2	TTCCAGTTGATCAAGGCTGGCCAGAGTCTGACGGT
AQUACULTURE SIGNIFICANCE PATHOGENS (*Edwarsiella tarda*)	16S	species-specific gene	16S Et P1	ACGGCCCGCAAGGTTAAACTTCAAATGAAATTGAC
			16S Et P2	CGCGGTAAAACGATGTCGATTTTGGAGGTTGTGCC
	gyrB	gyrase B	gyrB Et P1	TCTACTTCTCGACCATGAAAGACGACATTGGTGTC
			gyrB Et P2	AAGCGCAATGATCGTGAAGACCATTTCCACTATGAGGGT
			gyrB Et P3	TAGCAACGTGGTCGAGTTCCAGTATGACATCCTGG
	flavodoxine	electron-transfer protein	flavodoxine Et P1	ACTTGATCCTTATTGAGAAATTCGTGTTATCCACAAAGGA
			flavodoxine Et P2	TGGGATCACCGATCTTCCACAGTAAATGATCCTGCGTAGT
			flavodoxine Et P3	ATTCCAGAGGATCTTGCAGAGGAATGGCTGATCGCGTG

	invA	needle complex export protein	invA Et P1	CGGGATTAGTTAATGGTTATCCCTTGGTCGGAACG
			invA Et P2	ATGATCAGACTCAGGGCGTCGATATTACCTTCGCC
			invA Et P3	TAATACTAGCGCAGGTACGACCCATGTGAGTGCGA
	sepA	secreted protease	sepA Et P1	GGCGCTCAGTACCGCATATGATTTGTTTTATGAATT
			sepA Et P2	CGAGTAGATCAAATCACACCAGATATGAAGCGTTACC
			sepA Et P3	TTTGGCTCAGATAAAGTTCGCTCACTGGATCACCCA
	katB	virulence factor	katB Et P1	GGCGACTATCCAAAATGGGATCTGTACATCCAGGT
			katB Et P2	ATAGCCATATGACGCAGGATCTGGTGACGGCAATC
			katB Et P3	TCTGGATCCACAGCAGGTTGAACAGATTCAAGGTA
	pagC	virulence membrane protein	pagC Et P1	CTATGCCGATACGGTCAATGCCTTGGCCTATGGCG
			pagC Et P2	GAAGGCGCGCTATAATTCTCTGCTGATAGGGCCGG
			pagC Et P3	TGCAAAATGATGTGGATTCGACGACCCACTGGTGG
	pvsE	Vibrioferrin biosynthesis protein	pvsE Et P1	GTCATGGAGGTGCTGGACATCAAGCGCAATCTGGG
			pvsE Et P2	TCACCTGCAGATAGAGATGGTCAATCTCCGCGGCG
	QseC	sensor kinase	QseC Et P1	TCCGCAACCTACTGGATAACGCGATCCGCTACAGC
			QseC Et P2	CAGCTGTTGACCCTATCGCGCCTCGACTCGCTATC
			QseC Et P3	TCAACACCCTATTTGCCCGTACCAACGCGCAGATG
AQUACULTURE SIGNIFICANCE PATHOGENS (*Listonella anguillarum*)	*ftsZ*	bacterial cell division protein	ftsZ La P1	AAATGGGCCATGCTATGATGGGTAGTGGAGTGGCT
			ftsZ La P2	GTATTACCTTACTTGAAGCATTTGCGAGTGCGAAT
	chiA	chitinase gene	chiA La P1	AGGGGTGATGCCAGCTTCATTAAGTGATCCAACCG
			chiA La P2	GCTAATAAGTTAGTCTTAGGTACGGCTATGTATGGCC
			chiA La P3	TGTGGATGAAAATGGCGAACCTTATAAAGGCCCTGCC
	empA	species-specific gene	empA La P1	CGACTTCTTTGTTGCAACCAATGAGCCTGCACGGC
			empA La P2	TGCGTTTGGACGAAAACCAAATAGCTCAAATGGTT
			empA La P3	TGCGGATCTTGTTACAGAAAACGAGCGTGCTCAAT
	trh	thermostable direct hemolysin-related hemolysin	trh La P1	CTAAAACTCTACTTTGCTTTCAGTTTGCTATTGGCTTCTA

	rtxA	virulence gene	rtxA La P1	AAGTATTCGACACTTCTCCAAAATGGTTTTCCCAT
			rtxA La P2	ATGTCGTTATTTCCATCGTCGGCGGAATAATTTCC
			rtxA La P3	CGCCATAAGCATGAATTTTGCCGCCCATACCGATG
	rtxB	virulence gene	rtxB La P1	AACAAGCATTAAAACAAGAGGATGAGCGAGTATGA
			rtxB La P2	CAGAGCAAGGCACGCATCTACAACTTCTCGCAGAG
			rtxB La P3	TACCGTTATTACCATCGCACACCGCCTCTCTACGG
	rtxD	virulence gene	rtxD La P1	AACCATACAATTGAGGTCGATGGTACCTCTGTTGC
			rtxD La P2	TAGAGACTCGACAATGGATGAGCAACTTGGGTTGG
			rtxD La P3	ACGCGTTACGGCACCTTATCGGCAACATTGGTTCA
	rtxE	virulence gene	rtxE La P1	GGTCAACTTTCTCTCCTTCAAATGGGTCAGGTATAA
			rtxE La P2	GTGCAGGACTTTACGCACGTTTATGGGAACAACAAG
			rtxE La P3	TCGACGATTCGTCATGCCGATAACATTATCGTCAT
	rtxH	virulence gene	rtxH La P1	AACACTCGATACTGTTTGGACAAATTTCTCAGACAT
			rtxH La P2	TGATTCTGTCCAACAAAATAGAGAGAGAAAACACCTTTG
			rtxH La P3	CCGTATCACTTTCCGCCATAATACCGTTTGCCATGTTGG
AQUACULTURE SIGNIFICANCE PATHOGENS (*Photobacterium damselae* subsp. *piscicida*)	*ompU*	outer-membrane porins	ompU Pdp P1	AGTTCTACGTTCTACTTACAACTACATGGAAAACATGGAT
			ompU Pdp P2	CTACGTAGCAGGTCAATACCAGAACGCTCGTAACATCGGT
			ompU Pdp P3	AAGCAGGGTTTTGCATCTGTATCTTACACATCTGGCGATT
	toxR	control of the expression of outer membrane protein	toxR Pdp P1	TGCAAGTCGTAACGCCAAATAACAATCCAATTATGA
			toxR Pdp P2	TCCTGGCATTATTGATCCCTCTTGCAAGTTACTTTGC
			toxR Pdp P3	GGTCACAGGCTCGCGCTCACTTTTATCCATTATCGCT
	phospholipase	hydrolyzing enzyme	phospholipase Pdp P1	CTCACACCCACCATATTCTTGCTGAAGAAGTCGCT
			phospholipase Pdp P2	TGGTTCAGACAGTTATATGTTTTGGGGTGTCACCC
			phospholipase Pdp P3	ACCGCAGTTCATCTAAAGACTATTTAATGAGTCATGC

AQUACULTURE SIGNIFICANCE PATHOGENS (*Photobacterium damselae* subsp. *damselae*)	*pyrH*	UMP kinase	pyrH Pdd P1	GCAACGCTATATACTCAGCTAAGCTATCAAGATGTTCTTG
			pyrH Pdd P2	TTGATGGTGTATTTACAGCCGATCCGGTTAAGAACCCAGA
			pyrH Pdd P3	TGATGCAATTAGCCAGCTTCGTCAAGGTCGCGTGG
	dly	virulence gene	dly Pdd P1	ACTGCGATTTGCTATTAATATCACATCAGCGTCATTAC
			dly Pdd P2	GGTTGTAATTCCGATAGAGATTCACCATCCAAACATAGTT
			dly Pdd P3	GGGTTAACCCCAAATGAGCTAATGTAATGATCAAGATTAA
AQUACULTURE SIGNIFICANCE PATHOGENS (*Yersinia ruckeri*)	*gyrB*	gyrase B	gyrB Yr P1	TTCTCTACTATGAAAGATGACATCGGTGTGGAAGT
			gyrB Tr P2	CCAGACCTTCACCAATAACACCGAATTTCAATACGAAAT
	p1	secretory protein	Metalloprotease Yr P1	CCTTTGTTTATCACTCGCACTAGAGTATAAAACTCGACA
			Metalloprotease Yr P2	TATTCGATTAAATACCGCCACTGGCTGATTAATTCGATC
			Metalloprotease Yr P3	GGTCAGGCATTCCCCGACTTCTTGGTTAAAATTGTCGG
	pilV	fimbrial biogenesis	pilV Yr P1	ACACTAGTGATGTCCGCATCGACCTAACCTCTTAA
			pilV Yr P2	CAGTCGCGTCACGGTTATGCTGGTAGGCAGTTCAT
			pilV YR P3	GGACATTACTGGGGTTATCAGAATTTTGGTATTGCTC

Table 3: The DNA microarray target genes and the corresponding selected probes.

The final microarray design is illustrated in Figure 1. Probes were grouped in the device on the basis of the function coded by their target gene: in red are indicated genes responsible for antibiotic resistance, in green are represented those coding for genes that are part of mobile genetic elements, in dark blue are the genes typical of faecal contamination. in the lower part of the microarray are located all the 16S RNA specific probes, in particular the yellow ones recognise pathogens of clinical interest such as *Vibrio cholerae, Vibrio parahaemolyticus*, etc.; while the light blue ones targeted pathogens of veterinarian interest like *Yersinia ruckeri* and *Aeromonas hydrophila*.

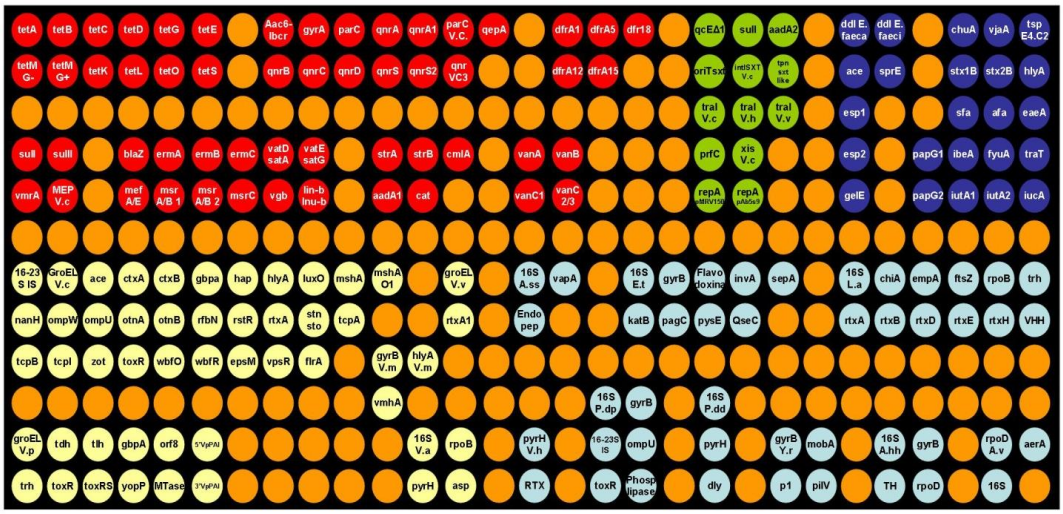

Figure 1: The DNA microarray design.

For each selected gene, one to fifteen probes, each consisting of 35-40 nucleotides, complementary to its nucleotide sequence were designed based on published sequences (NCBI GenBank database) and in-house sequencing data. The quality of the 1600 oligonucleotide-probes was evaluated with the Oligo Explorer program.

The Combimatrix DNA microarray was synthesized in collaboration with the Biotechnology Department (University of Verona) using a semiconductor-based electrochemical-synthesis process. Each oligonucleotide probe is synthesized via a platinum electrode that is independently controlled by the synthesizer's computer. Synthesis is based on established phosphoramidite chemistry and occurs at thousands of sites simultaneously according to a computer algorithm that activates only specified electrodes. Microarray probes are generally followed by a poly-T spacer at the 5' end, in order to improve the accessibility of the probes to the target DNA. This was not done on our probes because they are longer than 20 nucleotides and in this case there is no need of a spacer sequence. An oligonucleotide complementary to a known 16S rRNA sequence was included in the array as a positive control, and an oligonucleotide that did not

recognize any bacterial genes was present as a negative control, to avoid both false-positive and false-negative results. Printed slides were dried and stored at room temperature in the dark.

Figure 2: The DNA microarray ready for use.

DNA preparation (extraction and fragmentation): for the set-up of a specific protocol it was necessary: i) to prepare the DNA samples and ii) to optimize the hybridization conditions (see Figure 3).

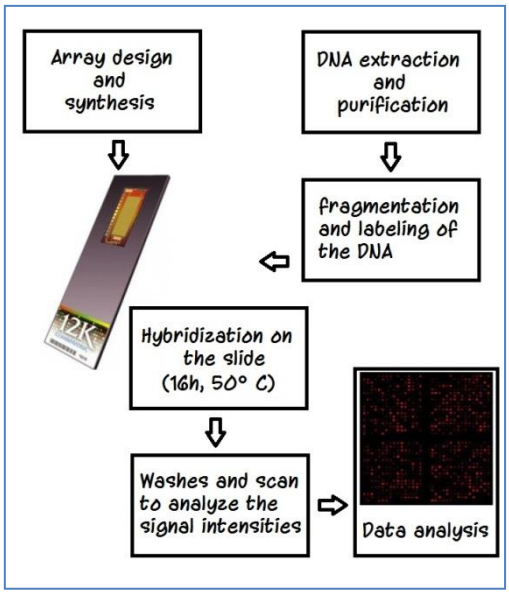

Figure 3: Schematic representation of the protocol set up for the microarray procedure.

The genomic DNA was extracted from 1 ml fresh culture by the phenol-chloroform method previously described supplied with an RNAse treatment. Particular DNA conditions were requested for a good hybridization: a DNA concentration of at least 100 ng/µl in more than 50 µl, a high level of DNA integrity that was verified by gel electrophoresis (1% agarose) and a high purity degree. To assure this, two parameters were verified using a NanoDrop 1000 Spectrophotometer (*Nanodrop Technologies*):

- the ratio of the absorbencies at 260 and 280 nm ($A_{260/280}$) that should be between 1,7 and 2. It indicates the DNA purity and the absence of proteins contamination.
- the ratio of the absorbencies at 260 and 230 nm ($A_{260/230}$) that should be higher than 2. It indicates the absence of phenol contamination; the DNA labelling will be proportional to this index.

The DNA was fragmented by sonication (7°C for 1000 seconds) using the Covaris Ultrasonicator S220 to obtain segments of approximately 150 bp.

SPECIFICITY

The array specificity was evaluated hybridizing separately the genomic DNA directly extracted from cultures of a number of *Vibrio, Enterococcus, E. coli, Aeromonas* and *Photobacterium* species; a total of 43 different strains were used for these experiments. To extract the DNA, the standard phenol-chloroform protocol previously described was used. A mix of different genomic DNAs extracted from the listed bacterial species, expected to be present in the marine waters, was used in a single sample to evaluate the recognition efficiency in presence of complex bacterial populations.

SENSITIVITY

To estimate the sensitivity of the device, i.e. assessing the overall DNA detection limit of the device, a serial dilution (700 ng, 350 ng, 150 ng and 100 ng) of bacterial genomic DNA from each of the strains was prepared and hybridized in the same conditions.

VALIDATION WITH NATURAL SAMPLES

The validation has been performed on bacterial strains inoculated in natural marine samples without previous PCR amplification. To evaluate the possibility of using the device with real samples, sea water, marine sediment and mussel samples obtained from the Adriatic Sea were used as matrices to add bacterial DNA and subsequently re-extract it. The three chosen bacterial strains *V. cholerae, V. parahaemolyticus and E. coli* were suspended in water and adjusted to a density of approx. 1.8 x 10^8 CFU/ml each. Different bacteria concentrations (10^8, 10^7 and 10^6 CFU/ml) were used to inoculate environmental samples and DNA was then extracted using the standard phenol-chloroform protocol.

Labelling and hybridation:

To label the DNA, the ULS™ Labeling Kit for CombiMatrix arrays (with Cy5) (*Kreatech Diagnostics; Amsterdam, The Nederlands*) was used. Cy5 fluorophore belongs to the cyanine family and, alone or in combination with Cy3, is commonly used in a wide

variety of biological applications. They are usually synthesized with reactive groups on either one or both of the nitrogen side chains so that they can be chemically linked to either nucleic acids or protein molecules. Cy5 emits fluorescence in the red region (~650/670 nm) and absorbs in the orange region (~649 nm). 1,6 µl of Cy5 fluorophore were added to 1,6 µg of purified and fragmented nucleic acid and incubated for 22 minutes at 85°C. Some DNA spikes sequences were added (2ul); they are labelled DNA fragments complementary to some control probes inserted in the microarray. They are added in a known specific concentration, used for the instrument calibration. Starting from the intensities of the signals revealed on the corresponding probes, and knowing the exact spikes' amount hybridized, it is possible to quantify the fluorescence signal of the samples. After the labelling reaction, excess of fluorophore was removed; the Degree of Labeling (DOL) and the final DNA concentration were measured on Nanodrop 1000 Spectrophotometer. DOL≥2% were accepted.

Hybridizations occurred on a Combimatrix CustomArray 4x2K chip, according to the manufacturer's instructions (CustomArray 4x2K microarray protocol): a hybridization solution (see Figure 4) was mixed to the DNA samples in order to reduce the non-specific interactions between DNA and probes.

Table 8. Hybridization Solution

Reagent	Volume for 30 µl	Final Concentration
2X Hyb Solution Stock	15 µl	6X SSPE, 0.05% Tween-20, 20mM EDTA
DI Formamide (for RNA targets)[a]	7.5 µl	25%
Labeled targets: DNA or fragmented RNA	Varies (up to 6 µl)	15-67 ng/µl recommended
Salmon sperm DNA (10mg/ml)[b]	0.3 µl	100 ng/µl
1% SDS	1.2 µl	0.04%
Nuclease-free water	to 30 µl	
Total Volume	30 µl	

Figure 4: The employed hybridization solution.

The DNA plus the hybridization solution were denaturated (3 minutes at 95°C, plus 1 minute in ice), and about 700 ng of labelled DNA were then hybridized on the slide that was maintained at 50 °C for 16 h. after hybridization the time that the slide remained at room temperature was minimized to prevent cross-hybridization.

Acquisition and data analysis: Post-hybridization washes aimed to eliminate all the probes non-bounded to the target. The slide was washed sequentially with a washing solution (6x, 3x, 0.5x), PBST solution, PBS solution and finally water (see Figure 5).

Table 2. Wash Solutions		
Step	Solution	For 10 ml
6X SSPET Wash	6X SSPE, 0.05% Tween-20	3 ml 20X SSPE 50 µl 10% Tween-20 6.95 ml Nuclease-free water
3X SSPET Wash	3X SSPE, 0.05% Tween-20	1.5 ml 20X SSPE 50 µl 10% Tween-20, 8.45 ml Nuclease-free water
0.5X SSPET Wash	0.5X SSPE, 0.05% Tween-20	250 µl 20X SSPE 50 µl 10% Tween-20 9.7 ml Nuclease-free water
PBST Wash	2X PBS, 0.1% Tween-20	2 ml 10X PBS, 100 µl 10% Tween-20, 7.9 ml Nuclease-free water
PBS Wash	2X PBS	2 ml 10X PBS, 8 ml Nuclease-free water

Figure 5: The employed washing solutions.

The slide was scanned using the Axon GenePix 4400A (*Axon Corporation, CA)* scanner to analyze the fluorescence emitted by the duplex probe-target (laser beams from 440 to 540 nm), adjusting the laser power to avoid spots' saturation. Microarray scanners contain two lasers, emitting light at wavelengths suitable for exciting the fluorescent dyes used as labels. A confocal microscope is attached to a detector system and records the emitted light from each of the spots. Spatial resolution of these systems can be as low as of 3–5 µm, although 10 µm of resolution is sufficient for conventional microarrays. During the hybridization, labelled probes are expected to form duplex with their complementary targets producing a fluorescent signal. The intensity of the light signal is proportional to the number and the length of duplex formed. Considering the large quantities of data, a computerizing data processing is necessary. In our case, data were extracted from the obtained image using CombiMatrix imager software that extracts primary data from scanned microarray slide images and normalizes them to remove the influence of experimental variation, so that meaningful conclusions can be made. The cut-off value for determining whether or not a target probe was recorded as positive was assigned as the average signal intensity of the negative controls (threshold) plus one standard deviation. A signal was considered positive, and therefore the specific gene was considered to be present in the sample, when at least 75% of all probes associated to the target gene gave a signal higher than the cut-off value.

The slide was stripped to re-use it up to 4 times for other hybridizations; the stripping solution (ethanol+ethanolammine) is employed to disrupt the interactions between probes and DNA and to remove all the DNA fragments. The slide was scanned again after the stripping procedure to assure its cleanliness.

Results

Sample preparation

Several problems were encountered during the set up of DNA extraction: using the classical phenol-chloroform extraction protocol the obtained DNA resulted to be fragmented and disrupted, and thus not in the ideal form to be hybridized (see Figure 6).

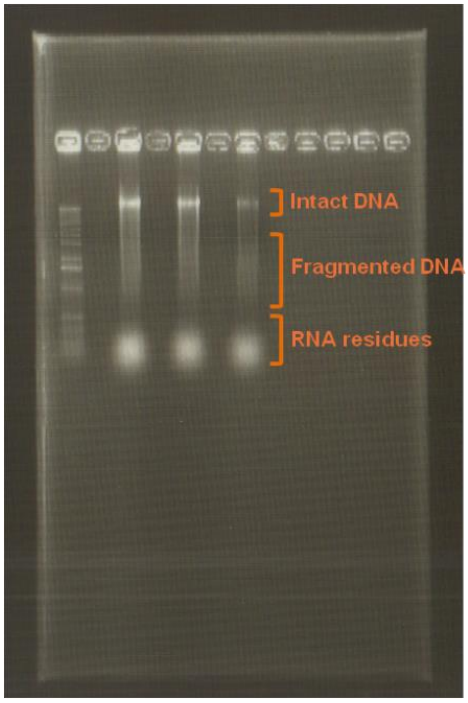

Figure 6: 1% agarose gel of DNA extracted by phenol-chloroform classical method.

In addition, the DNA purify level was above the cited values of absorbencies. To solve those problems, other protocols were tried: extraction by lysis buffer, automatic extraction, kit-mediated DNA extraction and also use of centrifugal filter units (*Millipore*). All those attempts failed in obtaining good parameters and integrity without losing big quantities of nucleic acid. The only successful treatment was the one employing the phenol-chloroform method supplied with an RNAse treatment. This ladder was then used for all DNA samples' preparations.

Also the fragmentation step required many attempts in order to finally obtain fragments of 150 bp. The use of heat (95°C-8 minutes) to fragment DNA samples lead to fragments of only 50 bp, those ones could not be hybridized because they would bind a-specifically to the probes. Then the fragmentation by sonication using the Covaris instrument was tried, in particular different intervals of treatment were tested: 1500 seconds, 1300 seconds, 1000 seconds and 600 seconds, obtaining fragments of 88 bp, 100 bp, 160 bp and 210 bp. The chosen time of fragmentation was then of 1000 seconds.

Validation of the DNA microarray

Specificity of microarray hybridization.

A series of experiments were performed to verify the recognition efficiency of the microarray, using nucleic acids directly extracted from cultures of 43 different strains listed in Table 2. A signal was considered positive, and therefore the specific gene was considered to be present in the sample, when at least 75% of all probes associated to the target gene gave a signal higher than that revealed by the negative control. The results obtained indicate that the designed probes are specific for their corresponding target genes and genes were detected only in the samples where the corresponding DNA was included. In particular, genes of *V. cholerae, Aeromonas, Photobacterium, Edwarsiella, Yersinia e Listonella* were always detected correctly; for *V. parahaemolyticus* this was possible only in presence of high bacterial DNA concentrations. Some problems were there when hybridizing *V. harveyi* samples, because of some cross reaction with its probes. This problem was eliminated substituting some of the probes when synthesizing the second microarray. For *E. coli* and *Enterococcus* the originally designed probes were not sufficiently specific, so new ones were created after a deeper study of the corresponding sequences to substitute the first ones.

Additionally, the test made with the mix of nucleic acids showed that good detection results are possible also when the sample contains DNAs from many different bacterial species. Thus we can assume that the device can be employed in field studies because it maintains a high specificity even in presence of complex bacterial populations.

When hybridizing the negative control sample, no significant signal was detected with any of the probes, confirming the optimal probes' design and the specificity of our device. Taken together, these results indicate that the designed probes were specific for the corresponding targets.

Determination of the microarray sensitivity.

To estimate the sensitivity of the microarray, we have used four quantities of genomic DNA extracted from some of our target bacteria: 700 ng, 350 ng, 150 ng and 100 ng. The signal intensities obtained with the three concentrations have been analyzed and compared to determine the minimum detectable DNA amount. A decrease of the signal intensity comparable to the decreasing quantity of DNA hybridized was observed. From the first to the last DNA quantity, a 3% loss of information was observed and some few genes were no more detected. This percentage is very small, and for this reason the limit of the microarray sensitivity was established in 150 ng of nucleic acid. This was the minimum amount that gave signal intensity significantly higher than the background

level, when compared with the negative control. However, to be sure of all the targeted genes detection, a dose of 200-300 ng would be preferred. This quantity corresponds to about 10^5 bacterial cells, and represents a good result to propose this device as a suitable tool for the analysis of natural samples. The variations in probe sensitivity are difficult to predict but a possible explanation of their observation is that the secondary structure of ssDNA could interfere with the hybridization. Therefore to increase it and thus to improve the microarray performances, the number of probes for each target gene should be increased (Kim *et al*., 2010).

Reproducibility of the system.

Each experiment was repeated twice for each different sample applying the same conditions; then data were validated with statistical analysis obtaining a good correlation coefficient (0.93). This indicates that the system has a good reproducibility.

Validation of the array with natural samples.

To validate the microarray, some experiments using natural samples have been performed. In particular we have tested: sea sediments coming from coastal areas, seawater obtained from the Adriatic Sea and shellfishes (such as mussels), that are known to be a reservoir of bacteria due to their water-filtering activity. The experiments using real environmental samples have also defined the detection ability of this device in presence of natural inhibitors, typical of environmental samples.

Different bacteria concentrations were used to inoculate environmental samples: a sufficient quantity of DNA for the whole microarray processing was obtained only when the inoculated bacteria in a sea water sample were at least at a concentration of 10^7 CFU/ml. To bypass this problem, lower bacterial concentrations were added to the natural sample that was subsequently inoculated in an enrichment media promoting the growth of *Vibrio* and *Aeromonas* strains. Both the sample of DNA from high bacterial concentration introduced in seawater, and the natural samples after enrichment gave positive hybridization signals, indicating that the device can be used to detect microorganisms in natural samples such as sea water and sediments. The natural samples were previously analysed by cultivation and PCR to assess the microbial population present in them, before performing the hybridization. The results obtained using the PCR approach coincided with those obtained using the DNA microarray tool.

It is important to note that the enrichment step, set up to obtain good recognition signal even with poor natural samples, modifies the real bacterial concentrations. Thus a

quantitative analysis could not be done if employing this protocol. In addition, the microbial population composition could be altered after this step, because a strain's growth could be promoted or discouraged by the enrichment medium, and thus the final bacterial mix could be different in its structure from the original one.

Important technical problems have been encountered when a sufficient quantity of purified DNA has to be extracted directly from sediments or from mussels inoculated with faecal indicators and marine bacteria. This is due to the presence in those matrices of contaminating substances such as humic acids that reduce the efficiency of DNA extraction.

Microarray applications on environmental strains

Our device was also used to determine the presence or absence of a number of virulence and antibiotic resistance genes in environmental strains isolated from fish farms during a previous study (Labella *et al*., 2013). Strains were tested with the microarray following the previously described protocol, and multiplex PCRs were set up as a validation tool.

APPLICATION 1: Table 4 illustrates the results of the screening of 7 multi-resistant environmental strains isolated from water and sediment samples in aquaculture sites as regards a number of antibiotic resistance determinants. In 3 samples out of 7 (43%) the AR genes were detected both by multiplex PCR and microarray (samples 10CP, 15CP, 143AS) while in two cases the genetic determinants SXR and qcEdelta1 were detected only with the microarray device but not confirmed by the PCR approach (samples 1VB and 65CP). Finally, in sample 149AS the microarray failed in detecting the parC gene instead revealed by multiplex PCR.

APPLICATION 2: A total of 10 environmental strains isolated from water, plankton and sediment samples obtained in the Venetian Lagoon during the period 2006–2009 were also screened with the DNA microarray and the results were validated with multiplex PCR. In particular, we looked for the presence of virulence genes (showed in light blue), fitness genes (showed in blue) and mobile elements genes (showed in purple) (see Table 5). Three of the genes included in the virulence battery (*yopP, tdh,* and *trh*) are involved in *V. parahaemolyticus* virulence, while regarding *V. cholerae*, the gene *MTase* coding for a methyl-transferase, the *ctxA* gene for the cholera toxin, the *nanH* gene, encoding a neuraminidase, and the virulence-activator factor *toxR* were considered. In this group have been added genes detecting two pathogenicity islands (VPI-2 from *Vibrio cholerae*

and Vp-PAI of *Vibrio parahaemolyticus*) and the regulator of the *V. harveyi* bioluminescence *LuxO*.

As regards the fitness genes, two *V. cholerae* genes were chosen: *flrA* is involved in the regulation of *V. cholerae* flagella synthesis, while *VpsR* is a transcriptional regulator involved in biofilm formation and environmental persistence.

The set of genes carried in mobile genetic elements included: the class1 integrase, the *TraI* gene involved in conjugation process and two genes typical of ICEs elements: the integrase gene *Int ICE* and the *traC* gene involved in ICE's transfer.

All the analysed strains except w22 AD were positive for at least one of the screened genes; of these, 7 strains carried virulence genes, with the most frequent gene isolated being *nanH* (5 strains). No strains carried the *ctxA* gene. Seven other strains carried one or more fitness genes, with *flrA* being the most frequent one. It should be noted that virulence and fitness genes typical of human pathogenic *Vibrio* species are present, in variable proportion, in other members of this genus such as *V. anguillarum* and *V. harveji*. The results obtained show clearly that the most part of our strains (6 out of 10) presents at least one gene characteristic of MGEs, underlining the high prevalence and importance of the phenomenon of HGT.

Some strains result to be particularly interesting; the water sample w12 AD for example is positive for the *nanH, luxO, toxR* genes, all conferring enhanced virulence and fitness properties to the bacterium harbouring them, plus the flagella synthesis regulator *flrA* and the class 1 integrase. Strain 39 AD presents the *trh* gene, coding for a thermostable related haemolysin but is defective of the *tdh* gene (thermostable direct haemolysin); the opposite happens to strain p48 Ve in which the *tdh* factor was detected but not the *trh*. This strain also show to be positive for the presence of the *yopP* gene (component of Type II secretion system T3SS) and of an MTase (DNA methyltransferase). Those results are explained considering that the strain harbours also the *Vibrio parahaemolyticus* pathogenicity island (Vp-PAI); this 80-kb pathogenicity island contains in fact two *tdh* genes and a set of genes for the type III secretion system (T3SS2). Looking at strains sed40 AD and sed43 AD, we can see that they have exactly the same gene profile and both of them are *V. alginolyticus;* we can assume that they have to be considered as the same strain and thus they should be renamed in a unique one.

Finally, strain 50 Chio seems to contain both a class 1 integrase and a *traI* gene, confirming the presence of a mobile integron.

Sample	sample type	AMP	TET	TIM	SULF+TIM	FLU	Positive signal in array	Positive signal PCR
1 VB	water	R	R	R	R	S	blaZ, tetC, SXT, dfrA1,	blaZ, tetC, dfrA1,
10 CP	water	R	R	R	R	R	tetM, ermB, aac(6')Ibcr, qcEdelta1, sulI, SXT	tetM, ermB, aac(6')Ibcr, qcEdelta1, sulI, SXT
15 CP	water	R	R	R	R	R	tetM, qcEdelta1, intSXT, repA pAb5S9	tetM, qcEdelta1, SXT, repA pAb5S9
143 AS	water	R	R	R	R	S	tetA, tetC, parC, qcEdelta1, sulI, dfrA1	tetA, tetC, parC, qcEdelta1, sulI, dfrA1
149 AS	sediment	R	R	R	R	S	tetA, tetC, qcEdelta1, sulI, dfrA1	tetA, tetC, parC, qcEdelta1, sulI, dfrA1
65 CP	sediment	R	R	R	R	R	tetA, tetM, aac(6')Ibcr, qcEdelta1, sulI, aspVa	tetA, tetM, aac(6')Ibcr, sulI, aspVa
AS1	water	R	R	R	R	S	floR, StrA, StrB, SXTintI, luxO, GroEl, gbpA, toxR, ompW	floR, StrA, StrB, SXTintI, luxO, GroEl, gbpA, toxR, ompW

Table 4: APPLICATION 1: Presence of AR genes in environmental strains as detected by DNA microarray and multiplex PCR; in red are marked those genes detected only by one of the two methods.

Bacterial strain	*yopP*	*tdh*	*trh*	*MTase*	*ctxA*	*Vp-PAI*	*nanH*	*VPI-2*	*luxO*	*toxR*	*flrA*	*VpsR*	Class 1-Integrase	TraI-MGE	*Int ICE*	*traC ICE*	*Species*
w12 AD							+		+	+	+		+		+		*V. cholerae*
w22 AD											+						*Photobacterium*
p2 AD													+				*Shewanella spp*
w51 Manf						+	+										*V. alginolyticus*
39 AD			+							+	+	+	+				*Vibrio spp*
sed40 AD							+	+	+		+					+	*V. alginolyticus*
sed43 AD							+	+	+		+					+	*V. alginolyticus*
w42 Ve							+					+			+		*V. anguillarum*
p48 Ve	+	+		+		+											*V. parahaem*
50 Chio											+		+	+			*V. harveyi*

Table 5: APPLICATION 2: Screening of a group of environmental bacterial strains for the presence of virulence genes using the DNA microarray.

Discussion

Our recent data, indicating the presence of a high percentage of multi-resistant bacterial strains in fish farms (Labella *et al*., 2013), supports the view of environmental bacteria as a reservoir of antibiotic-resistance genes that, if transferred to other bacteria sharing the same ecological niche, might constitute a risk for human health. As a consequence, the ability to detect and identify both bacteria and their main virulence genes is increasingly becoming important for environmental surveillance, clinical medicine and bio defence (Kim *et al*., 2010). At present, the incidence and transfer mechanisms of antibiotic resistance genes in the marine environment are not well known. It is mandatory to monitor the possibility of gene exchange among marine microflora members and between marine bacteria and bacteria of medical interest transitorily present in seawaters.

In this project, we designed a microarray containing specific probes for more than 200 genes of veterinarian and medical interest to be searched in marine bacteria and faecal bacterial indicators. We target marine bacteria and fish pathogens as a model because conventional diagnostic procedures can be very challenging to correctly identify these fastidious microorganisms. The limitations of non-molecular methods not only affect veterinary diagnostics but also severely limit our ability to study the ecology of these economically important pathogens.

This device represents a more efficient approach in comparison to PCR and other molecular tools, because it allows parallel hybridizations of samples on a unique surface and permits independent detection of multiple genes. The particularity of this microarray is that DNA extracted from pure culture or from natural samples is directly used for hybridization without additional amplification. This was done in order to show the potential use of the device in aquaculture sites without the need of a PCR step. Planar DNA microarrays are composed of spatially registered, immobilized DNA probes (typically synthetically produced oligonucleotides) that are complementary to the target genes. Probes appear as ‘‘spots’’ in the final image where each spot represents a unique probe sequence and spots are usually 100–200 μm in size and located within 200–500 μm of each other (see Figure 7).

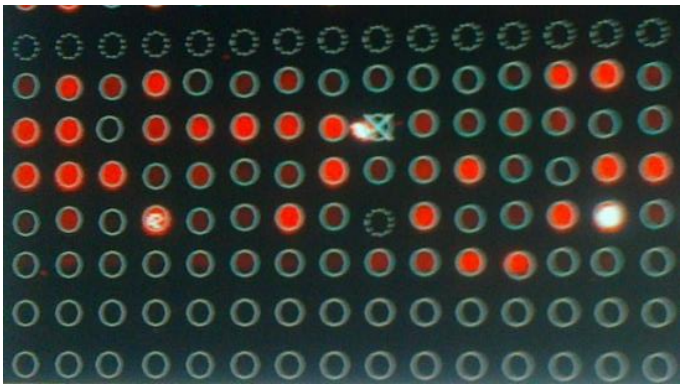

Figure 7: Photography of the array spots as they appear on the screen after the scanning procedure.

Depending on the objective, targets may be PCR products, genomic DNA, total RNA, cDNA, plasmid DNA, or oligonucleotides. The DNA target molecule to be analyzed is fluorescently labelled and hybridized by base pair matching to the complementary recognition probe that is fixed on a solid support. Direct or chemical labelling with Cy-3 or Cy-5 fluorescent dyes is the most common mean for detecting targets on microarrays. Once post-hybridization steps are completed, a high-resolution scanner is used to acquire the fluorescence to determine the presence/absence of the corresponding gene. The sequences of probes on the microarray are specific and as a consequence the detection signals generated upon hybridization provide the basis for bacterial identification.

Although the detection time needed to complete the analysis of samples is 10 hours longer than using conventional PCR methods, the procedure is shorter than that of cultivation and represents a more efficient and complete approach. Any PCR-based detection system, although potentially very sensitive, must still confront a number of challenges inherent with sampling complex substrates. It should be considered that, either using a PCR technique or a microarray to detect pathogens in water, a number of factors reduce the sensitivity of the assay. In addition, considering environmental samples where the bacterial concentration can be as low as few CFU, the detection by immune-based or molecular methods is not possible. The proposed enrichment step can overcome this problem, making use of the DNA microarray even when bacterial concentrations are very low, situation that is frequent when analysing natural samples. Another aspect to be taken into account is the possible genetic variation in 16S rRNA among species that represents a subject of debate. González *et al*. in 2004 believed that a high degree of genetic similarity for 16S rRNA genes across species might compromise the specificity of PCR detection. The strategies of probe design and microarray technology we used in this study can overcome the obstacles mentioned above, as seen in Chang *et al*. (2012). We

deliberately designed specific probes based on polymorphic regions of the 16S rRNA gene with the highest possible degree of variation. In addition, the length of each probe was designed to be as short as 30 nucleotides. According to a hybridization rule-of-thumb of 10–15%, a DNA duplex will form between targets and their complementary probes if genetic dissimilarity is <10–15%, *i.e.*, three nucleotides of the 30-mer probe in our case. When genetic dissimilarity exceeds 10–15%, particularly when base mismatches are distributed systematically or randomly across the probe sequence, then the target+probe duplex is less likely to form.

The main advantage of the described microarray is the possibility of simultaneous detecting hundreds of genes, the high specificity and the sequence-based detection of target genes. In fact contrariwise to agarose gel analysis of PCR products, detection did not rely solely on the length of the products but also required the fragments to be complementary to the probes.

To the best of our knowledge, this is the first oligonucleotide-based chip containing such a large variety of probes for a high diversity of genes of environmental, economical but also clinical interest. The microarray and the dedicated protocol set up to improve its potentialily might provide a specific and sensitive tool for detection of marine pathogens. This system could detect as little as 200 ng of genomic DNA which is equivalent to 10^5 bacterial cells. This is not as sensitive as other arrays reported in literature, but in our case bacteria or natural matrices are directly used for hybridization without a PCR amplification step. It should be noted that while we obtained good results with water samples, there are still a series of concerns when working on sediments and mussels.

As concern the lack of detection of a number of the selected genes, several factors could determine false-negative results on microarrays: as an example, direct capturing of 16S rRNAs with surface-immobilized oligonucleotides depends upon their secondary structure that can limit the access to the binding sites. Moreover, the use of a solid support can reduce the binding efficiency due to unfavourable steric interactions mediated by the solid matrix (Chang *et al*., 2012). We should also consider that, depending on the sample matrix, the preparation of nucleic acids can represent a significant challenge. Matrices can be very "dirty", thus another concern during the extraction is the possible co-extraction of inhibitors. It must be noted that the microarray downstream detection ability is deeply influenced by the upstream samples collecting and processing (Call, 2005). The setting up of protocols for sample enrichment before hybridization is a useful tool to overcome the problem of low bacterial concentration, and consequently, small amounts of bacterial DNA, in natural samples. However, this step in some way false the

sample composition both from the quantitative and qualitative point of view and does not solve the problem of the non-culturable bacteria.

This type of array gives valuable information about the classes of genes that we might expect to be ‘‘dispensable’’ within a species, and could be used to fingerprint strains relative to the reference strain. Hakenbeck *et al*. (2001) used a similar type of whole genome microarray and found that up to 10% of *Streptococcus pneumoniae* genes appeared altered between tested strains and a single reference strain. This type of array can serve both as a fingerprinting tool and as a tool for identifying new genetic markers that may be amenable to PCR-based assays (Call *et al*., 2003).

Talking about a possible use of the microarray technique in bacteria quantification, we have to remember that Wu *et al*. in 2001 demonstrated that this method has limitations with respect to bacterial enumeration in complex communities because the hybridization signal can be confounded by both target abundance and hybridization efficiency. That is, a low-abundance genetic marker that has high genetic similarity to a microarray probe might produce a stronger hybridization signal compared with a higher-abundance marker that has a poor genetic match to the same microarray probe. Only real-time PCR can give an estimation of the nucleic acid quantity present in the sample and allows a quantification of the number of cells present in it.

Another possible application for this device is the analysis of changes in the bacterial community over long periods of time. Using this microarray, variations in bacterial species composition and in gene incidence associated to climate and environmental changes could be monitored over time and also from the spatial point of view. This will enable comparisons among different marine micro-environments. The possibility of targeting so many genes will give both a panoramic view of the marine bacterial community composition in the study area and an estimation of the incidence of genes involved in virulence and AR. It would be possible to sample in different geographical areas (coast, aquaculture centres) to compare the bacterial species present, or analyze the climate-induced variations by sampling under different environmental conditions (season of the year, humidity level, temperature), or even focusing on specific sites and following the bacterial communities over the years.

In conclusion, this study presents the development and validation of a new DNA microarray containing specific probes for the detection of more than 200 genes of many marine bacteria and faecal indicators of veterinarian and medical interest. This device shows a high specificity in detecting target genes and works also in complex samples

containing nucleic acids from different origins. It also has a good sensitivity when working with bacterial strains and water but not sufficient when working with natural samples such as sediments and shellfishes. Those characteristics make it suitable for applications in food safety, epidemiological surveillance and also environmental analysis thanks to the proposed enrichment protocol and/or PCR amplification before hybridization.

PART 2:

TRANSFER OF MOBILE GENETIC ELEMENTS BETWEEN MARINE ALLOCHTONOUS AND AUTOCHTONOUS BACTERIA & MOBILIZATION OF NON-MOBILE GENETIC ELEMENTS

Introduction

Antibiotic resistance

Antimicrobial resistance is an increasingly serious threat to global public health. It develops when a microorganism no longer responds to a drug to which it was previously susceptible. As a consequence, standard treatments are not effective and infections are harder to control, with a higher risk of spread of the infection, prolonged stays in hospital and added social and economic costs. The problem has been also faced in recent times by WHO, noticing that antibiotic resistance has reached alarming levels worldwide. In its global report on antibiotic resistance, called "Antimicrobial resistance: global report on surveillance 2014" WHO wanted to provide an accurate picture of the magnitude of this phenomenon and to define some surveillance guidelines. The report focuses on the importance of an integrated surveillance of AR bacteria carried by food-producing animals and in the food chain (WHO global report on antibiotic resistance, 2014).

The term "multidrug resistant" refers to microbes with enhanced morbidity and mortality due to multiple high levels of resistance to the antibiotic classes recommended for their treatment; as a consequence the therapeutic options for these microbes are reduced, and periods of hospital care are extended and more costly.

A little antibiotic story

The successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first employed. The most costly example in terms of morbidity and mortality is given by bacteria. Bacteria are known to be extremely adaptable thanks to their high population numbers, their genomic plasticity and the possibility of exchanging genetic material. It seems not so strange, as a consequence, that during the decades they have developed many mechanisms permitting to resist to external adverse conditions, including the presence of antibiotics. They were discovered in the late 19th century (see Figure 8), but only half a century later the first successful treatment was found with the introduction of antibiotics. The definition of "antibiotic" was first proposed by S. Waksman, the discover of streptomycin, and it denotes any class of organic molecule that inhibits or kills microbes by interaction with bacterial targets. Their discovery was a turning point in human history; however their use has been accompanied by the rapid appearance of resistant strains. Medical pundits are now warning of a return to the pre-antibiotic era (Davies *et al*., 2010).

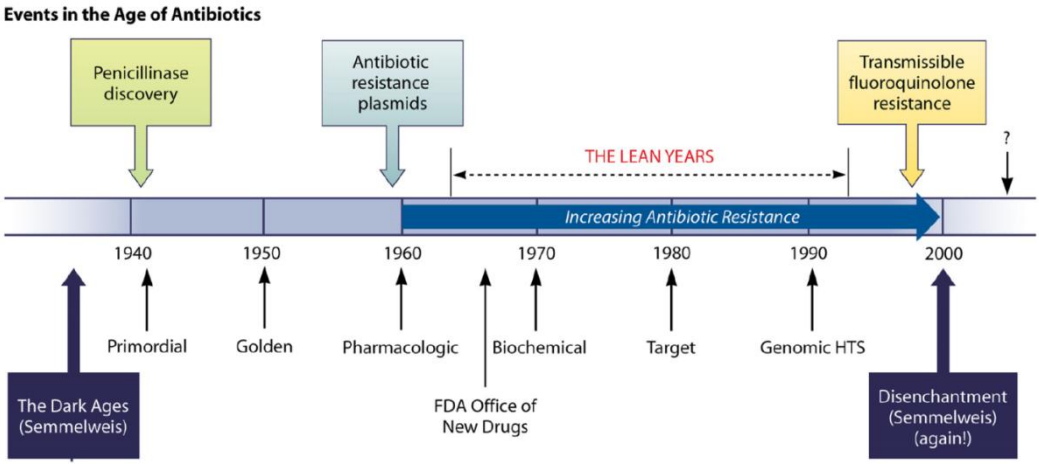

Figure 8: History of antibiotic discovery and concomitant development of antibiotic resistance (Davies et al., 2010).

The indiscriminate use of antibiotics in medicine and agriculture has favoured the spread of highly resistant bacteria, as a way to adapt and survive in presence of a selective pressure, the same strategy that bacteria have improved to adapt to environmental changes. This problem was first revealed in Japanese communities and hospitals in the 1950s when a *Shigella* outbreak was found to be resistant to usual antibiotics. Primary resistance is naturally present in some microorganisms such as *Pseudomonas aeruginosa* that is resistant to penicillin G; secondary resistance develops during the treatment with an antibiotic (Kummerer, 2009). It was discovered in the '40s by Newcombe that the exposure of bacteria to drugs determine a selection of pre-existing resistant clones, and as a consequence that is not the antibiotic itself that induces the appearance of resistant strains.

Development of antibiotic resistance in bacteria

Environmental microorganisms might represent a reservoir of resistance genes potentially transferable to human pathogens. For instance, the quinolone resistance gene *qnrA* originated in the water-borne bacteria *Shewanella algae*; considering that quinolones are synthetic drugs, the existence of these determinants indicates that AR genes can have other functions in the original host (Poirel, 2005). Quinolones prescribed in human therapy are also extensively used in aquaculture, it is thus possible that sub-inhibitory concentrations of quinolones in water may select for waterborne *S. algae* strains and therefore enhance transfer of this naturally occurring quinolone resistance determinant to *Enterobacteriaceae*. This consideration emphasizes the possible role of the aquatic

environment in emergence of drug resistance and underlines the importance of monitoring this habitat.

While studies of AR in clinical bacterial strains have been extensively conducted, only in recent years detailed studies on AR in the environment have been published. To this end two different methodologies are generally applied: one search for any potential gene conferring resistance using functional genomics, the other looks for resistance genes present in human pathogens by PCR. The first characterizes any gene causing resistance and hence study the potential environmental "resistome", while the second analyses the AR genes already acquired by human pathogens (Martinez, 2012). As a consequence they give different information: functional metagenomics finds new resistance mechanisms while PCR inform us about genes' stability and reservoirs, useful to evaluate the potential risks for human health. The resistance "mobilome" includes all the AR genes that are already present on MGEs, whose transfer depends on the mobilization and integration ability of the MGE they are located in.

Antibiotic resistance is a multifactorial problem and many aspects should be considered. Bacteria can acquire resistance through several mechanisms: mutations in the antibiotic target leading to targets no more able to bind the antibiotic, the synthesis of enzymes modifying or degrading the antibiotic or changes in cell permeability or production of efflux pumps that clear the agent from a cell. Once those mechanisms are established, they can be introduced in other members of the bacterial population via vertical gene transfer (by cell division) or by horizontal gene transfer-HGT (by conjugation). HGT can determine a rapid spread of the resistance genes in the environment, having negative effects on both terrestrial and aquatic organisms, included humans. Antibiotics are known to promote the transfer of mobile genetic elements. For example the treatment of *E.coli* with fluoroquinolones determines the expression of the Shiga toxin and the excision of the phage encoding it (Wong *et al*., 2000). The SOS response induced by antibiotics has as a consequence the mobilization of many genes. Beaber *et al*. in 2004 showed that SXT was transferred and integrated with a higher efficiency if the donor was exposed to low concentrations of the antibiotics SXT gives the resistance to. Again, the transcriptional activator necessary for this process are up regulated via SOS. The expression of acquired antibiotic resistance genes is deeply regulated, thus reducing the biological cost associated to the expression of those genes and promoting their dissemination. For example, the expression of antibiotic resistance genes is silenced until the exposure to the corresponding drug. This fact represent a perfect evolutionary strategy: "switchable" genes render bacteria fitness-neutral in the absence of the selective agent.

Maintenance and fixation of AR genes

Acquiring AR determinants involves also a fitness cost and the establishment of a successful gene transfer chain is possible only in presence of selective pressure for the determinant. Therefore, unless resistance is selected it is unlikely that MGEs with AR genes will be fixed in the population of environmental bacteria. Only in presence of antibiotics in the environment (due to wastewaters, aquaculture sites, farms or industries), a positive selection for those elements can be envisaged. Finally, the possibility for a resistance gene to be transferred to human pathogens depends on whether the donor's habitat is close to human-linked ecosystems. In particular the phenomenon will be higher in those habitats where human pathogens and environmental strains co-exist in presence of contaminating antibiotic residues (Martinez, 2012).

Maintenance of AR genes in habitats with no antibiotic pressure can be favoured by second-order selection processes. MGEs generally contains more than one AR gene, and then the presence of one antibiotic will select for the whole pool of AR genes present in the MGE. Also other elements can be present, such as genes coding for production of toxins, bacteriocins and toxin/antitoxin systems; if they confer an advantage in a particular ecosystem then a co-selection of the AR gene will be established even in absence of the antibiotic. In such a way resistance genes can evade elimination even in absence of the specific antibiotic (Martinez, 2012).

Antibiotic resistance in the environment

More than 70% of the earth surface is covered with water, representing thus the largest habitat of our planet and hosting a large diversity of life. It is not surprising that initially antibiotic resistance genes have been found in marine bacteria, even in samples collected 500 km offshore and at depths of 8200 m (Aminov, 2011). Antibiotics are present in the environment at very low concentrations, not sufficient to kill bacteria or block their cell division. The exposure to those substances promotes also the selection of bacteria presenting a high mutation rate (mutators) because this characteristic increases the opportunity of generating, through mutations, a favourable genotype, permitting the bacteria survival. Antibiotics select not only strains resistance to themselves, but also, promoting the mutators spread, select indirectly strains that can develop resistance toward non-related antibiotics.

It has been seen in a study by Baharoglu and Mazel in 2011 that the treatment of *V. cholerae* cells with sub-lethal concentration of many different antibiotics determines an up regulation of genes controlled by SOS system. Moreover, a high spontaneous mutation frequency was observed in bacteria treated with low antibiotic concentrations that was not

observed in strains lacking SOS regulon. For these reasons, sub-MIC drug concentrations have an important role in SOS response induction, even if the phenomenon is not seen in all bacteria and with all antibiotics (see below) (Baharoglu and Mazel, 2011).

The spread of antibiotic resistance is becoming also a problem for consumers, because food can be a vehicle of AR genes. In animal agriculture the use of antibiotics in food animals promotes the development of resistant bacteria that can then be transferred to humans. If they survive the acid pH of the stomach they can potentially determine a disease in the consumer. Even if they don't survive outside of the live animal, their AR genes may be available for uptake by other bacteria via HGT. A study of Durso *et al*. in 2012 showed that 0.7 to 4.04% of the metagenome sequences of environmental samples are genes involved in antibiotic resistance (Rodriguez-Rojas *et al.*, 2013).

For all these reasons, low environmental concentrations of antibiotics should be monitored, and RecA and LexA (the regulators of SOS response) should be considered as targets for the reduction of the resistance mutation rate in environmental bacteria. In general, sub-inhibitory concentrations of antibiotics increase the frequency of HGT, both *in vitro* and *in vivo*, resembling to a positively regulated mechanism of switch. This leads to a new concept of "hormesis": low concentrations may regulate a set of genes in target bacteria; higher concentrations determine a stress response and extremely high quantities are lethal (Aminov, 2011). Despite the fact that some countries have enacted legislations to limit the non-therapeutic use of antibiotics especially in food animals, their use in aquaculture and agriculture is still widespread (Aminov, 2011).

Mobile genetic elements

Natural selection promotes the evolution of strategies that increase the adaptation rate; in eukaryotes the genetic variability is mainly due to sexual reproduction, which involves chromosomal recombination, and to mutations that are rare events and that determine only localized changes in the genome. In prokaryotes other factors are responsible of evolution: point mutations, high levels of recombination and the transfer of genetic material between different species and even different genera. This process, referred to as Horizontal gene transfer (HGT), represent a cornerstone of bacterial evolution, involving much broader changes in their genome (Hacker *et al*., 2001). It explains the genetic bacterial diversity and rapid acquisition of resistance determinants seen in epidemic strains and their variability among different outbreaks in the same areas (Ceccarelli *et al*., 2006). The impact of lateral gene transfer on bacterial evolution is confirmed by the fact that foreign DNA represents up to one-fifth of the bacterial genome. The first portrayal of HGT was made by Griffith in 1928, who described the transformation of avirulent

pneumococci into virulent ones due to the addition of factors coming from dead avirulent microorganisms (Aminov, 2011).

HGT of mobile genetic elements (MGE) is the most important mechanism of dissemination of multidrug resistance among bacteria. Bacteria can exchange their genes via conjugation, transduction or transformation; the first two require plasmids or viruses to transport the DNA to the recipient cell, while transformation is a process of exogenous DNA acquisition through the cell membrane. The majority of the transferred DNA is part of the flexible bacterial gene pool that comprises phages, plasmids, transposons, integrons, genomic islets, integrative and conjugative elements (ICEs) and genomic islands (GIs) (see Figure 9). Those elements show some characteristics such as a different G+C content and codon usage and encode additional functions that are not essential for bacterial growth. The core gene pool comprises the bacterial chromosomal genes encoding proteins with a basic role for the bacterium and thus indispensable for its survival (translation, metabolism, architecture). The number of genes in a bacterium that belong to the flexible pool vary from 18% (*E. coli* K12) to 1% (*Mycoplasma*) of the total genome (Hacker *et al*., 2001).

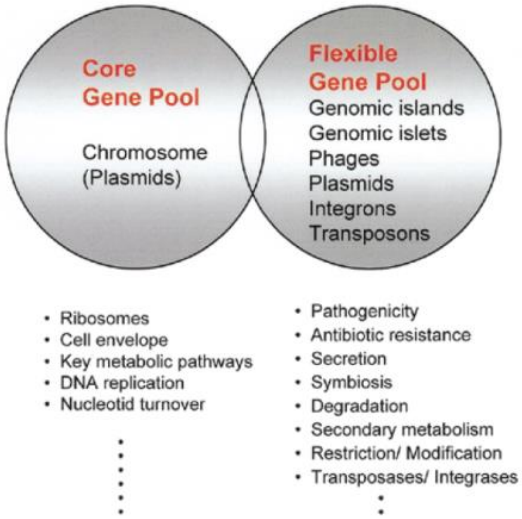

Figure 9: The two DNA pools of bacterial genome and their functions (Hacker et al., 2001).

Analyzing the genomes of many bacterial species, lots of cases of possible HGT events have been revealed; as an example, 600kb of *E. coli* genome may have been acquired via this process (Dahlberg *et al*., 1997).

Considering that open oceans are oligotrophic environments with an organic carbon concentration of about 1 mg/liter, it is not surprising that marine microorganisms have developed a complex physiological strategy to remain viable even in condition of

nutrients deprivation. Enteric bacteria, introduced in sea water by wastewater effluents, can also survive but are not able to multiply there. The HGT can represent then a system for dissemination of novel genetic information in the marine environment, contributing to the spread of plasmids carrying antibiotic resistance genes and novel gene combinations from genetically manipulated organisms used in agriculture or in other anthropogenic activities. Some studies suggests that some of the plasmids found in marine bacteria were introduced to the marine environment via terrestrial bacteria, as similar plasmids can be found in bacteria from septic tanks and bilge water. As a consequence, those plasmids have possibly arrived to humans determining clinical concerns in the treatments of infection even in the hospital practice, and an exponential rise of multi-resistant strains over the past three decades. This spread represents an example of molecular evolution over a very short time scale (Goodman *et al*., 1993). It has been demonstrated that stress conditions determine an increased rate of HGT, for example UV radiations or starvation affect the mobility of MGEs. In particular, the SOS response induced by DNA damaging agents such as mitomycin C and antibiotics, leads to the expression of transfer-activator genes, resulting in a 300-fold increase of HGT rates (see regulation SXT transfer; Beaber *et al*., 2004). As a consequence, the use of SOS response-inducing antibiotics may result in a co-selection of other antibiotic resistance genes located in the same MGE.

- Plasmids

Plasmids are ubiquitous vectors for HGT and in several cases carry genes for antibiotic resistance. Mobility is an essential activity for plasmid fitness and together with the capability of DNA replication constitute the function needed for plasmid survival. They can spread by conjugation if they carry two sets of genes: mobility genes (MOB) allowing conjugative DNA processing and membrane-associated mating pair formation (MPF) genes, forming of a type 4 secretion system (T4SS), providing the mating channel. A plasmid that codes for its own set of MPF genes is called self-transmissible or conjugative, because it encodes all the machinery for its mobilization (see Figure 10): relaxosome components, type IV coupling protein T4CP (for the connection between the relaxosome and the transport channel) and the components of the mating channel that assemble a T4SS which transports the relaxase protein bound to the DNA. If it has its own relaxase but it uses an MPF already present in the cell, it is called mobilizable. Some plasmids are called non-mobilizable because they are neither conjugative nor mobilizable. They spread by natural transformation or by transduction (Smillie *et al*., 2010).

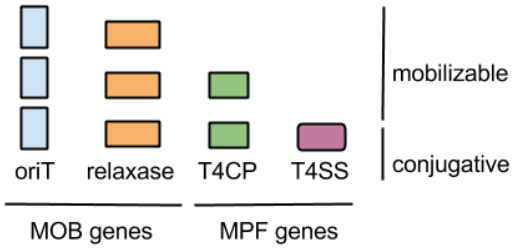

Figure 10: Genetic constitution of transmissible plasmids. Conjugative plasmids code for the four components of a conjugative apparatus: an origin of transfer (oriT), a relaxase, a type IV coupling protein (T4CP) and a type IV secretion system (T4SS). Mobilizable plasmids contain just a MOB module and need the MPF of a co-resident conjugative plasmid to become transmissible by conjugation (adapted from Smillie et al., 2010).

The only protein component of the conjugative machinery that is common to all transmissible, i.e., conjugative or mobilizable, plasmids is the relaxase. The relaxase is a key protein in conjugation, since it recognizes the origin of transfer (*oriT*), a short DNA sequence which is the only sequence required to be conjugally transmissible. The relaxase catalyzes the initial cleavage of *oriT* in the donor, to produce the DNA strand that will be transferred, and the final ligation of the transported DNA in the recipient cell. Non-transmissible plasmids can move by transduction, natural transformation or co-integration in mobile plasmids, but these mechanisms occur at a lower frequency than conjugation. Thus the persistence of non-mobile plasmids has to be explained by the potentially useful genes (for the host) that they carry. In this view, plasmids with no mobility may survive for long periods of time by natural selection. Finally, non-mobilizable plasmids larger than 300 kb are considered to be on their way of becoming secondary chromosomes (Smillie *et al*., 2010).

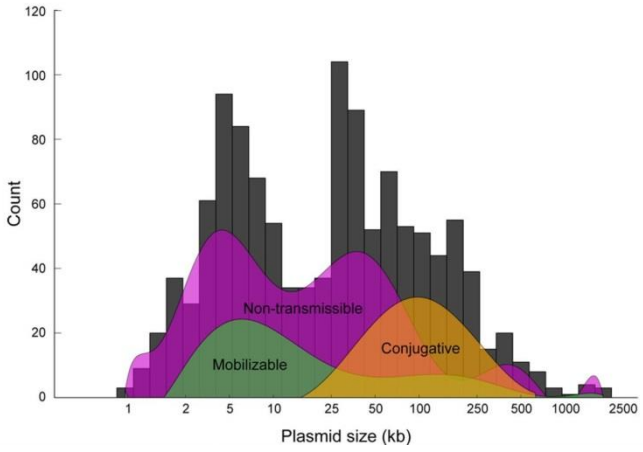

Figure 11: Distribution of conjugative, mobilizable, and non-conjugative plasmids according to plasmid size (Smillie et al., 2010).

- Integrons

A bacterium presenting an integron can capture and express environmental genes taken up as part of mobile elements termed gene cassettes. They have been found in clinical samples but also in environmental strains, especially those coming from aquatic areas and soil. Integrons are in fact important mechanisms for the acquisition of antibiotic resistance genes in many bacteria included *V. cholerae*; more than 70 different antibiotic-resistance genes have been characterized till now. Integrons are not autonomously mobile but are frequently linked to other MGE such as transposons, genomic islands, ICEs and plasmids that confer them mobility. They can thus move within or between bacterial genomes as part of the MGE they reside in, in a process called hitchhiking. Many studies suggest they are involved in horizontal dissemination because they have been found in different species, areas and times but always with the same structure (Domingues *et al*., 2012).

Five classes of integrons have been identified according to substantial differences in the integrase sequence located in the 5' conserved sequence (5'-CS); a 98% of sequence identity is required between the integrases belonging to a certain class. However, it has been seen that many evolutionarily-related integrons share a low degree of sequence similarity in the *intI* gene; it seem thus possible that *intI* has been transferred not only vertically but also via lateral gene transfer between different phylogenetic groups.

The most clinically relevant integrons are those from class 1, 2 and 3. In particular, class 1 integrons are the most frequent in clinical isolates, are responsible for a substantial proportion of multi-drug resistant nosocomial pathogens and deeply contribute to antibiotic dissemination. This class of integron consists of two conserved segments 3'-CS and 5'-CS separated by a variable region containing one or more gene cassettes made up of a single promoter-less gene and a recombination site, called 59-bases element or *attC* (Ceccarelli *et al*., 2006). The 3'-CS contains the *qacEΔ1* and *sul1* genes responsible of resistance to quaternary ammonium compounds and sulphonamides, and an open reading frame *orf5*. The 5'-CS contains the *intI1*gene coding for the integrase, the recombination sequence *attI1* site and the Pc promoter (see Figure 12).

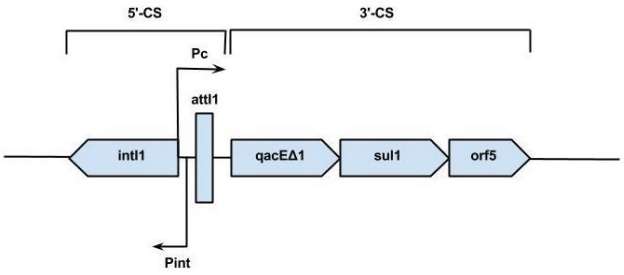

Figure 12: General structure of class 1 integron (adapted from Larouche and Roy, 2011).

Gene cassettes are small mobile elements presenting a single and promoter-less gene (generally conferring antibiotic resistance) and a recombination site *attC* located both at the end and at start of the cassette. Cassettes are linear when integrated, and circular when in the free form. Gene cassettes are inserted in the *attI* site via an IntI-mediated site-specific recombination between the two *att* sites (see Figure 13 a and b).

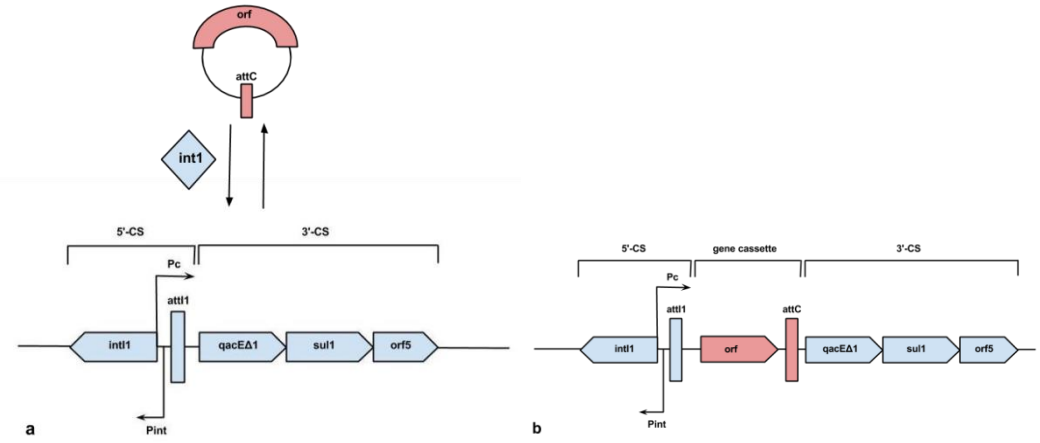

Figure 13: a) the integron-gene cassette recombination mechanism; b) the obtained integrin with the gene cassette (adapted from Boucher et al., 2007).

The level of transcription of the cassettes depends on the distance to the promoter: the closer are generally expressed at higher level than the distal ones. Multiple insertion events are frequent and cassettes are generally inserted in the same orientation to assure the gene transcription starting from the Pc promoter, while the integrase gene is usually in the opposite orientation. An exception is given by the "inverted integrase integrons" whose *intI* gene has the same orientation as the gene cassettes (Boucher *et al*., 2007).

Class 1 integrons are generally found in Gram positive bacteria isolated from human clinical samples, or in Gram-negative bacteria coming from a wide range of contexts such as animals, food products, soil and aquatic environments. They can be associated with transposons and/or conjugative plasmids that serve as vehicles for the intra and

interspecies transmission of genetic material. As such, they can be found in a variety of genetic contexts and among a large number of phylogenetically diverse Gram-negative and Gram-positive isolates.

Recently a new type of integron, named the super-integron (SI), has been found in chromosome 2 of *V. cholerae*. This element spans 126 kb and comprehends at least 179 gene cassettes. It shows some characteristics that distinguish it from known mobile integrons: a larger number of cassettes, a chromosomal location (they are considered to be sedentary), a mostly linear descent within a given group, i.e. there is no evidence of lateral gene transfer of the core, and a high homology observed between the *attC* sites of the cassettes. Moreover, the gene cassettes contained in SIs share a common characteristic: the presence at their boundaries of two core sites (CSs) in the same orientation with a sequence that is the target for the recombination process. SIs represent a potent system for rapid adaptation to the unpredictable changes of the environment, by allowing bacteria to scavenge foreign genes increasing their fitness (Rowe-Magnus *et al*., 1999).

- ICEs

ICEs are a large class of self-transmissible genetic elements that are mediators of HGT among prokaryotes. They encode many properties including drug resistance and they transfer by conjugation, similarly to conjugative plasmids but unlike plasmids ICEs do not replicate autonomously. They also show a similarity with temperate bacteriophages because they integrate in the bacterial chromosome and can replicate within it; moreover they can excise, if external conditions are favorable, and form a circular intermediate that can be transferred by conjugation. ICEs are usually named with the prefix ICE followed by an abbreviation for the bacterial species, three letters to indicate the country and a number to distinguish the isolates (Burrus *et al*., 2006). The term ICE was introduced in 2002 by Burrus *et al*.; the first one to be identified was *Tn916* isolated in 1980 from *E. faecalis*, then during the past decade an increasing number of ICEs have been described.

The ICE family **SXT/R391** is one of the largest known and is composed of more than 40 members, found both in clinical and environmental strains of γ-proteobacteria. SXT elements are also called 'constin' i.e. conjugal, self-transmissible, integrating elements. R391 was originally derived from a 1967 South African *Providencia rettgeri* isolate, and it can reside together with SXT in the same host. A cell that contains one of these two ICEs can acquire a copy of the other ICE, yielding tandem arrangements of SXT and R391 in the host chromosome. Tandem repeat structures are often excellent substrates for recombination and exconjugants derived from donor strains containing such tandem

arrays sometimes contain hybrid ICEs with genes from both R391 and SXT (Garriss *et al*., 2009).

Vibrio cholerae O139, the first non-O1 serogroup of *V. cholerae* to give rise to epidemic cholera, is characteristically resistant to the antibiotics sulphamethoxazole, trimethoprim, chloramphenicol and streptomycin. Resistances to these antibiotics are encoded by this 62 kb self-transmissible, conjugative, chromosomally integrating element designated as 'SXT element' that is involved in the emergence of multidrug-resistant *V. cholerae* strains. The resistance to the 4 antibiotics (sulfamethoxazole (Su), trimethoprim (Tm), chloramphenicol (Cm) and streptomycin (Sm)) is due to the presence of the correspondent resistance genes (respectively *sullI*, *dfr18*, *floR*, *strA/B*, see Figure 14) (Burrus *et al*., 2006) (Ceccarelli *et al*., 2006).

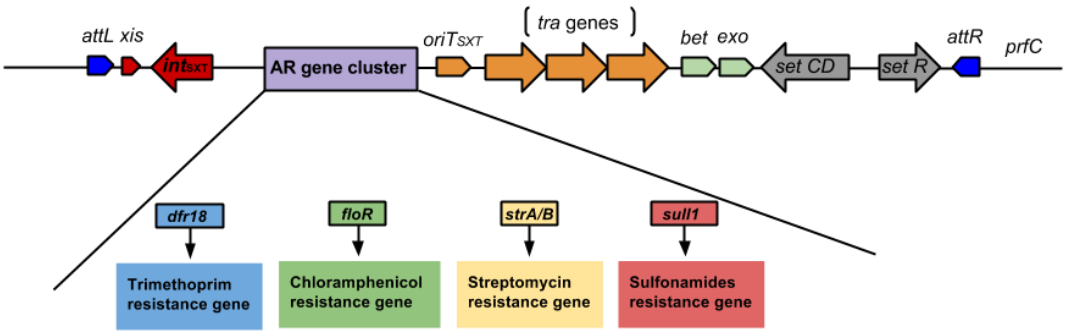

Figure 14: Representation of the core structure of the SXT/R391 ICE; ORFs are color-coded as follows: genes encoding the conjugative transfer machinery (tra genes and $oriT_{SXT}$), orange; genes involved in DNA recombination and repair (bet and exo), light green; site-specific excision and integration genes (xis and int_{SXT}), red; genes involved in regulation (setCD and setR), grey; cluster of antibiotic resistance genes, purple (adapted from Wozniak et al., 2009).

SXT encodes resistances to many antibiotics, including sulfamethoxazole and trimethoprim, that abbreviated give the name SXT. Comparative analysis of 13 SXT/R391 genomes done by Wozniak *et al*. in 2009, revealed that they share a conserved core region of 47kb containing a syntenous set of 52 genes involved in regulation, excision, integration and transfer with a 97% sequence identity and they are clustered with genes with similar function. The core is interrupted by clusters of variable genes encoding a large array of functions, conferring to the host specific properties such as antibiotics/heavy metals resistance, motility, biofilm formation regulation, aromatic compounds degradation, nitrogen fixation and toxin-antitoxin systems that prevent the ICEs' loss.

Those elements have become widespread in Asia and Africa: currently almost all Asian *V. cholerae* clinical isolates contain SXT-related ICEs. Recently SXT has been isolated even in other countries such as Spain and Mexico (see Figure 15); these findings suggest a long-term association of ICEs with *Vibrios* and bacteria in general terms (Burrus *et al*., 2006).

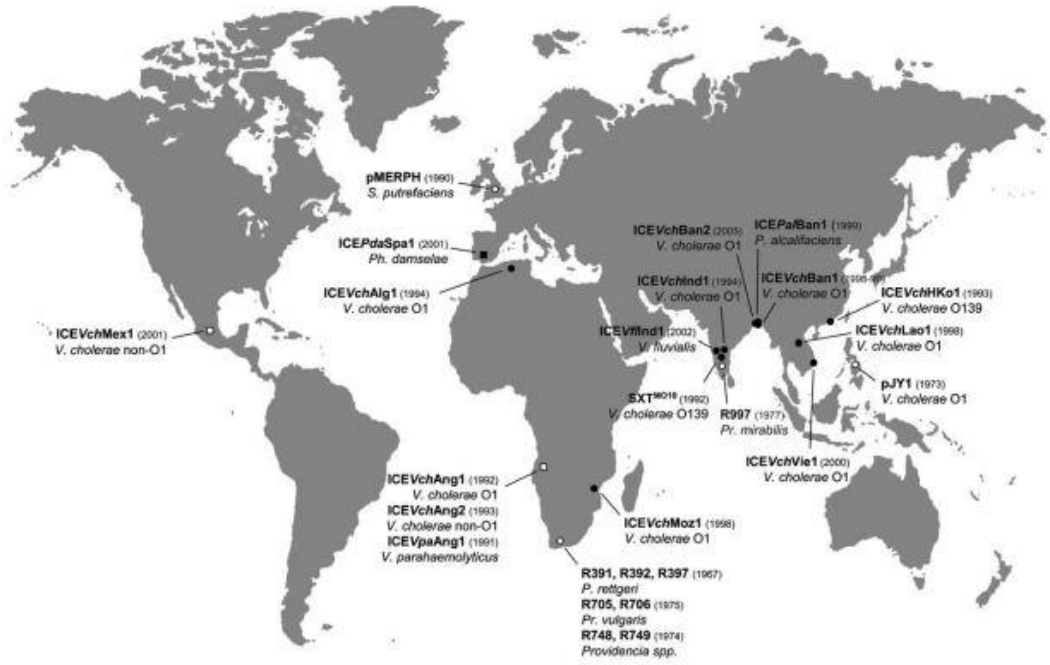

Figure 15: The worldwide distribution of SXT-related ICEs (Burrus et al., 2006).

The study of Rodriguez-Blanco *et al*. (2012) revealed a trend of gradual emergence of SXT/R391-like ICEs and report the isolation of these elements in 5 more aquatic bacteria such as *Vibrio splendidus* and *Shewanella haliotis*, thus contributing to the broadening of their known host range.

SXT TRANSFER MECHANISM

The SXT transfer requires a series of steps (see Figure 16):

1. **Excision from the host chromosome**. Both the excision and the integration of SXT require an SXT-encoded tyrosine recombinase Int_{SXT}. Int_{SXT} mediates the ICE excision from the host chromosome, recombining the flanking *attL* and *attR* sites to regenerate *attP* and *attB*, the sites of integration located on the plasmid and on the chromosome. The excision from the chromosome is facilitated by the recombination directionality factor (RDF) Xis. Then, SXT forms a circular molecule and the relaxase Mob, which is part of a multiprotein complex called relaxosome, recognizes the origin of transfer (*oriT*).

2. The Mob protein generates a nick in one strand and becomes covalently bound to the 5' end of the nicked strand.
3. **Conjugative transfer**. the single strand of SXT interacts with the type IV coupling protein (T4CP) which generates the energy for its translocation through the mating pore, a type IV secretion system (T4SS) formed by proteins related to the ones used by the IncA/C plasmids.
4. Once transferred in the recipient cell, the Mob protein ligates the single-stranded DNA molecule and the complementary strand is synthesized.
5. **Integration of the transferred molecule into the host chromosome:** Integration in the recipient cell's chromosome into the 5' end of the *prfC* gene is mediated by recombination between nearly identical 17bp sequences found both on the chromosome (*attB*) and on the plasmid-like form (*attP*). The integration provides a novel 5' coding sequence and a promoter for RF3 expression (Bordeleau *et al*., 2012).

SXT encodes exclusion systems that inhibit redundant transmission of ICEs between donors. The gene *eeS* codes in fact for inner membrane proteins that are dispensable for transfer from the donor cell.

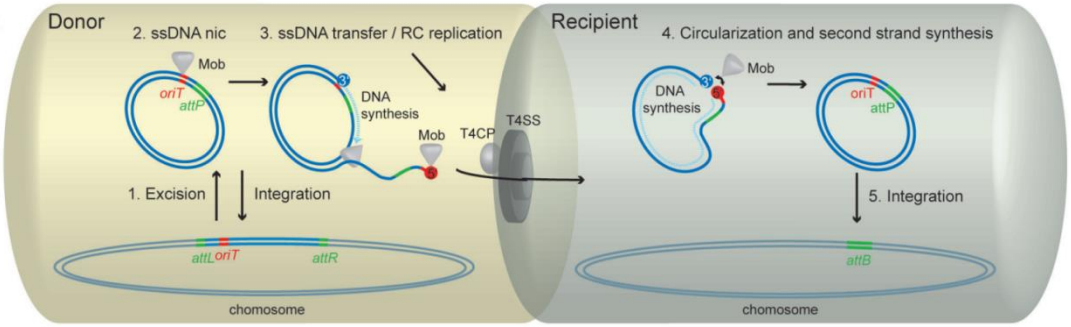

Figure 16: Schematic representation of SXT conjugative transfer (Bordeleau et al., 2012).

The members of SXT family have a great genomic syntheny in particular in genes involved in mobilization. All the ICEs that encode an integrase gene similar to int_{SXT} and that integrates into the 5' end of *prfC* are considered part of the SXT/R391 family because this it is considered a defining feature of those elements (Daccord, Ceccarelli and Burrus, 2010).

A work of 2008 by Taviani *et al*. reported a group of bacteria harbouring ICEs but lacking the typical resistance profile of SXT-related elements, this was due to the absence of the gene cluster responsible of the antibiotic resistance. The existence of ICEs lacking the resistance genes confirm the hypothesis of the presence of cryptic genetic information

in them, encoding for favorable factors besides drug resistance. ICEs in fact can contain genes involved in nitrogen fixation; can participate in the mobilization of pathogenicity islands determining a change in bacteria virulence, or even code for DNA repair systems. SXT and ICEs should then not be considered as just means of resistance transmission but also as vectors for genetic information, both between the *Vibrio* genus and towards other species (Taviani *et al*., 2008).

Finally it has to be noticed that SXT elements are able to mobilize non-mobilizable plasmids in *trans* and chromosomal DNA in a directional fashion in *cis* from strain to strain (see GIs). In particular, SXT-mediated mobilization is not dependent upon excision of the element itself from the chromosome, but relies on the recognition of a similar *oriT* by the ICE-encoded relaxase. We can conclude that SXT may have an important role in cross-species gene transfer, presumably any DNA sequence within 500 kbp of the 3' end of *prfC* could be mobilized by them, providing an alternative pathway for virulence genes' mobilization. For this reason bacteria harboring an SXT element have a huge potential of promoting horizontal gene transfer and this can explain the widespread dissemination of these elements in bacterial populations (Hochhut *et al*., 2000).

REGULATION OF SXT TRANSFER

The pathway regulating SXT transfer is very similar to the one that governs the lytic development of phage λ and is well shown in Figure 17. Under non-inducing conditions SetR, the SXT coded master repressor, binds to 4 operators and represses the expression of a subset of genes, including *setC* and *setD* that are the key elements in the transfer mechanism. In fact they encode transcriptional activators that promote the expression of *int* and of *tra* that are necessary for SXT mobilization. An increase in SXT transfer has been seen in presence of DNA-damaging agents such as mitomycin C, UV light and ciprofloxacin that promote the SOS response. As illustrated in Figure, those agents, alleviating SetR repression (probably stimulating its autocleavage), induce the expression of *setC* and *setD*, promoting SXT transfer. All SXT-related ICEs contain those elements, thus the basic scheme of regulation can be applied to all of them (Burrus *et al*., 2006).

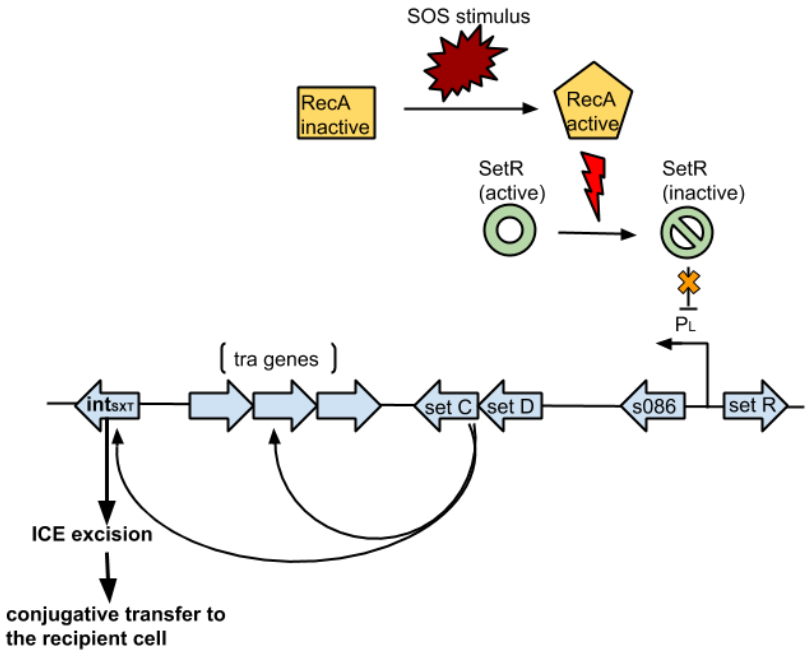

Figure 17: Effects induced by SOS response on SXT transfer (adapted from Beaber et al., 2004).

- Genomic islands

Genomic islands (GIs) are a superfamily of mobile elements constituted by DNA segments 10 to 550kb long, flanked by short direct repeats and inserted adjacent to tRNA loci; they can be acquired by HGT. All GIs contain a recombination module that consists of: a site-specific recombinase (integrase) of the tyrosine recombinase family, two attachment sites *attL* and *attR*, and sometimes a recombination directionality factor RDF (see Figure 18) (Boyd *et al.*, 2009).

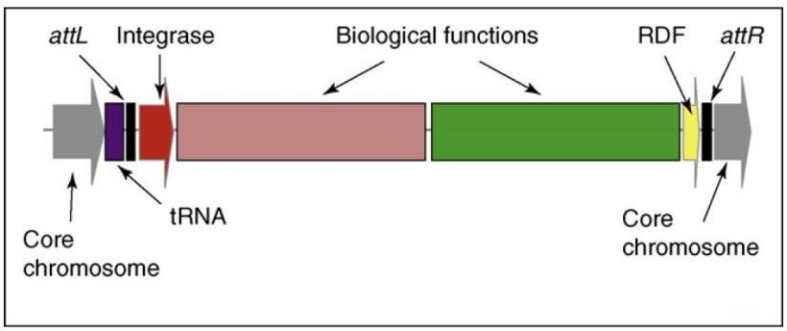

Figure 18: Representation of the main components of a genomic island. Grey arrows represent core chromosomal genes. The purple box represents a tRNA gene, and black boxes represent attachment sites (attL and attR). The red arrow indicates an integrase gene and the yellow arrow indicates RDF. The other rectangular boxes represent genes with diverse biological functions, which differ between island regions (Boyd et al., 2009).

The sequences of GIs display certain properties that mark them as atypical compared to the overall genome of the organism in which they are found. Among them, the presence of a large chromosomal region only in a subset of isolates of a species and absence in closely related isolates, presence of mobility genes such as integrases and transposases, association with a tRNA gene, flanking direct repeat sites, a G+C content which differs significantly from that of the host organism, and instability in the chromosomal insertion sites (Murphy and Boyd, 2008).

A study of 2012 made by Fernandez-Gomez *et al.*, revealed that GIs are present in 94% of the studied bacterial genomes, composed by 70 selected marine bacteria. They found that 70% of the GIs contained MGE, such as transposons, integrons and phage-related genes. Approximately 55-60% of the GIs genes are hypothetical proteins, versus 28% in the rest of the genome. Genes present in the GIs were also annotated assigning them to functional categories using the Clusters of Orthologous Groups (COG) method; as expected the most common group was category L (replication, recombination and repair), that represents the 20% of the total (see Figure 19) (Fernandez-Gomez, 2012).

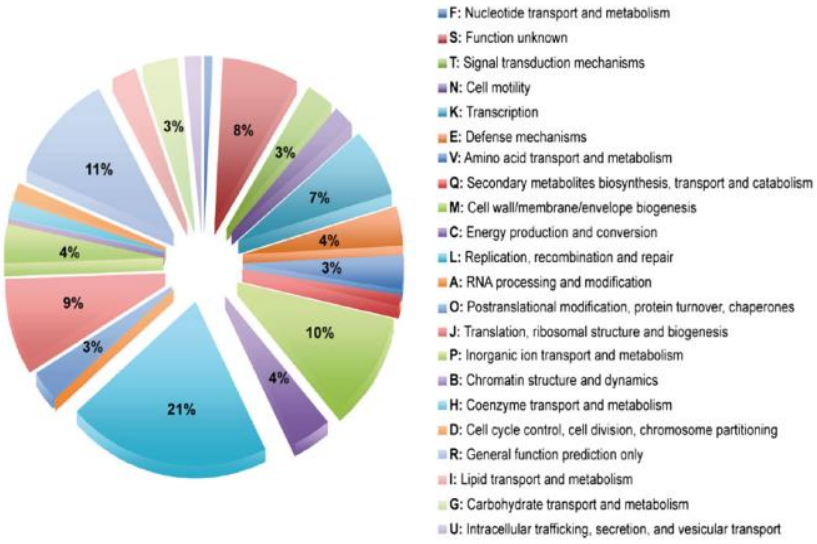

Figure 19: Distribution of annotated genes within the GIs according to their COG category (Fernandez-Gomez, 2012).

When a Gls contains genes conferring to the bacteria fitness advantages, like colonization properties or virulence factors that promote the survival and the pathogenicity in the host, it is referred to as Pathogenicity Island. The term "pathogenicity island" was first used by Hacker to describe unstable regions of *Escherichia coli* isolates different from any other described integrative element. GIs includes genes for a multitude of functions such as: resistance to antibiotics (resistance islands), enhancement of the survival rate of the

bacterium (fitness islands), ability to utilize carbon and nitrogen sources (metabolic islands), ability to break down novel compounds (degradation islands) (Domingues *et al*., 2012).

Finally GIs can contain genes associated with biological functions, such as translation and transcription's regulation. It is then proposed that GIs have a role in increasing fitness under disadvantageous conditions by providing not only novel genes, but also modulating their transcription and regulation (Fernandez-Gomez, 2012).

In most cases their exchange mechanisms are unknown, but Murphy and Boyd in 2008 suggested that their transfer depends on the presence of mobilizing self-transmissible elements and involves uptake by transformation or hitchhiking with mobile elements such as phages, plasmids or ICEs. They reported the excision and circularization of three GIs found in seventh pandemic *V. cholerae* isolates, included VPI-2. In addition we have other examples of GIs mobilizable by mobile elements, such as the *Staphylococcus aureus* pathogenicity island (SaPI) that can be transferred in presence of a phage, or the pathogenicity island found in many *Enterobacteriaceae* that is physically linked and mobilized by ICEs (Daccord *et al*., 2010). The vast majority of GIs doesn't have any known mechanism of transfer and are considered non-self-transmissible.

Recently a new family of small mobilizable genomic islands (MGIs) has been identified in vibrios; their core structure is restricted to $oriT_{MGI}$, and 4 conserved genes: int_{MGI}, *cds4*, *cds8* and *rdfM* (Daccord *et al*., 2012).

They can be mobilized by SXT/R391 ICE using a *cis*-acting $oriT_{MGI}$ sequence, mimicking $oriT_{SXT}$ that can be recognized by the SXT machinery, despite the 37% divergence between the two sequences (Daccord *et al*., 2012). The frequency of co-transfer was only slightly lower than the ones of each element. The structural organization of those elements on the MGI is showed in Figure 20: int_{MGI} is adjacent to *attL*, and *rdfM* is on the opposite side, near the *attR* gene. The integrase gene *intV2* (VC1758) and the RDF gene *vefA* (VC1809) of the *Vibrio* pathogenicity island 2 (VPI-2) are organized in a similar manner.

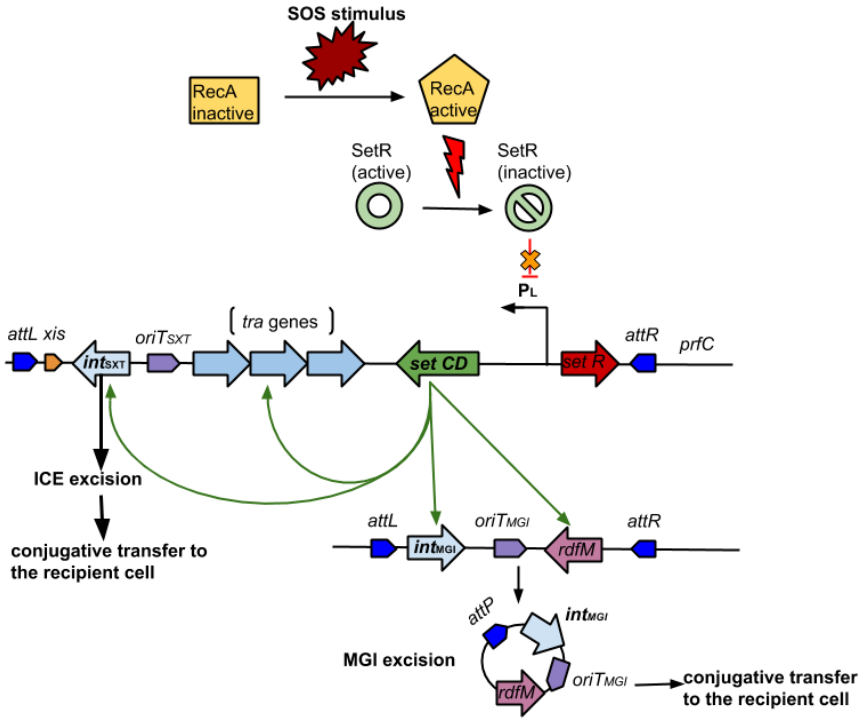

Figure 20: Model of ICE-mediated mobilization of a MGI.

They showed that while the MGI-encoded integrase (int_{MGI}), alone is sufficient to promote its integration into the chromosome, the MGI-encoded RDF RdfM is necessary for the excision process. RDFs are Xis excisionases, i.e. small proteins playing architectural roles in recombination events catalyzed by a recombinase. Both int_{MGI} and *rdfM* are activated by SetCD regulator encoded by SXT. As a consequence, MGI cannot excise from the chromosome of a cell without SXT-induced SetCD activation, but once in a recipient cell it can autonomously integrate through the action of int_{MGI} expressed at low level even in absence of SetCD (Daccord *et al*., 2012). Once the MGI is excised as circular molecule, $oriT_{MGI}$ is recognised by the ICE-encoded relaxase to be translocated into the recipient cell as ss-DNA through the mating pore encoded by the ICE.

This process allows MGIs to establish in the host cell and to be maintained in its progeny even in absence of SXT element; this mechanism prevents MGI loss due to the absence of a mobilizing ICE. This is a clear example of how a non-self-transmissible element can take advantage of the conjugative machinery of other MGEs to successfully be transferred (Daccord *et al*., 2012). Finally it is to say that MGIs besides promoting genomic plasticity through their own mobilization, can also mobilize at least 1Mbp of DNA located at the 5' of their integration site.

In conclusion, the proposed model of mobilization of MGIs by SXT is the following: in absence of an ICE the GI remains integrated avoiding loss during cell division that could

occur to the plasmid-like form. When the ICE is present in the bacterium, and if DNA is damaged, MGI excises thanks to the ICE machinery. In particular SetCD acts as a transcriptional activator of genes for transfer (*tra*) and excision (*int* and *xis*) (Daccord *et al*., 2010).

SALMONELLA GENOMIC ISLAND 1

Salmonella enterica serotype Typhimurium phagetype DT104 (hereafter referred to as *Salmonella* DT104) is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (commonly abbreviated ACSSuT). It has recently emerged in a number of countries, it is the second most prevalent phagetype of *S. enterica* Typhimurium in England and Wales and it is increasing in the USA and Canada. Outbreaks of multidrug resistance (MDR) Typhimurium DT104 have also been reported in poultry, beef, cheese, and swine in numerous countries. The genes conferring the multidrug resistance have been identified and they were reported to be the same and in the same order in different isolates, suggesting a dissemination by HGT of the multidrug resistant region. Boyd *et al*. in 2000 using a genomic walking approach identified the genetic element responsible for the MDR: the *Salmonella* genomic island 1 (SGI1), a 43-kb chromosomal mobile element. As shown in Figure 21, SGI1 is located between the *thdF* gene and a cryptic retronphage or the *yidY* gene, at 3.89 Mb in the *Salmonella* genome (Boyd *et al*., 2000). It is flanked by two 18-bp direct repeats DR-L and DR-R which appears to be a duplication of the 3' end of the *thdf* gene, involved in thiophene and furan oxidation in *E. coli.*

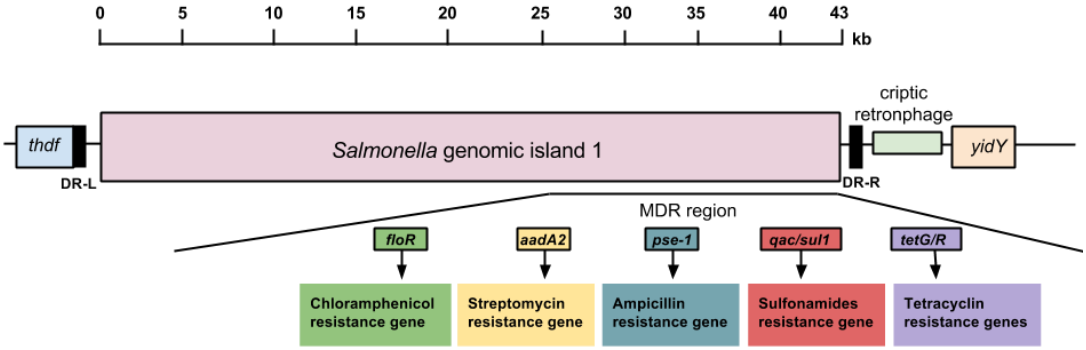

Figure 21: Representation of the SGI1 canonical structure.

The duplication indicates a site-specific recombination event that results from the insertion of plasmids or prophages. In addition to the MDR region, it contains at least 25 open reading frames (ORFs), including an integrase gene *int* and an excisionase gene, some of which showed similarity to genes commonly found on conjugative plasmids. The

majority of Typhimurium DT104 isolates shows similar genetic makeup of the MDR region, that when in its entire form is comprised of the *floR* and *tetR-tet*G genes bracketed by two class 1 integrons both carrying a *qac/sul1* gene and a *pse-1* or *aadA2* cassette, clustered on a 16-kb region of the Typhimurium DT104 genome (Boyd *et al*., 2001). Many variant SGI1-like elements exist, probably due to various assembly of this island with other elements such as plasmids. in particular a high variability has been seen in the MDR region, determining different antimicrobial resistance patterns associated to SGI1; this suggests that a high level of recombination occurs in this particular region of SGI1 (Boyd *et al*., 2002).

Boyd *et al*. in 2000 showed that SGI1 is not an autonomous MGE, because it can't excise from the chromosome; for its conjugal transfer an helper plasmid of the IncA/C family is in fact required (Douard *et al*., 2010).

VIBRIO PATHOGENICITY ISLANDS

As described from Hacker *et al*. in 1997, a pathogenicity island is a large instable chromosomal region encoding several virulence genes, it is present in pathogenic isolates and absent from non-pathogenic ones, it shows a G+C content different from the rest of the genome, it is associated with a tRNA gene, it has repeated sequences near the integration site and it contains a bacteriophage-like integrase (Jermyn and Boyd, 2002). *V. cholerae* O1 and O139 serogroup isolates harbor four PAIs: *Vibrio* pathogenicity island-1 (VPI-1), VPI-2, *Vibrio* seventh pandemic island-I (VSP-I), and VSP-II.

VPI-1 contains the TCP cluster that allows the bacteria to colonize the human intestinal tract; it is 41 kb in size and includes 13 kb of previously unidentified DNA. It is hypothesized that all the genes on the VPI-1 are important in disease, either having a direct role in cholera pathogenesis or an indirect role in the transfer and mobility of the VPI-1, thereby creating the potential for the emergence of new epidemic and pandemic strains.

This VPI contains genes such as *tcpA* that encodes an important colonization factor, the toxin-coregulated pilus (TCP), *toxT*, *tcpP*, and *tcpPH* that encode regulators of virulence genes, genes that may be required for the transfer and integration of the VPI and DNA of uncharacterized but potentially important function (Karaolis *et al*., 1998).

VPI-1 is generally found in clinical isolates and sporadically in environmental ones; it can move through phage transduction or co-mobilization along with the self-transmissible replicon SXT. When bacteria acquire the VPI-1, they become susceptible to CTXΦ transduction because the phage uses as receptor TcpA that is located on the VPI-1. It has been speculated that the acquisition of this mobile element has allowed those bacteria to

colonize the human intestine thus becoming pathogens; moreover the VPI-1 possession allows bacteria to become toxigenic after the CTXΦ infection. Finally the secretion of CT determines the excretion of lots of bacteria from the host determining the survival and spread in environment, and being a selective advantage over nonpathogenic strains (Karaolis *et al.*, 1998). Considering that for the CTX acquisition the TCP receptor encoded on the VPI-1 is needed, the VPI-1 presence is the initial and essential factor for the emergence epidemic strains (Karaolis *et al.*, 1998).

VPI-2 is a 57,3 kb chromosomal region; all toxigenic O1 and O139 isolates contain it, while it is absent in non-toxigenic non-O1/non-O139 isolates. It has been discovered by Jermyn and Boyd in 2002 that, studying a number of toxigenic and non-toxigenic isolates, founded that only the toxigenic ones contained the *nanH* gene that is encoded by a region then named VPI-2. It consists of 52 open reading frames (ORFs), VC1758 to VC1809, on the *V. cholerae* N16961 genome.

VPI-2 contains several genes as shown in Figure 22: a type-1 restriction modification system (*trmf*) protecting the bacteria from viral infections (ORFs VC1763 to VC1769), a *nan-nag* gene cluster involved in sialic acids metabolism which may act as a carbon and nitrogen source (ORFs VC1773 to VC1784), and a region homologous to Mu phage (*faghe*) (ORFs VC1791 to VC1799). In particular, *V. cholerae* neuraminidase may also form part of the mucinase complex that hydrolyzes intestinal mucus, enabling the bacterium to move readily to the epithelium.

Figure 22: schematic representation of the canonical structure of the VPI-2.

The region is quite instable, in fact some isolates contain only a part of it: the regions between VC 1760 (helicase) and VC1789 (IS911-like element) are frequently deleted. It has been suggested by Jermyn & Boyd in 2005 that the size differences and instability of VPI-2 may be associated with the presence of a Mu-like phage, because Mu phage insertion and replication occur by transposition and can thus induce chromosomal rearrangements. The VPI-2 is located in chromosome 1 within the 3' end of a serine tRNA gene, this locus is a conserved landmark for the insertion of mobile genetic elements such as bacteriophages and pathogenicity islands (Jermyn and Boyd, 2002). Thanks to some comparative sequence analysis they found out that *nanH*, and thus the *nan-nag* genes, were horizontally transferred between the *Vibrio mimicus* and *Vibrio*

cholerae, hypothesizing that VPI-2 was present in the most recent common ancestor of *V.mimicus* and transferred to *V. cholerae* soon after these species diverged. Considering that both species occupy similar environmental niches, it is possible that *V. mimicus* acts as an environmental reservoir of novel DNA for *V.cholerae* and the gene exchange could represent an emerging theme in the genus evolution (Jermyn and Boyd, 2005). A possible explanation of the re-emergence of El Tor biotype is could be the presence of VPI-2 in it and the deletion of this island from O139 serogroup (Jermyn and Boyd, 2002). The VPI-2 could thus have an important role in pathogenesis both directly influencing bacteria virulence and indirectly transferring genes that improve bacteria survival in different niches (Jermyn and Boyd, 2002).

VSP-I is a 16-kb region spanning ORFs VC0175 to VC0185, it is present only in the O1 El Tor and O139 serogroup isolates.

VSP-II is a 27-kb region integrated at a tRNA-methionine locus; it encodes homologues of an RNase H1 protein, a type IV pilus, a DNA repair protein, two transcriptional regulators, two methyl-accepting chemotaxis proteins, and a P4-like integrase.

Cooperation of MGEs

The cooperation and interaction of different mechanisms of HGT can be seen in many examples of mobility among the genetic elements normally not mobile on their own. For example the MDR genomic island SGI1 is specifically mobilized *in trans* by the conjugative IncA/C plasmid family at frequencies ranging from 10^{-3} to 10^{-6} transconjugants per donor. These MGEs may thus also contribute to the spread of the antibiotic resistance genes contained in other elements among enteric pathogens and potentially more widely.

These multidrug resistance plasmids are widely distributed among *Salmonella* and other enterobacterial isolates from agricultural sources; they were first identified more than 40 years ago from fish infected by antibiotic-resistant *Aeromonas hydrophila* and *Vibrio* spp. Resistance to beta-lactams, aminoglycosides, chloramphenicol, folate pathway inhibitors (sulfonamides and trimethoprim), quinolones and tetracycline is commonly conferred by these large plasmids (ca. 140 to 200 kb) (Carraro *et al.*, 2014).

BLAST analyses revealed that most of the conserved core SXT/R391 genes are also present in IncA/C conjugative plasmids as shown in Figure (Wozniak *et al.*, 2009). To date, the closest known relatives of the SXT/R391 transfer proteins are found in the IncA/C plasmids (Figure 23).

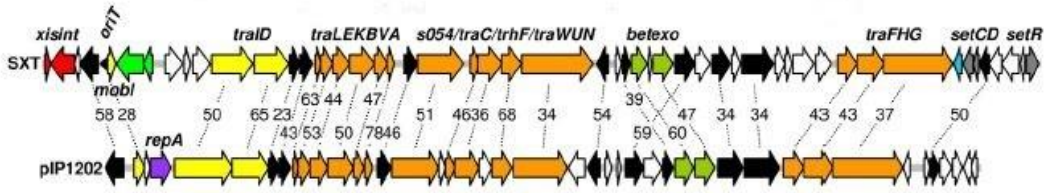

Figure 23: Alignment of the conserved core genes of SXT/R391 ICEs with the genome of the IncA/C conjugative plasmid pIP1202 from Yersinia pestis. ORFs are colour coded as follows: DNA processing, yellow; mating pair formation, orange; DNA recombination and repair, green; integration/excision, red; replication, purple; regulation, grey; entry exclusion, blue; homologous genes of unknown function, black; genes without corresponding counterparts in ICEs and pIP1202, white. Numbers shown in the middle represent % identity between the orthologous proteins encoded by SXT and pIP1202. (Wozniak et al., 2009).

Every predicted SXT transfer protein is encoded by the IncA/C plasmid pIP1202 isolated from *Y. pestis* and the identities of these predicted protein sequences vary from 34 to 78%. Furthermore, there is perfect synteny between the gene clusters encoding the respective conjugative machineries of these two mobile elements (yellow and orange genes in Figure). Despite the extensive similarity of the SXT and IncA/C conjugative transfer systems, these plasmids lack homologues of *setR* and *setD/C* as well as *int/xis*, suggesting that regulation of conjugative transfer differs between these elements. The similarity of IncA/C plasmids and SXT/R391 ICEs is not limited to genes important for conjugal DNA transfer. Ten genes of unknown function (shown in black in Figure), are similar in the two elements and most of them have an identical locations. Both elements also contain homologs of *bet* and *exo* (shown in green in Figure); these are the only known homologs of the λ Red recombination genes found outside of bacteriophages. Together, the similarity of DNA sequences and organization of SXT/R391 ICEs and IncA/C plasmids suggests that these elements have a common ancestor. The fact that the contents of the hotspots in the two classes of elements are entirely distinct suggests that their evolutionary paths diverged prior to acquisition of these variable DNA segments (Wozniak *et al*., 2009).

The genus *Vibrio*

Vibrios are Gram-negative curved bacteria, motile by a single polar flagellum. They are part of the Vibrionacee family that includes 4 genera: *Vibrio, Photobacterium, Plesiomonas* and *Aeromonas,* which differ by many properties, such as pathogenicity in

humans, G+C content, the presence of a polar flagellum, the requirement of sodium for their growth and many others.

They have a respiratory and fermentative metabolism, and include both halotolerant and halophilic bacteria, depending on their requirement for NaCl for optimal growth.

Vibrios are commonly isolated from shellfish with 100-fold higher concentration in filter-feeding organisms such as oysters. In fact their persistence in marine environments is due to the adherence to living and non-living substrates; moreover they show a deep sensitivity to temperature shifts. Water temperature represents in fact the most important factor regulating their distribution and abundance, in association with salinity; for this reason they are usually isolated from environment in warm seasons. They are widely distributed in the environment and can occupy many ecological niches; their presence depends on physical, chemical and biological factors: nutrient availability, salinity, and temperature and host presence. Finally they have a deep role in biodegradation, nutrients regeneration and bio-geochemical cycling (Belkin and Colwell, 2005).

The genus *Vibrio* includes more than 60 species and three species defined as human pathogens: *V. cholerae, V. parahaemolyticus* and *V. vulnificus*. However, other species are in some way associated with foodborne diseases. *Vibrio* infection outbreaks are generally due to consumption of food or water contaminated with faeces and sewage. In particular, *Vibrio cholerae* serogroups O1 and O139 are responsible of epidemic/pandemic diarrhea outbreaks in many parts of the world. *Vibrio vulnificus* is a common halophilic pathogen of marine and estuarine waters but is able to invade human hosts via wound infections or raw fish consumption. This bacterium causes one of the highest mortality rates but a low number of cases is reported (generally less than 40 per year in the USA). Between 1996 and 2005 a 41% increase in cases has been reported. In particular, infection due to this pathogen have been reported in Denmark, Germany, Sweden, Spain, Greece and Belgium, and it has been isolated both from shellfish and water and associated with plankton and copepods (Baker-Austin *et al*., 2010). *Vibrio parahaemolyticus* is an halophilic inhabitant of estuarine and marine environments; its virulence and pathogenicity are less serious than *V. vulnificus* and fatality is rare. However this type of infection is common worldwide and determine the first cause of infection related with seafood consumption in the USA (10.000 cases per year in the USA).

Other *Vibrio* species defined as non-pathogenic for humans but rarely associated with infections in humans have been found: *Vibrio alginolyticus, Vibrio fluvialis* and *Vibrio mimicus*. *Vibrio anguillarum, Vibrio harvey* and *Vibrio salmonicida* represent pathogens

for fish and marine inhabitants. Overall in USA, *Vibrio* species are annually the cause of about 8000 illnesses (Mead *et al*., 1999).

Vibrio cholerae and cholera

Vibrio cholerae is the etiological cause of pandemic cholera, a human gastrointestinal disease manifested by severe diarrhea, dehydration and electrolyte imbalance. It can survive both in aquatic environments and in human gastrointestinal tract thanks to its ability of switching on and off a set of virulence genes that allow a great adaptability. Its endemic nature in some areas illustrates that transmission occurs when human waste contact domestic water; in facts the Vibrio-associated infections are due to consumption of contaminated food/water or to exposure of skin lesions to contaminated water.

Cholera is a multifactorial process: starting with the bacteria colonization that is followed by expression of virulence factors and toxin action (see Figure 24). Infection is often mild or subclinical but one on 20 infected people show symptoms very severe such as dehydration and shock.

The infection usually starts with the ingestion of water or food contaminated by the bacterium; human volunteer experiments showed that high inoculum levels close to 10^{11} CFU are necessary to survive passage through the stomach acid barrier, to colonize the small intestine and to cause diarrhea. Otherwise even a smaller dose can be very dangerous: only 10^{6} CFU are sufficient to determine an acute diarrhea. It is a high dose, probably such quantities are necessary because of the exposition of bacteria to the acid pH of gastric compartment, to growth inhibitory substances like bile salts and organic acids, and to innate immune factors, such as complement secreted by intestinal epithelial cells and defensins produced by Paneth cells (Reidl and Klose, 2002).

If the bacterium survives the gastric barrier of the stomach it enters the intestinal lumen where the low-level expression of TCP enables the microorganism to adhere to the mucosal. The adhesion constitutes a second signal promoting the colonization of the small intestine's epithelia (proliferation to concentrations of 10^{8} CFU per gram) and the production of the enterotoxin cholera toxin (CT). CT is responsible of the cholera symptoms: a massive secretory diarrhea (up to 20 liters per day in adults) and vomiting start within 12-24 hours causing a severe dehydration that can lead to hypotensive shock and death by dehydration, aiding the bacterial expulsion from the infected host, and facilitating transmission to new hosts (Schild *et al*., 2007).

Following attachment and colonization, CT enters the intestinal epithelial cells at the apical membrane level and affects the normal ion transport, leading to chloride secretion, water loss and diarrhea. It also activates the adenylyl ciclase at the cytoplasmic surface of

the basolateral membrane. Anyway the bacterium itself does not enter the mucosa even if it is possible a role in assisting toxin delivery through a mechanism similar to type II secretion. Both ToxR and TcpP are critical for all those events (Belkin and Colwell, 2005).

The CT is encoded by the *ctxAB* genes carried by the filamentous bacteriophage CTXΦ whose bacterial receptor is the toxin coregulated pilus TCP, an essential colonization factor in humans and animals.

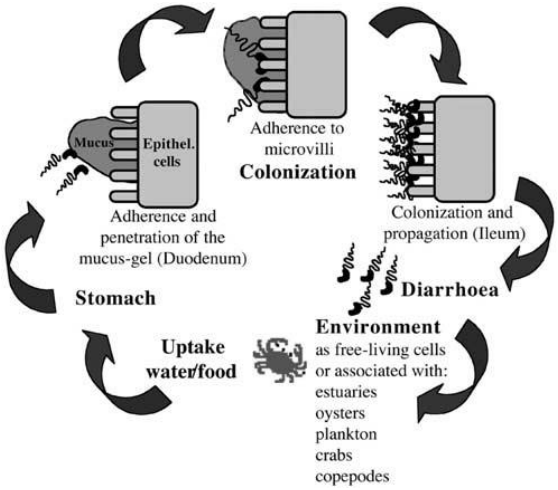

Figure 24: Infection cycle of V. cholerae (Reidl and Klose, 2002).

V. cholerae is a microorganism that normally spends a substantial part of its lifecycle outside the human host but, when introduced to humans, causes disease with measurable frequency. In the '80s it was shown by Colwell *et al*. that this pathogen is present in estuarine environments and that the human-human transmission is not the only way for the bacterial spread (see Figure 25).

In natural environment this bacterium is responsible of the remineralization of organic nutrients and its concentration in water depends on temperature, salinity and presence of planktonic organisms. *Vibrios* in fact have an increased growth at high temperatures (higher rates of *Vibrio* isolation in environment during warm seasons) and in presence of copepods. Although this, they can tolerate salinities near to 0% and till 45%, they can grow under high-pH conditions and remain stable in full sunlight.

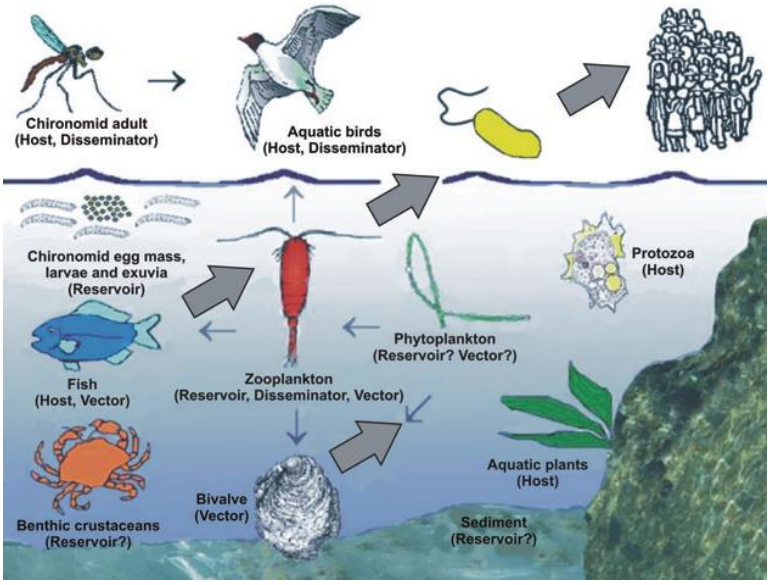

Figure 25: Vibrio cholerae reservoirs in the environment (Vezzulli et al., 2010).

Environmental reservoirs can act as both disseminators of the pathogen across large geographic areas and vectors of infection. It has been suggested that they can be responsible for the start of an epidemic and critical to disease's endemicity. In a field trial, Colwell and co-workers demonstrated a reduction 48% in cholera cases in rural Bangladesh (Colwell *et al*., 2003) by using a simple filtration method which was developed earlier by the same group (Huq *et al*., 1996). Finally reservoirs can represent an evolution system for bacteria if we consider the "sit and wait" hypothesis that predicts that virulence is positively correlated with pathogen persistence in the external environment. In fact, long-lasting persistence reduces pathogen dependence on host-to-host contact for transmission and propagation, enabling it to evolve to higher virulence levels; this can explain the alternance between long non-host phases and violent cholera epidemics (Vezzulli *et al*., 2010).

We can now understand the life cycle of this pathogen: when it is inside the human body it activates early genes, critical for the gastro-intestinal tract colonization, for example TCP genes. Later it induces the late genes that contribute increasing the fitness in the phase of transition to water. In the aquatic environment the osmolarity and carbon source drop need a change in the transcriptional pattern; this is made before leaving the host thanks to a pre-induction mechanism. Understanding the basis of bacteria-host interaction can lead to the comprehension of the persistence mechanisms, permitting the setup of appropriate defense strategies.

The virulence characteristics of *V. cholerae* are largely attributed to a series of horizontal gene transfers that, starting from a benign marine organism determined the evolution of the above-mentioned into a human pathogen. Recent studies showed in fact that genes

homologues to those coding for virulence factors are present in environmental strains and the gene exchange from clinical to marine bacteria that constitute an environmental reservoir, have probably taken place in aquatic environment. This phenomenon is possible because most of virulence genes are located in mobile genetic elements and can thus be exchanged between bacteria thanks to quorum sensing properties.

The genus *Salmonella*

Bacteria belonging to the *Salmonella* genus are Gram-negative, rod-shaped, facultative intracellular anaerobes. The genus belongs to the family *Enterobacteriaceae* and based on DNA sequences, the closest related genera are *Escherichia, Shigella,* and *Citrobacter*. analysis of DNA homology later revealed that the genus consists of two species: *Salmonella enterica* and *Salmonella bongori*. The latter is a commensal of cold-blooded animals, whereas the former is found in reptiles and warm-blooded animals and divided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae and indica*. These subspecies are further classified into more than 50 serogroups based on the O antigen structure, which reflects variation in the exposed part of the LPS, and then further divided into > 2500 serovars based on the variation in flagellin (H antigen) (Lan *et al*., 2009). Subspecies *enterica* has far more serovars than the others and contains around 60% of known *Salmonella* serovars that inhabit the intestinal tract of humans and warm-blooded animals.

Many *Salmonella* serovars are quite promiscuous as they are not host-specific and can cause disease in a wide range of hosts. Others are highly adapted to a specific host, such as S. Typhi and S. Gallinarum, which cause systemic illness in humans and poultry, respectively. Most infections result from the ingestion of contaminated food products, such as poultry, eggs and dairy products and, in children and animals, from direct faecal-oral spread. Humans infected with *S. enterica* subspecies *enterica* show one of 2 major clinical syndromes: non-typhoidal salmonellosis (gastroenteritis) or typhoid fever (systemic disease).

Typically acquired by oral ingestion of contaminated food or water, *Salmonella* has to survive passage through the gastric acid, evade killing by digestive enzymes, bile salts, opsonisation by secretory IgA, defensins and other antimicrobial peptides as well as other innate immune defence mechanisms to gain access to the underlying epithelium and deeper tissues. The gastric acid would normally reduce the inoculum size significantly, however, *Salmonella* have an adoptive acid-tolerance response, which may increase their survival through the stomach.

Salmonella infections can cause important morbidity, mortality and economic burden and are particularly severe in infants, the elderly and immunocompromised individuals. Although the outbreaks of human salmonellosis are frequently limited to single cases or confined to one family, the global data of *Salmonella* infection are impressive. In Canada about 8,000 cases of human salmonellosis are reported yearly by the National Laboratory for Enteric Pathogens. In the United States salmonellosis accounts for more than 40,000 reported cases, 500 deaths and financial costs are greater than $ 50 million each year (Lan *et al*., 2009).

During the last decades antimicrobial resistance in *Salmonella* developed tremendously. The development of resistance in *Salmonella* toward antimicrobial agents is attributable to one of multiple mechanisms, including production of enzymes that inactivate antimicrobial agents, through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of efflux pumps and modification of cellular targets for drugs. Many strains, especially serovar Typhimurium, are multidrug resistant to five or more antimicrobial agents originating from the chromosomally located *Salmonella* Genomic Island 1 (SGI-1) (Boyd *et al*., 2001).

Anthropogenic activities & bacteria

The dominant role of humans in the generation of environmental reservoirs of AR cannot be disputed: since the 1940s increasing amounts of antibiotics have been manufactured, used clinically, released into the environment and widely disseminated thus providing constant selection and maintenance pressure for populations of resistant strains in all environments. It is estimated that millions of metric tons of antibiotic compounds have been released into the biosphere over the last half-century. Many are the possible uses of antibiotics: the growth promotion and/or prophylactic use in animals and aquaculture sites, therapeutic use in humans, pest control in agriculture, use as biocides in toiletries, hand care and household cleaning products, and also the maintenance of culture sterility and selective use in research and industry. It should be noted that therapeutic use in humans accounts for less than half of all applications of produced antibiotics (Davies *et al*., 2010).

Food-related bacteria represent a heterogeneous group coming from many different environments all related to food handling or production (see Figure 26). Considering that very few species are human pathogens, for many years no studies were set up on their prevalence, spread and AR. However, an increasing interest has been recently observed, especially because of the discovery of a gene transfer mechanisms between them and the normal human flora and/or medically important bacteria.

The possible interplay between bacterial pathogens and bacteria of the normal flora needs to be deeply analysed, to avoid both direct transfer of AR bacteria to humans and indirect transfer of drug resistance genes by normal flora bacteria (Sorum and l'Abée-Lund, 2002).

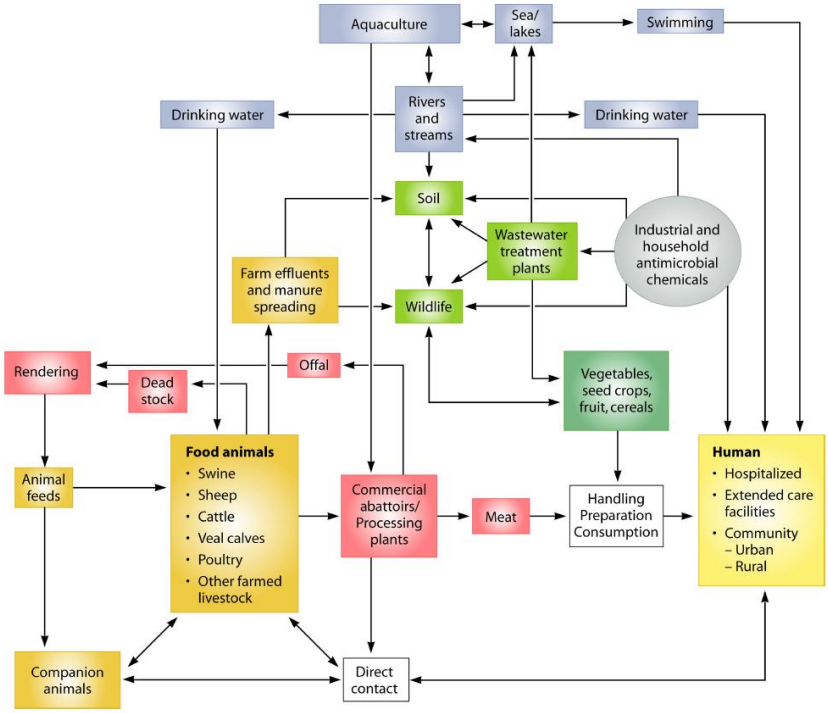

Figure 26: The numerous ecological niches linked with food-related bacteria. Arrows represent the documented or hypothetical routes of bacterial transfer (Davies et al., 2010).

Industrial aquaculture is a growing industry in all developed and also developing countries, due to the worldwide decline of ocean fisheries stock. Between 1987 and 1997 global production of farmed fish more than doubled in weight and value, and actually those products account for over one-quarter of all fish consumed by humans. This raising is accompanied by potentially damaging practices such as the use of large amounts of antibiotics, done in order to limit the spread of infections due to the high fishes' density, the poor hygienic conditions and the failure to isolate infected animals (Naylor *et al.*, 2000). As a result, an increasing use of prophylactic antibiotics has been applied, giving them to fishes as part of their food or occasionally in baths and injections. The uneaten food and the faeces, containing antibiotics, accumulate at the bottom of the pens, diffusing in sediments and are thus able to be transported by currents to even distant sites (Sorum and l'Abée-Lund, 2002).

This massive use of antibiotics can lead to a passage of those drugs into the environment, where they can be ingested by wild fish and other organisms. Residual antibiotics remain in the sediments and act as a selective pressure, altering the microflora and the plankton composition in those niches; those shifts can be amplified by the eutrophication produced in the aquaculture environment. Alterations of the ecological equilibrium have to be monitored because they can create situations impacting both fish and human health by promoting for example algal blooms and anoxic environments (Cabello, 2006).

The other consequence of the massive use of antibiotics is the selection for AR bacteria, that has resulted in an increased antibiotic resistance in environmental bacteria and in fish pathogens as showed by many studies (Sorum and l'Abée-Lund, 2002; Labella *et al*., 2013). The emergence of AR undermines the effectiveness of the prophylactic use of antibiotics in aquacultures, posing also the question of the possible passage of AR determinants to human-related bacteria, including pathogens. AR determinants have in fact the potential of being transmitted to other bacteria by HGT mechanisms such as conjugation and transduction, because of the high viruses concentration in seawater and sediments. Not unexpectedly, exchange of genes for resistance to antibiotics between bacteria in the aquaculture environment and bacteria in the terrestrial environment, including bacteria of animals and human pathogens has recently been shown. In fact it has been demonstrated that some fish pathogens such as *Aeromonas* can transmit and share determinants for resistance to antibiotics with pathogens such as *Escherichia coli* isolated from humans (L'Abée-Lund and Sorum, 2001). Similarly, different research groups have successfully transferred R-plasmids harbouring different type of class 1 integron from *Aeromonas* to *E.coli* (Rhodes *et al*., 2000; Schmidt *et al*., 2001).

Those dangerous possibilities have determined a drastic restriction of the antibiotics use in aquacultures in many countries such as Norway and Usa. Restrictions include: control of the prescription of therapeutic antibiotics, total elimination of their prophylactic use and proscription of the use of drugs that are used in human treatments. Those practises and the use of vaccines have drastically reduced the antibiotics' use in aquacultures in developed countries, indicating that a different management of those industries is compatible with economical profit. However, the use of drugs is still unrestricted in many aquaculture industries in China and Chile, where statistics says that annually about 100 metric tons of quinolones are used in veterinary medicine, with potentially dangerous consequences affecting human and animal health on a global scale (Cabello, 2006).

The transmission of AR genes between bacteria of the aquatic and terrestrial environment has been showed by the emergence of plasmid-mediated quinolone resistance among

human gram negative bacteria and the potential tracing of the origin of these determinants to the aquatic bacteria *Shewanella algae* and *Vibrio* (Nordmann and Poirel, 2005).

At the same time this phenomenon leads to an accumulation of residual drugs in commercialized fish and shellfish products. As a consequence, we assist to an undetected consumption of antibiotics by consumers of fish with four risk factors: possible alteration of their normal flora, enhanced susceptibility to allergies, toxicity problems and selection of AR bacteria.

Materials & Methods

Culture media:

- TCBS agar (Thiosulfate-Citrate-Bile-Sucrose Agar), for the selective isolation of *Vibrio* strains. It is prepared according to the formula of Kobayashi *et al*. (1963). Sodium Citrate, Sodium Thiosulfate and Oxbile are selective agents, providing an alkaline pH to inhibit Gram-positive organisms and suppress coliforms. Sucrose is the fermentable carbohydrate. Sodium Thiosulfate is also a sulphur source, and acts with Ferric Citrate as an indicator to detect hydrogen sulphide production. Bromthymol Blue and Thymol Blue are pH indicators.

  Formula / Liter
  Yeast Extract ..... 5 g
  Enzymatic Digest of Casein ..... 5 g
  Enzymatic Digest of Animal Tissue..... 5 g
  Sodium Citrate ..... 10 g
  Sodium Thiosulfate ..... 10 g
  Oxbile ..... 5 g
  Sodium Cholate ..... 3 g
  Sucrose ..... 20 g
  Sodium Chloride ..... 10 g
  Ferric Citrate ..... 1 g
  Bromthymol Blue ..... 0.04 g
  Thymol Blue ..... 0.04 g
  Agar ..... 14 g

- BH agar (Brain Hearth Agar), a rich medium for the cultivation of a wide variety of microorganisms. The current formula is a modification of Hayden (1923), using dehydrated infusions of pork brain and pork heart tissue.

  Formula / Liter
  Brain Heart Infusion (Solids) ..... 8 g
  Enzymatic Digest of Animal Tissue ..... 5 g
  Enzymatic Digest of Casein ..... 16 g
  Dextrose ..... 2 g
  Sodium Chloride ..... 5 g

Disodium Phosphate ..... 2.5 g
Agar ..... 13.5 g

- TSB (Tryptic Soy Broth), a general purpose medium supporting the growth of a variety of bacteria. If it is supplemented with agar it is called TSA (Tryptic Soy Agar)

  Formula / Liter
  Enzymatic Digest of Casein ..... 15 g
  Enzymatic Digest of Soybean Meal ..... 5 g
  Sodium Chloride ..... 5 g
  Agar ..... 15 g

- SS agar (Salmonella Shigella Agar): it is used for the isolation of *Salmonella* spp. and some strains of *Shigella* spp. It is a modification of the Desoxycholate Citrate Agar described by Leifson in 1935. Beef Extract and Enzymatic Digest of Casein provide sources of nitrogen, carbon, and vitamins. Bile Salts, Sodium Citrate and Brilliant Green inhibit Gram-positive bacteria, most coliform bacteria, and inhibit swarming *Proteus* spp., while allowing *Salmonella* spp. to grow. Sodium Thiosulfate and Ferric Citrate permit detection of hydrogen sulphide by the production of colonies with black centers. Neutral Red is the pH indicator.

  Formula / Liter
  Beef Extract ..... 5 g
  Enzymatic Digest of Casein ..... 2.5 g
  Enzymatic Digest of Animal Tissue..... 2.5 g
  Lactose ..... 10 g
  Bile Salts ..... 8.5 g
  Sodium Citrate ..... 8.5 g
  Sodium Thiosulfate ..... 8.5 g
  Ferric Citrate ..... 1 g
  Brilliant Green ..... 0.00033 g
  Neutral Red ..... 0.025 g
  Agar ..... 13.5 g

PCR primers:

targeted gene	primer sequence	positive reference strain	length of the PCR product	reference
floR	F1764:5'-cctttgtcgctttccg F1765:5'-acccacatcggtagga	*Vibrio cholerae* SXT	634bp	Targant *et al*., 2010
strA-strB	FwStrB:5'-cccgtctggcaatgaaactt RvStrA:5'-attgctaacgccgaagagaa	*Vibrio cholerae* SXT	250bp	Doublet *et al*., 2008
SXT element *intI* integrase	Sxt286F:5'-ctgtggccaatcatcaactc Sxt287R:5'-cgaccgagatgggctaagtg	*E. coli* SXT	1034bp	Rodriguez-Blanco *et al*., 2012
SGI1 integrase *int*	intFw:5'-ccctaactcgaaggggctcc intRev:5'-acggactttccgcagtgagg	*S*.Typhimurium DT104	1172bp	Doublet *et al*., 2008
thdf-int2 retron (*Salmonella* Typhimurium SGI1- identification)	U7L12:5'-acaccttgagcagggcaaag C9L2: 5'-agcaagtgtgcgtaatttgg	*S*.Typhimurium LT2	435bp	Boyd *et al*., 2001
yidY (SGI1 right junction in *Salmonella* non-Typhimurium)	104RJ:5'-tgacgagctgaagcgaattg 104D:5'-accagggcaaaactacacag	*S*. Agona 47SA97	450bp	Boyd *et al*., 2001
int2 retron (SGI1 right junction in *Salmonella* Typhimurium)	104RJ:5'-tgacgagctgaagcgaattg C9L2: 5'-agcaagtgtgcgtaatttgg	*S*.Typhimurium DT104	510bp	Boyd *et al*., 2000
thdf-int (SGI1 left junction)	U7L12:5'-acaccttgagcagggcaaag LJR1:5'-agttctaaaggttcgtagtcg	*S*.Typhimurium DT104	500bp	Boyd *et al*., 2000
groEL-pse1	int2:5'-ttctggtcttcgttgatgcc pse1:5'-catcatttcgctctgccatt	*S*.Typhimurium DT104	1338bp	Doublet *et al*., 2003

SGI1 16784–17839 (St4)	P134-L1:5'-aatcgacacgcgctgtattg P134-R1:5'-gtgtttgggcaagatcccag	*S.*Typhimurium DT104	1490bp	Mulvey *et al*., 2006
SGI1 24363–25201 (St6)	St2-GP24:5'-tcaagattcctatctgcagg St2-GP28:5'-agagttactagaccaagcgc	*S.*Typhimurium DT104	838bp	Mulvey *et al*., 2006
right junctions SXT-chromosome in *prfC*	SXTint:5'-tacaactgagcattggcgca SXTrw:5'-tcttcgtgtcccggagtatc	*Vibrio cholerae* SXT	436bp	this study
right and left SXT-chromosome junctions	Primer 4:5'-tgctgtcatctgcattctcctg Primer 5:5'-gccaattacgattaacacgacgg	*Vibrio cholerae* SXT	785bp	Hochhut *et al*., 1999
Insertion site (IS5') of *V. cholerae* VPI-2 in the chromosome	VPI-2ir1 Fw:5'-ggattcggtcgatactgtc VPI-2ir1 Rv:5'-tcgtagccttccattgc	*Vibrio cholerae*	1600bp	Jermyn and Boyd, 2002
Cluster *nan-nag*	nan-nag Fw:5′-gcaatcgaaaatcaagtctc nan-nag Rv:5′-gctgaccatccacgaataac	*Vibrio cholerae*	2520bp	Gennari *et al*, 2012
trmf gene of VPI-2	trmf Fw:5'-ccagacattccagacagata trmf Rv:5'-cggcgaatgagttacgagt	*Vibrio cholerae*	1041bp	Gennari *et al*, 2012
faghe gene of VPI-2	faghe Fw:5'-tgacacctttggctttccg faghe Rv:5'-ttattactggtgctgctgcg	*Vibrio cholerae*	1413bp	Gennari *et al*, 2012
Insertion site (IS3'a) of *V. cholerae* VPI-2 in the chromosome	1808Fw:5'-atatgagagcaagggaagtg 1808Rv:5'-tctcggtctaactcgtatgg	*Vibrio cholerae*	451bp	Jermyn and Boyd, 2002

Table 6: DNA primers used in this study and corresponding target genes.

Sampling and processing procedure

Sampling sites

Samples were collected in 2011 from four different fish farms in the Adriatic Sea, in particular between the Venetian Lagoon and the Gargano area in Puglia (Italy) plus an additional coastal site in Veneto. Geographical areas and sampling sites are indicated in Figure 27 and 28, and their characteristics are summarized in Table 7.

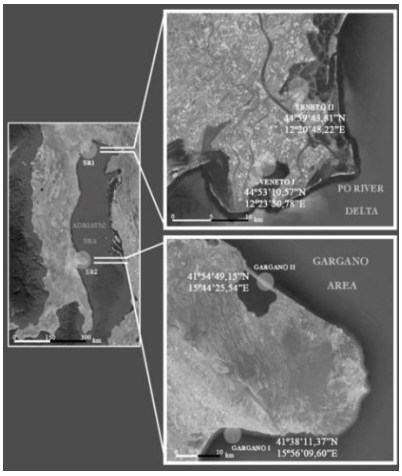

Figure 27: Sampling areas of the Adriatic Sea region from Italy coast. SR1: sampling region 1 corresponds to Po river delta (Veneto region, North Italy) where two aquaculture centres were studied (Veneto I and Veneto II). SR2: sampling region 2, corresponds to Gargano area (Puglia region, South Italy) where two aquaculture centres were sampled (Gargano I and Gargano II) (Labella et al., 2013).

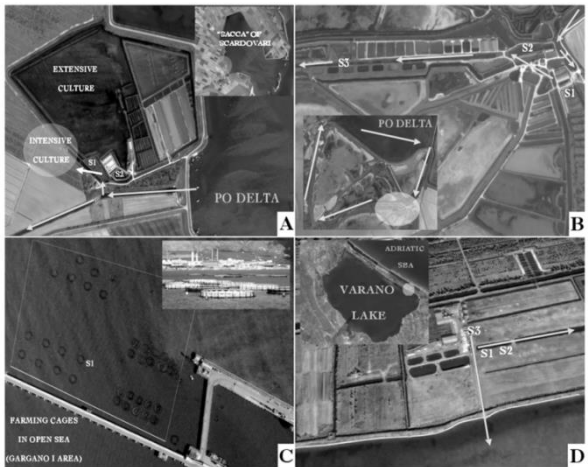

Figure 28: Aquaculture centres included in this study (from A to D: Veneto I, Veneto II, Gargano I and Gargano II). S1, S2 and S3 are the sampling locations across the fish farms from inlet to outlet of water circuit. Arrows represents the open water courses from the Adriatic Sea to the Varano lagoon (Labella et al., 2013).

Fish farm	Location	Distance from the coast	Water circuit	Water temperature at sampling	Cultured fish	Antibiotic therapy used
Veneto I	North Adriatic	Valley brackish lake	Closed: water taken from and let out in the lagoon	19-26° C	European seabass, Shi drum	None (experimental farm)
Veneto II	North Adriatic	Valley brackish lake	Closed: water taken from the estuary and released in the same place after 10 km	18-29° C	European seabass	May 2009, TIM + SUL May 2010, OXTET April 2011, FLU
Gargano I	South Adriatic	2 km	Open Sea	23° C	European seabass, Gilt-head seabream	Sporadic use of antibiotics before 2009–2010
Gargano II	South Adriatic	1 km	Open: water entering from the sea through a channel and remaining in the lagoon	29° C	European seabass, Gilt-head seabream	None (as declared by the manager). Extensive breeding of horses and sheep in the surrounding area

Table 7: Characteristics of the different aquaculture fish farms considered in this study.

The fish farm Veneto I is a small ‘‘valle di pesca’’ (valley brackish lake) located in the delta of the Po river and isolated from agricultural fields. Fishes are grown in ground tanks where the water is pumped in an autonomous circuit without thermoregulation. Water and sediment samples were obtained from the water entrance in the tanks (S1) and at the release point from the whole circuit (S2) (Figure 28A).

The aquaculture center Veneto II is also a ‘‘valle di pesca’’ dedicated mostly to semi-intensive culture of European sea bass. The centre captures water from a close estuary and releases the water into a channel which discharges again in the estuary (Figure 28B). Water released from the aquaculture centre and from other agricultural activities located in the area, is naturally filtered in such a way that the microbiological quality of water returning to the estuary is improved. The water temperature is not regulated and has a deep range of variations. Water, sediment and biofilm samples were obtained in this farm from three different sites as indicated in Figure corresponding to the water entrance (S1), the release points at the fish tanks (S2) and the final release point of the whole water circuit (S3).

The centre Gargano I is located in the open sea at about 2 km from the coastline (Figure 28C). Water samples were collected around the cages at a 3 m depth while sediment (the top 2 cm) was obtained directly under the cages by scuba divers.

Gargano II is situated in a lagoon in the area of the Gargano peninsula. The lagoon receives water from the sea entering via two channels passing through the land band that separate the lagoon from the Adriatic Sea. As shown in Figure 28D, water, sediment and biofilm samples were obtained from three sites: at the water entry channel (S3, control site) not influenced by the aquaculture activities, and in two points inside the fish

ponds, one at the point of fish feeding (S1) and the other at the outgoing water flux (S2).

Sampling procedure and sample pre-treatment

Water samples were collected from inlet water and outlet water entering or going out from the fishpond or fish tank at each aquaculture centre (see Figure 28). Two portions of sediment samples of the 5-cm top layer, from the same sites were sampled with a bottom Van Veen collector. Samples of biofilm were obtained by scrapping the internal part of the pipelines entering and/or going out from the fish tanks. The coastal marine samples were taken at Chioggia (point 45°12’02.35”N–12°18’08.47”E). Water samples was collected using a sterile Niskin plastic bottle at 1 m depth and surface sediment samples were obtained using a Van Veen grab. All samples were stored at 4° C, transferred to the laboratory and processed within 4–6 h. Water samples (1 L each) were filtered through 0.22-um nitrocellulose filters (Millipore Co., USA). Filters were then suspended in 300 ml of artificial

sea water (ASW) (Lleò *et al*., 2005). 5 g of sediment or biofilm was suspended in ASW, vortexed for 2 min and sonicated for 2 min at a low frequency (15 kHz for 30 s) to detach bacteria. Supernatants containing bacteria were then stored at room temperature for several minutes (Labella *et al.*, 2013).

Isolation of antibiotic resistant marine bacteria

Aliquots from each sample were plated in thiosulfate citrate bile salt (TCBS) agar and incubated overnight at 37° C. All the colonies isolated were cultured on Tryptone Soy Agar (TSA) supplemented with a specific concentration of the antibiotic. For tetracycline (TET), trimethoprim (TIM) and flumequine (FLU), concentrations slightly higher than the breakpoints, 8, 16 and 2 µg/ml, were used respectively (National Committee for Clinical Laboratory Standards, 2010). Similarly, for the association trimethoprim–sulfadiazide (TIM–SUL), 4 µg/ml of TIM and 76 µg/ml of sulfadiazine were mixed and used together. All the bacterial strains grown in plates containing antibiotics were subsequently tested three times to confirm antibiotic-resistant. Only those colonies growing in three separate tests in the presence of the different antibiotics were considered as resistant strains and were included in the study. Strains were stored at -80°C in MICROBANK (PRO-LAB diagnostics). MICs were determined according to the broth microdilution methodology of the Clinical and Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 2010) (Labella *et al*., 2013).

Strains were then grown at 37°C in Brain Heart infusion or on agar plates and they were tested again for their antibiotic susceptibilities, this time by the disk diffusion assay on Mueller-Hinton plates according to the guidelines of the Antibiogram Committee of the French Society for Microbiology. Susceptibility was determined using disks containing 30 antibiotics as previously described (Doublet *et al*. 2009). All antibiotic disks except for Florfenicol were purchased from Bio-Rad (Marnes-la-Coquette, France). Florfenicol disks were obtained from Schering-Plough Animal Health (Kenilworth, NJ). The antimicrobial susceptibility was determined by the Sirscan Micro micro-reader and with the use of SIRWEB database.

DNA extraction

The DNA extraction was performed using the following classical miniprep protocol (Wilson, 2001).

Bacteria incubated overnight at 37°C in a liquid medium were resuspended in TE buffer and lysed adding 10% SDS. Proteins were removed by digestion with proteinase K; cell wall debris, polysaccharides and remaining proteins were then eliminated by selective

precipitation with CTAB. High-molecular-weight DNA was washed with phenol-choloroform and recovered from the resulting supernatant by isopropanol precipitation. The DNA concentration and purity degree were verified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). The purified nucleic acid is stored at -20°C.

Primers design

The primers design was done using the program Primer3 (http://primer3.ut.ee/); in addition, to confirm primers' characteristics and accuracy, they were checked thanks to the program Oligo Explorer version 1.1.2.

PCR protocol

The Manual MasterTaq Kit (Eppendorf) was employed for all the PCR reactions. It contains a recombinant Taq DNA polymerase purified from *Escherichia coli* DH1, a 10x Taq Buffer with 15 mM magnesium, a separate 25 mM Magnesium Solution, and the special 5x TaqMaster PCR enhancer to help in case of difficult template DNA. TaqMaster is a buffer additive that improves thermostability and processivity of Taq DNA polymerase stabilizing the enzyme during PCR reaction. It also makes the enzyme less sensitive to exogenous PCR-inhibiting contaminants that are abundant in the case of natural samples.

Generally, we performed PCR with 600-800 ng/µl of DNA because our environmental samples contain many contaminants, and a higher DNA quantity can partially reduce the associated problems. 35 amplification cycles were employed and a final longer extension period (10-15 minutes) was chosen in order to optimize the amplification reaction.

Substance	Volume
water	22,6 µl
TaqMaster	10 µl
Buffer	5 µl
dNTPs (10mM)	1 µl
Primer FW	0,5 µl
Primer RW	0,5 µl
Taq (5u/µl)	0,4 µl
DNA	10 µl
TOTAL	50 µl

Table 8: PCR mixture.

PCR assays were carried out in a 50 µl PCR mixtures (see Table 8); they were incubated for 5 minutes at 95°C, followed by 35 amplification cycles. Each cycle consisted in: denaturation at 95°C for 45 seconds, annealing at the adequate temperature for 45 seconds, extension at 72°C for 45 seconds plus a final extension at 72°C for 10/15 minutes.
The obtained amplification products were then visualized using the GenoSmart2 imaging system (VWR) after 1.8-2% agarose gel electrophoresis and GelRed staining (Biotium).

DNA purification and sequencing

The PCR products were purified with the QIAquick PCR purification kit before sequencing, to remove the primers and other PCR residues. The purified PCR amplificates were then evaluated and quantified using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). For sequencing, the dried purified DNA was added with the forward primer; sequencing was carried out by BMR Genomics sequencing service (www.bmr-genomics.it).

Conjugation in liquid medium

The conjugation protocol was set up on the basis of many attempts done to find the optimum conditions favouring the bacterial gene exchange process (Figure 29).

- Day 1: MORNING inoculation of the bacterial strains in 5 ml BH medium, overnight incubation at 37°C in a shaker incubator.

  AFTERNOON one drop of the previous culture was taken and put it in 10 ml BH medium, overnight incubation at 37°C in a shaker incubator.
- Day 2: MORNING 4 drops of the overnight culture were put it in 10 ml BH medium, incubation at 37°C in a shaker incubator for 5.30 h.

  AFTERNOON 2 ml of the donor strain culture were put in a sterile tube together with 8 ml of the recipient strain culture, 5 ml of liquid medium were added (Figure 30, step 1); overnight incubation at 37°C without shaking.
- Day 3: centrifugation of the overnight culture (10 minutes, 4000 rpm), the obtained pellet was resuspended in 1 ml water. 100 µl of this suspension were inoculated in the solid medium additioned with the two antibiotics, one used to select against donor cells, and the other to select against unmated recipient cells (Figure 30, step 2). Serial dilutions were also plated into the appropriate selective media to determine the number of donors and transconjugants cells. At the same time, inoculation of the donor and recipient strains in both the solid medium with the two antibiotics

(negative control) and in the solid media with the single selective agent (positive control).

- Day 4: verification of the controls, and of the bacterial growth in the solid media with the 2 antibiotics (Figure 30, step 3); if transconjugant cells are present, they were purified and tested by PCR for the presence of the target genes (Figure 30, step 4). Inoculation of the transconjugants in both TCBS and SS solid medium was set up to the identification of the bacterial species; we also confirmed the identification using the Vitek 2 apparatus and an antibiogram was performed in most of the cases (Figure 30, step 5). The transfer frequency was calculated: it was expressed as number of transconjugants per donor cells in the mating mixture at the time of plating.

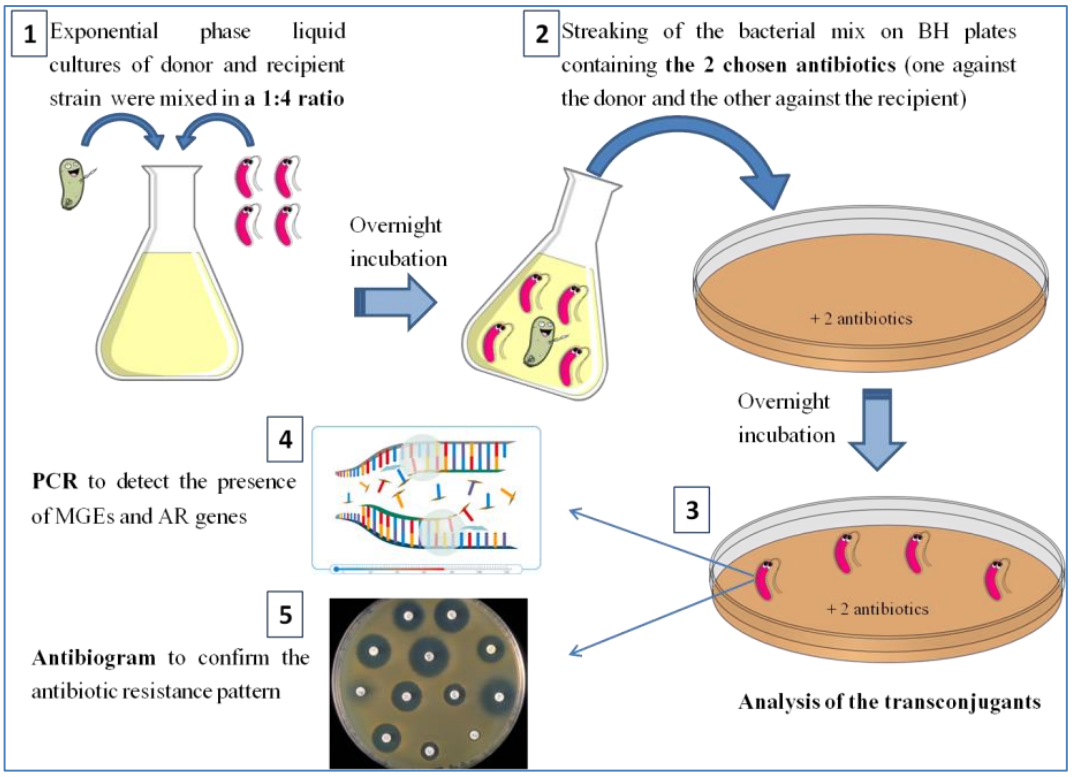

Figure 29: Schematic representation of the conjugation protocol used in this study.

Results

Preliminary tests on donors

A collection of 89 multi-resistant marine strains were tested for the presence of antibiotic resistant MGEs, namely the class 1 integron (*Int1* gene), the SXT element (SXT *IntI* gene) and for a number of *V. cholerae* and *V. parahaemolyticus* virulence genes.

From those 89 strains, 13 bacterial strains were chosen for this study (see Table 9), because of both their multi-resistant phenotype and their genetic properties. In fact they showed the presence of virulence genes such as *NanH* and the presumptive presence of mobile elements (pathogenicity islands and class 1 integron). The 13 AR strains were also tested by PCR for the presence of other mobile elements known to include antibiotic resistant genes, namely *Salmonella* genomic island 1 (SGI1) and the SXT integrative conjugative element (ICE) (primers are detailed in Table 6 in Materials & Methods section).

Bacterial species	Strain name	Resistance profile	Int1	NanH	tdh	floR	strA-straB	SXT IntI	SGI1 int
Shewanella algae	60 CP	CfpAmxTet	+	+	-	-	-	-	-
Shewanella paucimobilis	62 CP	SptStrTet	+	-	+	-	-	-	-
Vibrio parahaemolyticus	143a CP	SptAmxSssTim	+	-	-	-	-	-	-
Vibrio parahaemolyticus	144a CP	SptAmxTet	+	+	+	-	-	-	-
Shewanella algae	219 VB	SptAmxCfpSss	+	+	-	-	-	-	-
Vibrio alginolyticus	NPV3	AmxSssTet	+	+	+	-	-	-	-
Vibrio alginolyticus	NPV5	AmxTetTim	+	-	+	-	-	-	-
Vibrio alginolyticus	NPV6	AmxTetTim	+	+	+	-	-	-	-
Vibrio alginolyticus	NPV7	SptAmxSss	+	+	+	-	-	-	-
Vibrio metschnikovii	NPV18	SptStrAmx	+	+	-	-	-	-	-
Vibrio alginolyticus	NPV25	SptStrAmxSss	+	+	+	-	-	-	-
Vibrio mimicus	NPV32	SptAmxSss	+	+	-	-	-	-	-
Vibrio cholerae	AS1	StrSssTimChl	+	-	-	+	+	+	-

Table 9: The environmental strains used as donors in this study and their characteristics.

Donor strain choice

One of the 13 environmental isolates, the *V. cholerae* ctxA and ctxB negative AS1 strain, was particularly interesting in that it resulted positive for the SXT integrase *IntI* and it was simultaneously resistant to Streptomycin, Trimethoprim, and Sulfonamides, characteristics that are typical of SXT/R391+ strains (see Figure 30).

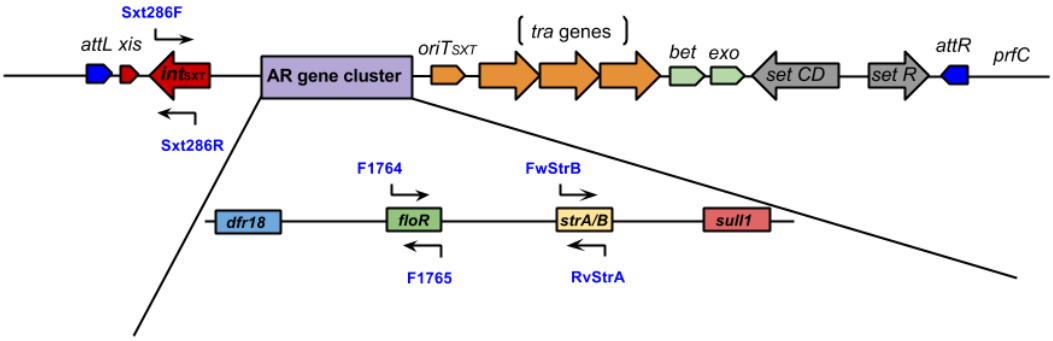

Figure 30: Representation of the core structure of the SXT/R391 ICE; the location of primers used in this study is indicated by arrows.

The SXT presence was confirmed by PCR using primers Sxt286F and Sxt286R (primers are detailed in Table 6 in Materials & Methods section) and, by the same technique, the strain was seen to be also positive for the *strA-strB* genes, conferring resistance to streptomycin.

Chosen recipient strains

The recipient strains were selected in the collection of the PGBA team and are showed in Table 10: *E. coli* K-12 J53-R (Rifampicin resistant), *E. coli* K-12 J53-S (Sodium Azide resistant), *Salmonella* Typhimurium LT2 (Rifampicin resistant), *Salmonella* Agona 47SA97 (resistant towards Streptomycin, Spectinomycin, Sulfonamides and Kanamycin because of the presence of SGI1-C), *Salmonella* Typhimurium DT104 S/960725 (Ampicillin and Sulfonamides resistant because of the presence of SGI1-B).

identification	**strain name**	**relevant genotype and resistance profile**	**reference**
E. coli	*E. coli* K-12 J53	Rifampicin	Institut Pasteur, France
E. coli	*E. coli* K-12 J53	Sodium Azide	Institut Pasteur, France
S. Typhimurium	*Salmonella* Typhimurium LT2	Rifampicin	McClelland *et al.*, 2001
S. Agona	*Salmonella* Agona 47SA97	SGI1-C deltaS023::kan, StrSptSssKan	Cloeckaert *et al.*,2000
S. Typhimurium	*Salmonella* Typhimurium DT104 S/960725	SGI1-B+, AmpSss	Cloeckaert *et al.*,2000

Table 10: Strains from the PGBA collection and used as recipients in the conjugation assays.

SGI1-C-containing strains display resistance to two antimicrobials: streptomycin and sulfonamides; this type of SGI1 has a single integron in the MDR region and not two as the classical form and it carries only the *qac/sul1* and *aadA2* genes (see Figure 31). On the other hand, SGI1-B confers resistance towards ampicillin and sulfonamides, due to the presence of the corresponding resistance genes *pse-1* and *qac*/*sul1* (see Figure 30). This *Salmonella* island has a single integron in the MDR region too.

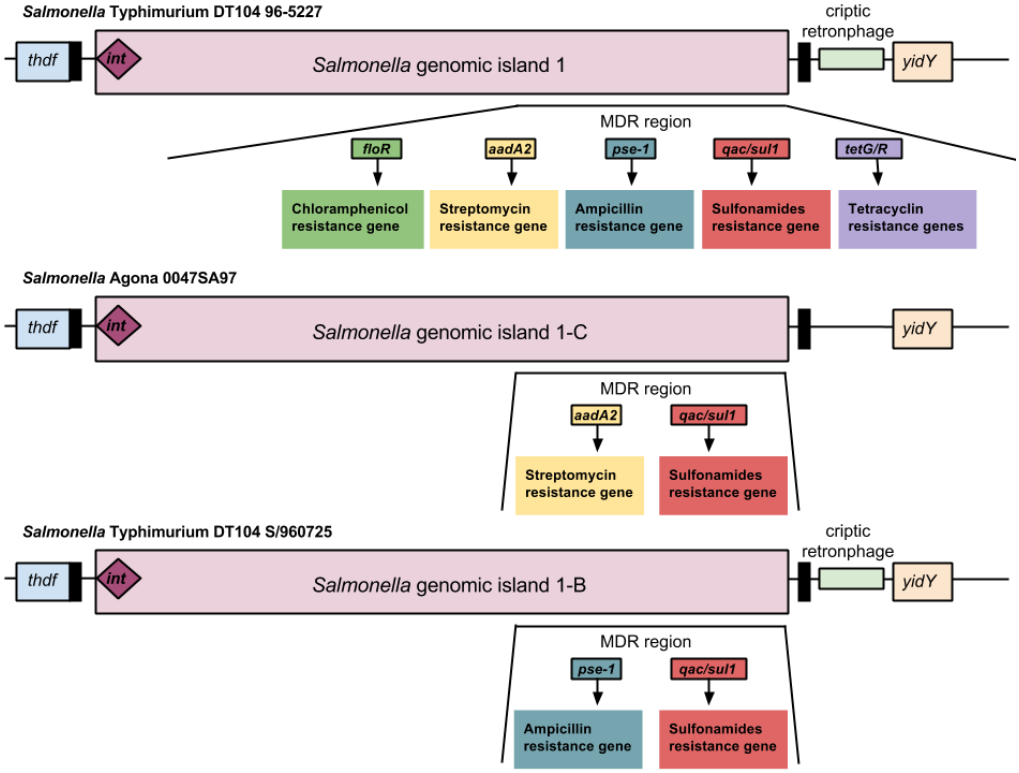

Figure 31: Schematic representation of SGI1 is shown at the top; the genetic organization of the MDR regions of the two strains used in this study is displayed below (adapted from Boyd et al., 2002).

All the donors and recipients strains were tested for their susceptibility towards Rifampicin, Sodium Azide, Ampicillin, Streptomycin, Spectinomicin and Kanamicin. Those tests were performed on agar plates containing different antibiotics concentrations, to determine the possible combination of antibiotics that should be used for the transconjugants selection. The following concentrations of antibiotics were chosen: Rifampicin (125 µg/ml), Sodium Azide (500 µg/ml), Kanamycin (50 µg/ml) or Ampicillin (250 µg/ml) were used to select against *Vibrio* donor cells. Streptomycin (400 µg/ml), Spectinomycin (50 µg/ml), Trimethoprim (240 µg/ml) were the antibiotics chosen to select against unmated cells.

The obtained transconjugant bacteria were tested for antibiotic susceptibility by the disk diffusion assay on Mueller-Hinton plates. The presence of SXT and antibiotic resistance

genes (*floR* and *strA-strB*) was confirmed by PCR. The SXT transfer frequency was determined by dividing the number of *E. coli* or *Salmonella* SXT+ transconjugants by the number of *Vibrio* donor cells.

CONJUGATION EXPERIMENTS IN CULTURE MEDIUM

Conjugation *Vibrio cholerae-E. coli*

We first tried to conjugate the selected 12 environmental strains, except *Vibrio cholerae* AS1, with the *E. coli* strain K-12 J53-R but in all mobilization assays no transconjugants could be obtained after 3 repeated attempts. Those results suggest that probably the resistance determinants of the donor environmental bacteria were not located on mobile elements and thus their transfer was not possible by horizontal exchange. We found out that the 12 strains used as donors presented the integrase gene *intI*, but were negative for the PCR targeting the SXT element. Recently a new type of integron, named the super-integron (SI), has been found in chromosome 2 of *V. cholerae*. It shows some characteristics that distinguish it from known integrons, such as a larger number of cassettes and a chromosomal location. Nothing is known concerning the gene cassettes expression of the super-integrons but it seems improbable that the upstream promoter could ensure the expression of distal cassettes. Probably some of the cassettes have their own promoter, allowing the transcription of genes located downstream (Rowe-Magnus *et al*., 1999). Interestingly, SIs have been found not only in the *V. cholerae* genome, but also in the genome of almost all the bacterial species represented by our 12 environmental strains: *V. mimicus, V. metschnikovii, V. parahaemolyticus, Shewanella* (Rowe-Magnus *et al*., 2001). The presence of the *intI* integrase in our 12 strains can be explained hypothesizing the existence of SIs in those strains, because the *intI* gene is nearly identical in SIs and in integrons.

In spite of these negative results, we tried to make some more assays, this time using the *Vibrio cholerae* SXT+ strain as a donor. Mobilization experiments were undertaken to determine whether SXT was transferable from a member of the *Vibrionaceae* family to a member of the *Enterobacteriaceae* family, in our case the chosen recipient was an *E. coli* strain (*E. coli* K-12 J53-R). These conjugations gave positive results: the *Vibrio-E. Coli* transconjugants showed an antibiotic resistance profile identical to that of the donor SXT strain (StrSssTimChl resistance) and the additional resistance to Rifampicin characteristic of the recipient strain (Table 11). The presence of SXT was confirmed by PCR targeting the SXT *IntI* gene (see Figure 32). By the same technique the transconjugant bacteria were screened for the presence of another gene included in the SXT, *floR*, determining the antibiotic resistance towards Chloramphenicol. Also this PCR assay gave positive signals,

identical to that of the donor SXT strain (see Figure 33). The conjugative transfer of SXT occurred at frequencies of about 10^{-5} transconjugants per donor.

DONOR STRAIN	RECIPIENT STRAIN	transconjugant strain	relevant genotype and resistance profile	SXT transfer frequency
Vibrio cholerae AS1 SXT+	*Escherichia coli* K-12 J53-R	*Escherichia coli* K-12 J53-R SXT+	**SXT+**, StrSssTimChlRif, *floR*+, *strA-strB*+	$5x10^{-5}$

Table 11: Transconjugant strains obtained in the conjugation assays in this study.

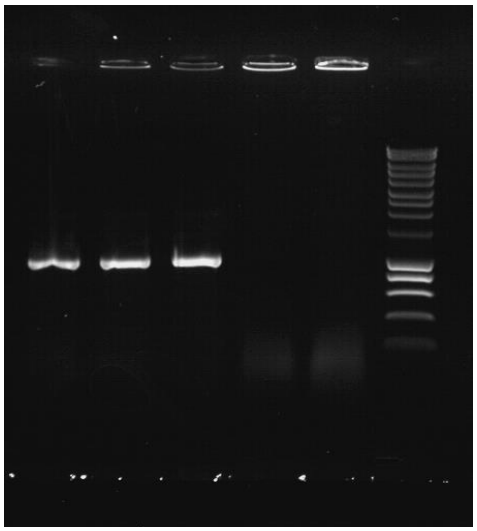

Figure 32: Amplicons obtained after PCR targeting SXT *intI* gene:
lane 1, *Vibrio* AS1 (donor strain)
lanes 2 and 3, two *E. coli* transconjugants
lane 4, *E. coli* K-12 J53 (recipient strain)
lane 5, negative control (no DNA)
lane 6, molecular weight marker.

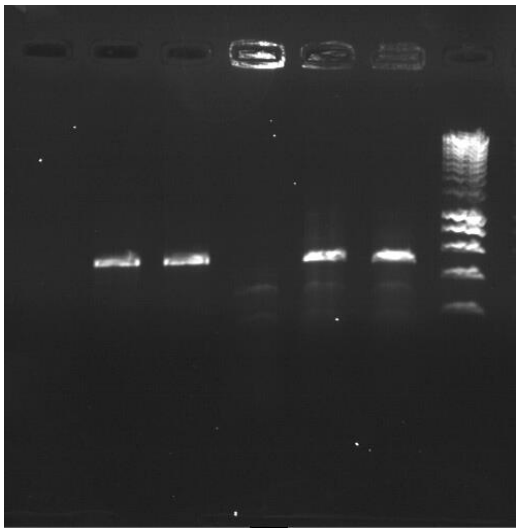

Figure 33: Amplicons obtained after PCR targeting *floR* gene:
lane 1, negative control (no DNA)
lane 2, positive control
lane 3, *Vibrio* AS1 (donor strain)
lane 4, *E. coli* K-12 J53 (recipient strain)
lanes 5 and 6, the 1.3 and 1.4 *E. coli* transconjugants lane 7, molecular weight marker.

Conjugation *Vibrio cholerae-Salmonella*

We have then gone further in the mating assays, conjugating the *Vibrio cholerae* AS1 strain with the *Salmonella* Typhimurium LT2, and with two *Salmonella* strains harbouring different versions of SGI1: *Salmonella* Agona 47SA97 SGI1-CdeltaS023::kan+ and *Salmonella* Typhimurium DT104 SGI1-B+.

In all the cited matings, the resistance markers of the donor strain (chloramphenicol, streptomycin, sulfamethoxazole, trimethoprim) were transferred into recipient cells as a linkage group and in association with SXT (see Table 12). This indicates that the cluster of

genes encoding resistance to the four antibiotics is located on the SXT element. The obtained transconjugant bacteria were tested for antibiotic susceptibility by disk diffusion assay; the presence of the SXT element and of antibiotic resistance gene *strA-strB* was confirmed by PCR (Figures 34-35).

DONOR STRAIN	**RECIPIENT STRAIN**	**transconjugant strain**	**relevant genotype and resistance profile**	**SXT transfer frequency**
Vibrio cholerae AS1 SXT+	*Salmonella* Typhimurium LT2	*Salmonella* Typhimurium LT2 SXT+	**SXT+**, StrSssTimChlRif, *thdf-int2* retron PCR+, *strA-strB*+	$6x10^{-3}$
Vibrio cholerae AS1 SXT+	*Salmonella* Typhimurium DT104 SGI1-B+	*Salmonella* Typhimurium DT104 SGI-B+ SXT+	**SXT+**, **SGI1+**, StrSssTimChlAmp, groEL-pse1+, *strA-strB*+	$4,6x10^{-3}$
Vibrio cholerae AS1 SXT+	*Salmonella* Agona 47SA97 SGI1-CdeltaS023::kan	*Salmonella* Agona 47SA97 SGI1-CdeltaS023::kan, SXT+	**SXT+**, **SGI1+**, StrSssTimChlKana, *strA-strB*+	$1,3x10^{-5}$

Table 12: Donor and recipient strains of each conjugation assay, strain name of the obtained transconjugants, genotype characteristics and SXT transfer frequency.

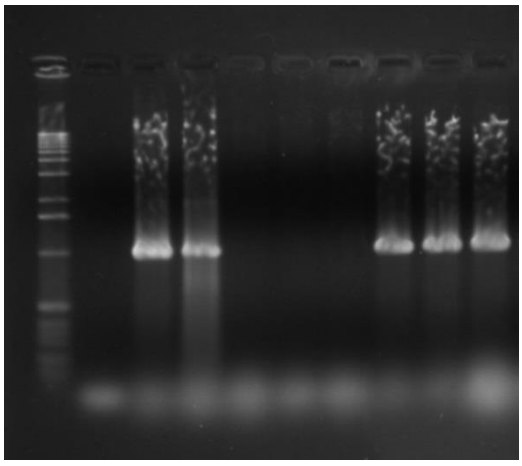

Figure 34: Amplicons of the PCR targeting *intI* SXT gene:
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, positive control
lane 4, *Vibrio* AS1 (donor strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lane 6, *Salmonella* Agona 47SA97 SGI1-CdeltaS023::kan+ (recipient strain)
lane 7, *Salmonella* Typhimurium DT104 SGI1-B+ (recipient strain)
lanes 8 to 10, the obtained transconjugants (*Salmonella* LT2 SXT+, *Salmonella* DT104 SGI1-B+/SXT+, *Salmonella* Agona SGI1-C+/SXT+).

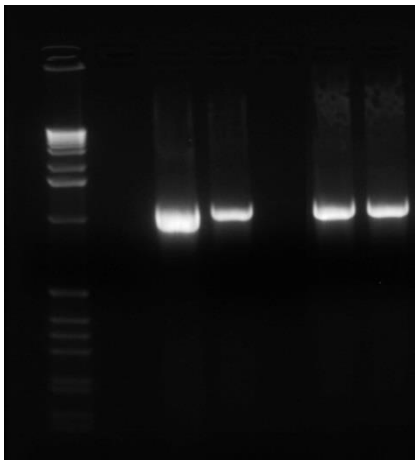

Figure 35: Amplicons obtained after PCR targeting *int* SGI1 gene:
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, *Salmonella* Typhimurium DT104 SGI1-B+ (recipient strain)
lane 4, *Salmonella* Agona 47SA97 SGI1-CdeltaS023::kan+ (recipient strain)
lane 5, *Vibrio* AS1 (donor strain)
lanes 7 and 8, the obtained transconjugants (*Salmonella* DT104 SGI1-B+/SXT+, *Salmonella* Agona SGI1-C+/SXT+).

All the obtained transconjugants showed the resistance profile of the SXT+ donor strain (StrSssTimChl resistance) and an additional resistance trait present in the recipient strain. PCR targeting the SXT element (Figure 34), the SGI1 *int* gene (Figure 37), the *thdf-int2 retron* (*Salmonella* Typhimurium SGI1- identification) and the *strA-strB* or *groEL-pse1* genes responsible for antibiotic resistance, gave the expected results. The SXT transfer frequency was of about 10^{-3} transconjugants per donor in the two first conjugations and of about 10^{-5} transconjugants per donor in the conjugation *Vibrio-Salmonella* Agona.

Conjugation *Salmonella-E. coli*

Having demonstrated that SXT can be transferred from a *Vibrio* strain different *Salmonella* serotypes, we decided to verify if the same element could be mobilized also from a *Salmonella* to an *E. coli*. We used as donor the previously obtained transconjugant strain *Salmonella* Typhimurium DT104 SGI1-B+ SXT+ that was conjugated with *E. coli* K-12 J53-S.

This conjugation gave positive results: the transconjugants showed an antibiotic resistance profile identical to that of the donor SGI-B/SXT strain (StrSssTimChlAmp resistance) and the additional resistance to Sodium Azide typical of the *E. coli* recipient strain (Table 13). The presence of SXT was confirmed by PCR. The conjugative transfer of SXT occurred at frequencies of about $6x10^{-5}$ transconjugants per donor.

DONOR STRAIN	**RECIPIENT STRAIN**	**transconjugant strain**	**relevant genotype and resistance profile**	**SXT transfer frequency**
Salmonella Typhimurium DT104 SGI1-B+ SXT+	*Escherichia coli* K-12 J53-S	*Escherichia coli* K-12 J53-S SXT+	**SXT+**, StrSssTimChlAmpSodiumazide, *strA-strB+*	$5,7x10^{-5}$

Table 13: Transconjugant strains obtained in the conjugation assay Salmonella-E. coli.

COMPARISON OF THE SXT TRANSFER FREQUENCIES IN CULTURE MEDIUM

If we compare the different SXT transfer frequencies obtained from the experiment in BHI medium we obtain the graphic showed in Figure 36. The average transfer frequencies (3 experiments for each mating) differ on the basis of the employed donors and recipients, and it is evident that the highest ones are obtained using the *Vibrio* strain as donor and the *Salmonella* DT104 or LT2 as recipients. In these cases the transfer frequency is higher than 10^{-3} transconjugants per donor, while in the other cases is definitely lower (10^{-5}).

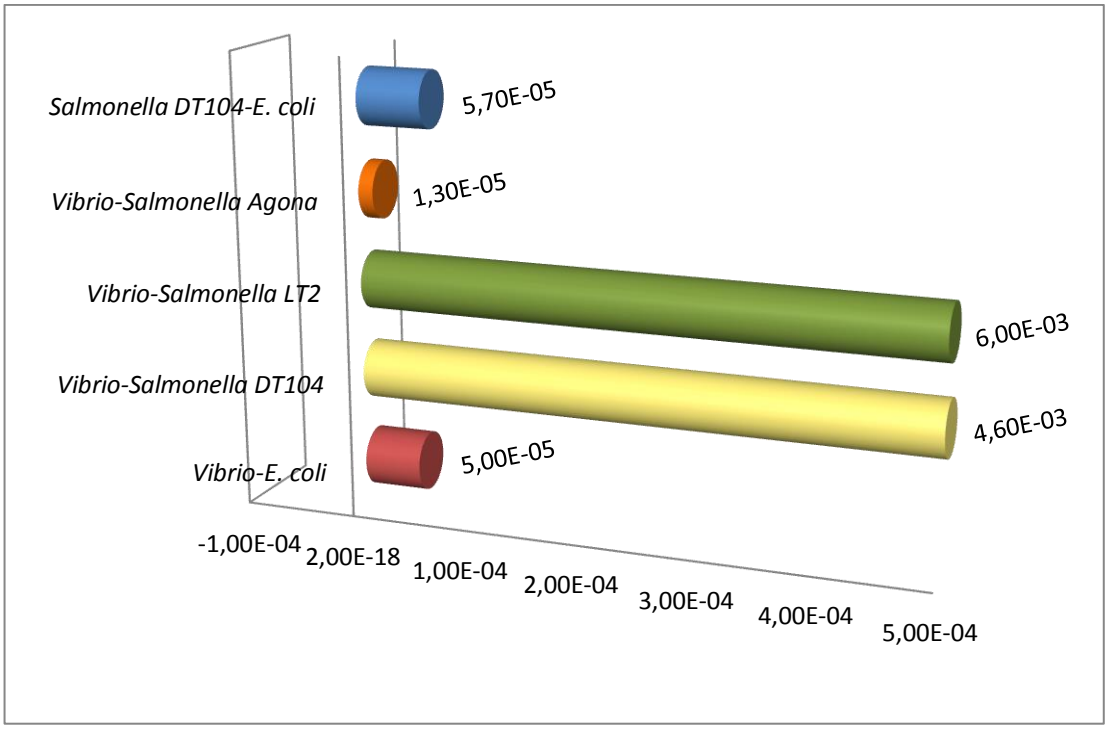

Figure 36: Comparison of SXT transfer frequencies obtained from conjugations in culture medium.

CONJUGATION EXPERIMENTS IN MARINE WATER MICROCOSMS

Conjugation *Vibrio-E. coli* and *Salmonella-E. coli*

In order to assess if the HGT occurs only in laboratory conditions and to estimate the HGT rates in natural environments, we set up conjugative transfers of SXT from *V. cholerae* AS1 SXT+ to *E. coli* and from *Salmonella* Typhimurium DT104 SXT+/SGI1+ to *E. coli* in a marine water microcosm at 20°C. This microcosm mimics the conditions present in the aquatic environment, in particular conjugations are done in filtered marine water supplemented with 1% BHI medium.

Data obtained indicated that the SXT element can be transferred in both cases with a frequency similar to that obtained in a culture medium (Table 14). Transconjugants were confirmed by PCR for the effective presence of SXT (Figure 37). This result confirms the possibility for AR-carrying elements to move between the marine autochthonous flora and human pathogens in the aquatic environment, suggesting that gene transfer is a natural phenomenon that takes place in the aquatic context.

DONOR STRAIN	RECIPIENT STRAIN	transconjugant strain	relevant genotype and resistance profile	SXT transfer frequency
Vibrio cholerae AS1 SXT+	*Escherichia coli* K-12 J53-S	*Escherichia coli* K-12 J53-S SXT+	**SXT+**, StrSssTimChlSodiumazide, *strA-strB*+	$4x10^{-5}$
Salmonella Typhimurium DT104 SGI1-B+ SXT+	*Escherichia coli* K-12 J53-S	*Escherichia coli* K-12 J53-S SXT+	**SXT+**, StrSssTimChlAmpSodiumazide, *strA-strB*+	$3x10^{-5}$

Table 14: Donor and recipient strains of each conjugation assay, strain name of the obtained transconjugants, genotype characteristics and SXT transfer frequency.

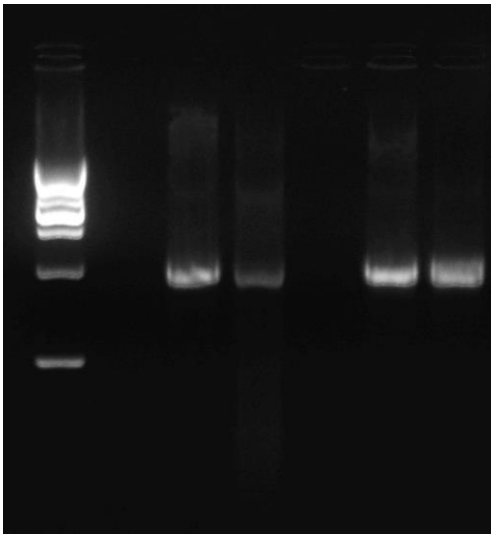

Figure 37: Amplicons obtained after PCR targeting SXT *intI* gene:
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, *Vibrio* AS1 (donor strain)
lane 4, *Salmonella* Typhimurium DT104 SGI1-B+ SXT+ (donor strain)
lane 5, *E. coli* K-12 J53 (recipient strain)
lanes 6 and 7, two *E. coli* SXT+ transconjugants obtained in marine water microcosms.

CONJUGATION EXPERIMENTS IN URINE MICROCOSMS

Conjugation *Vibrio-E. coli* and *Salmonella-E. coli*

We set up the same conjugative transfers of SXT (from *V. cholerae* SXT+ to *E. coli* and from *Salmonella* Typhimurium DT104 SXT+/SGI1+ to *E. coli*) in a biological fluid, specifically urine. This was done to mimic the conditions present in particular human districts, like bladder, and to verify the effective ability of SXT to be transferred even in this medium.

The positive results obtained show that the SXT element can be transferred between bacteria present in biological fluids, even if at a lower frequency than in culture medium (Table 15). As shown in Figure 38, transconjugants were screened by PCR to verify the SXT transfer. These data support the hypothesis that bladder and other human districts where bacteria are present (for example intestine) could be a hot spot for HGT.

DONOR STRAIN	**RECIPIENT STRAIN**	**transconjugant strain**	**relevant genotype and resistance profile**	**SXT transfer frequency**
Vibrio cholerae AS1 SXT+	*Escherichia coli* K-12 J53-S	*Escherichia coli* K-12 J53-S SXT+	SXT+, StrSssTimChlSodiumazide, *strA-strB+*	$1,4x10^{-5}$
Salmonella Typhimurium DT104 SGI1-B+ SXT+	*Escherichia coli* K-12 J53-S	*Escherichia coli* K-12 J53-S SXT+	SXT+, StrSssTimChlAmpSodiumazide, *strA-strB+*	$4,2x10^{-6}$

Table 15: Donor and recipient strains of each conjugation assay, strain name of the obtained transconjugants, genotype characteristics and SXT transfer frequency.

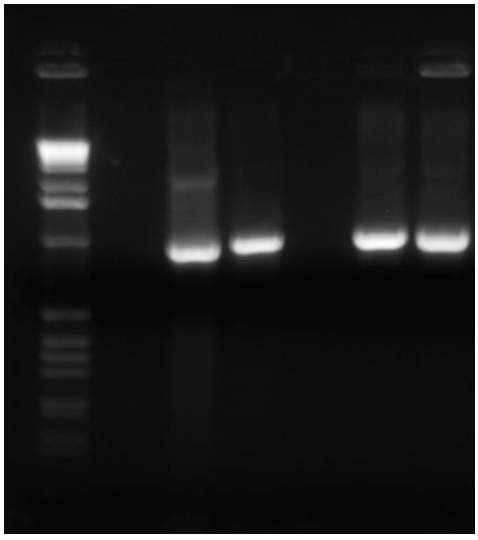

Figure 38: Amplicons obtained after PCR targeting SXT *intI* gene:
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, *Vibrio* AS1 (donor strain)
lane 4, *Salmonella* Typhimurium DT104 SGI1-B+ SXT+ (donor strain)
lane 5, *E. coli* K-12 J53 (recipient strain)
lanes 6 and 7, two *E. coli* SXT+ transconjugants obtained in urine microcosms.

COMPARISON OF THE SXT TRANSFER FREQUENCIES IN DIFFERENT MEDIA

We have then compared the SXT transfer frequencies (average of 3 different experiments) obtained from the conjugation *Vibrio cholerae* SXT+- *E. coli* and *Salmonella* DT104 SXT+/SGI1+ -*E. coli* in different environments: culture medium, marine water and urine.

In both types of conjugation the SXT transfer frequency seem not be deeply affected when performing the experiment in BHI medium or in seawater (Figure 39). On the other hand, a drastic reduction in SXT transfer frequency can be seen when moving to urine microcosms. One order of magnitude reduction is in fact registered in SXT transfer frequency when repeating the conjugation in urine medium (Figure 39).

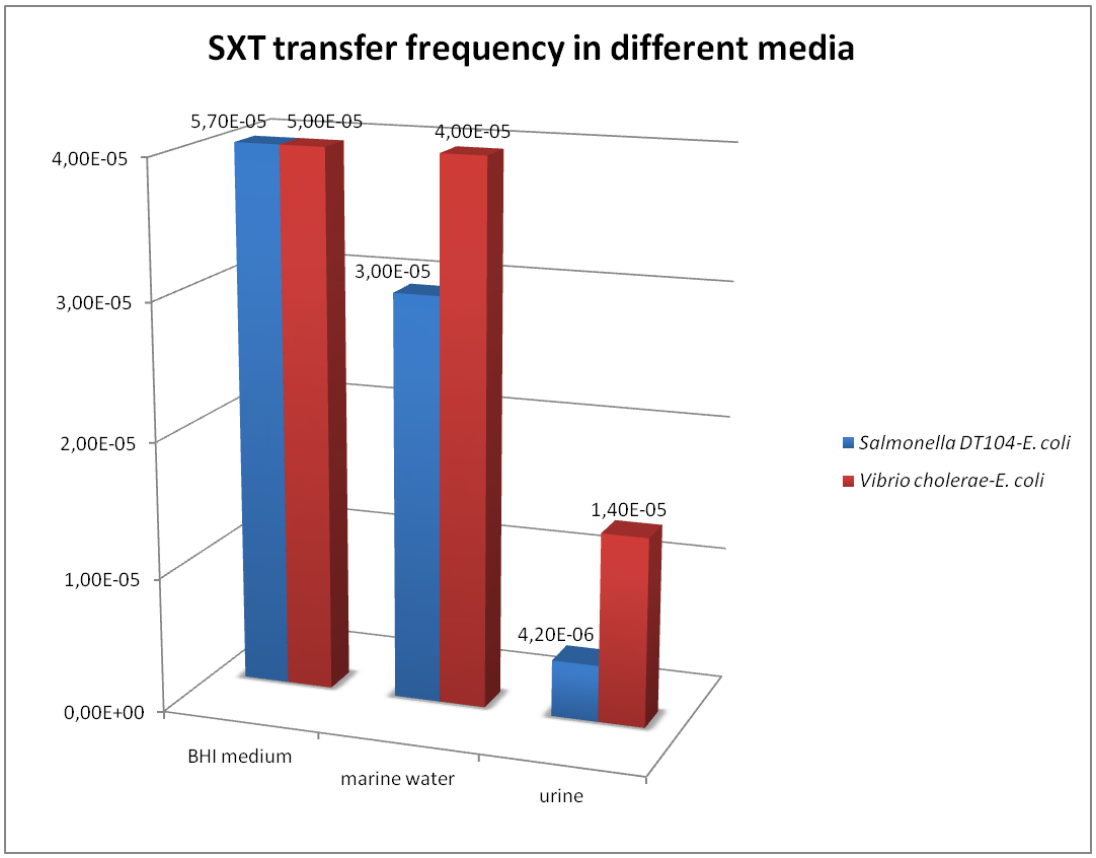

Figure 39: Comparison of SXT transfer frequencies obtained doing the conjugations in different media.

SXT integration in the chromosome

A study of Hochhut and Waldor of 1999 deeper analysed the integration site of SXT elements in both *V. cholerae* and *E. coli*; they showed that, in both species, SXT integrates into the 5' end of *prfC* locus. On the basis of this study, PCR primers complementary to the right end of SXT element and to the *prfC* sequence (SXTint and SXTrw, see Figure 40) were designed to detect the specific integration in this locus.

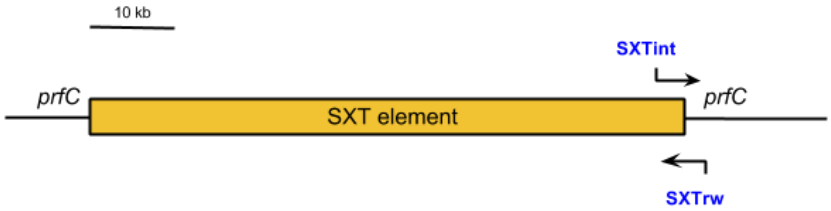

Figure 40: Representation of the SXT-element integrated into the prfC locus. SXT element is indicated by the yellow box while chromosomal DNA is the black thin line. The arrows indicate the primer pair SXTint and SXTrw used to demonstrate the SXT integration into prfC.

It was seen that the size of PCR product was the same for the *E. coli* SXT+ transconjugants and the *Vibrio* SXT+ donor strain, indicating that SXT effectively inserts in *prfC*. The same result was obtained applying the PCR protocol to the three *Salmonella* SXT+ transconjugants (see Figure 41). These results confirm that independently from the recipient strain used, SXT integrates specifically in this locus.

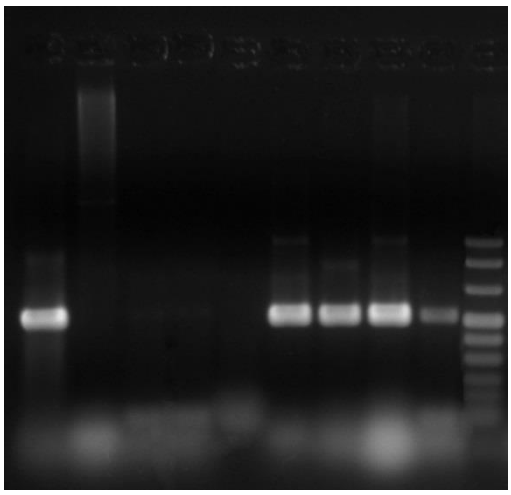

Figure 41: Amplicons obtained after PCR targeting the right junction *prfC*-SXT:
lane 1, *Vibrio* AS1 (donor strain)
lane 2, *E. coli* K-12 J53-S (recipient strain)
lane 3, *Salmonella* Typhimurium LT2 (recipient strain)
lane 4, *Salmonella* Agona 47SA97 SGI1-CdeltaS023::kan+ (recipient strain)
lane 5, *Salmonella* Typhimurium DT104 SGI1-B+ (recipient strain)
lanes 6 to 9, the obtained transconjugants (*E. coli* K-12 J53-S SXT+, *Salmonella* LT2 SXT+, *Salmonella* DT104 SGI1-B+/SXT+, *Salmonella* Agona SGI1-C+/SXT+)
lane 10, molecular weight marker.

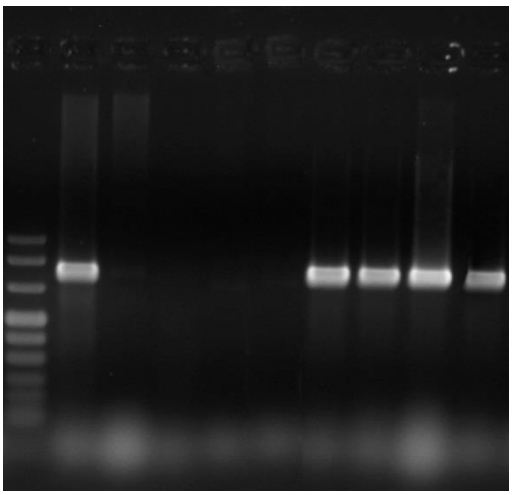

Figure 42: Amplicons obtained after PCR targeting the right and left SXT-chromosome junctions:
lane 1, molecular weight marker
lane 2, *Vibrio* AS1 (donor strain)
lane 3, *E. coli* K-12 J53-S (recipient strain)
lane 4, *Salmonella* Typhimurium LT2 (recipient strain)
lane 5, *Salmonella* Agona 47SA97 SGI1-CdeltaS023::kan+ (recipient strain)
lane 6, *Salmonella* Typhimurium DT104 SGI1-B+ (recipient strain)
lanes 7 to 10, the obtained transconjugants (*E. coli* K-12 J53-S SXT+, *Salmonella* LT2 SXT+, *Salmonella* DT104 SGI1-B+/SXT+, *Salmonella* Agona SGI1-C+/SXT+).

It has been previously demonstrated that SXT shows some similarities with bacteriophages (Hochhut et Waldor, 1999); phages integrate via a similar mechanism and they integrate and excise thanks to a circular extrachromosomal intermediate that in the case of SXT is not self-replicative. As a consequence, it is important to verify if also SXT uses the same mechanism. We tested then our *Vibrio* SXT+ donor strain and transconjugant SXT+ strains with a PCR protocol targeting extrachromosomal circular SXT. Primers oriented towards the right and left SXT-chromosome junctions (Primer 4 and Primer 5, see Figure 42) were used (Hochhut et Waldor, 1999). They amplify a product only if the right (*attR*) and left (*attL*) ends of the integrated element excise and circularize.

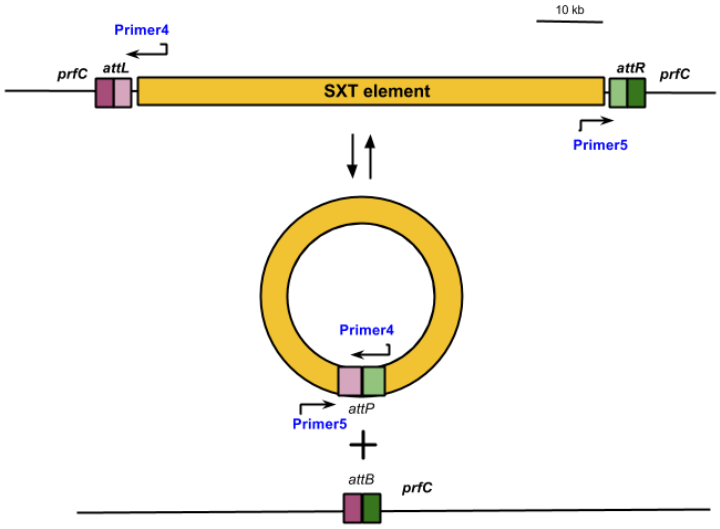

Figure 43: Representation of the integration and excision of SXT from the chromosome in the attB site. SXT element is indicated by the yellow box while chromosomal DNA is the black thin line. The arrows indicate the primer pair Primer 4 and Primer 5 used for the detection of the SXT circular form.

A PCR product of 785 bp was obtained for the donor *Vibrio* strain, the *E. coli* SXT+ transconjugants and the *Salmonella* SXT+ transconjugants, confirming that SXT excises and integrates via a circular intermediate (see Figure 43).

Mobilization of SGI1 by SXT

We have then gone further in our experiments and we focused our attention on the *Salmonella* Agona transconjugants SGI1+/SXT+ obtained using as a donor the *Vibrio cholerae* SXT+ and as recipient the *Salmonella* Agona 47SA97 that is a SGI1-C+ strain. SGI1 is not an autonomous MGE, in fact it cannot excise from the chromosome as showed by Boyd *et al*. in 2000. Conjugal transfer of SGI1 requires the presence of a helper plasmid

of the IncA/C family (Douard *et al*., 2010). These multidrug-resistance plasmids are widespread in *Salmonella* and other enterobacteria, and it was recently demonstrated that most of the conserved genes of the SXT family are also present in those plasmids, and that there is a high homology in the recombination genes regions (Wozniak *et al*., 2009) (see Figure 23 of the introduction chapter). Moreover, BLAST analyses done in recent years revealed a high sequence similarity between the IncA/C plasmid and the SXT/R391 element. Taken together, the similarity of DNA sequences and the organization of SXT/R391 ICEs and IncA/C plasmids indicate a probable evolution from a common ancestral genetic element (Wozniak *et al*., 2009).

For this reason we hypothesize that the SXT element, being to date the closest known relative of the IncA/C plasmids (Wozniak *et al*., 2009), could be able to mobilize the SGI1 in the *Salmonella* transconjugants presenting both SXT and SGI1. We decided then to set up a new conjugation assay to test the SGI1 *in trans* mobilization induced by SXT from the previously obtained *Salmonella* Agona transconjugant SGI1+/SXT+ (used as donor strain), to a *E. coli* or *Salmonella* recipient strain (see Table 16).

The SGI1 was mobilized only in one of the different types of conjugations, i.e. when both donor and recipient were *Salmonella* strains. Transconjugants were tested for antibiotic susceptibility by disk diffusion assay; the presence of the SXT element and of the different regions of SGI1 was confirmed by PCR (Figure 44 and Figure 45).

DONOR STRAIN	**RECIPIENT STRAIN**	**Transconjugant strain**	**Relevant genotype and resistance profile**	**SXT or SGI1 transfer frequency**
Salmonella Agona 47SA97 SGI1-CdeltaS023::kan, SXT+	*Escherichia coli* K-12 J53-S	*Escherichia coli* K-12 J53-S SXT+	**SXT+**, StrSssTimChlSodiumazide	$5x10^{-3}$
Salmonella Agona 47SA97 SGI1-CdeltaS023::kan, SXT+	*Salmonella* Typhimurium LT2	*Salmonella* Typhimurium LT2 SXT+	**SXT+**, StrSssTimChlRif, *thdf-int2* retron PCR+	$1,1x10^{-5}$
Salmonella Agona 47SA97 SGI1-CdeltaS023::kan, SXT+	*Salmonella* Typhimurium LT2	*Salmonella* Typhimurium LT2 SGI1-CdeltaS023::kan, SXT+	**SXT+, SGI1+,** StrSssTimChlRifKana**, SGI1 left junction+, SGI1 right junction in *S.*Typhimurium+, St4+, St6+**	$8,3x10^{-7}$

Table 16: Donor and recipient strains of each conjugation assay, strain name of the obtained transconjugants, genotype characteristics and SXT/SGI1 transfer frequency.

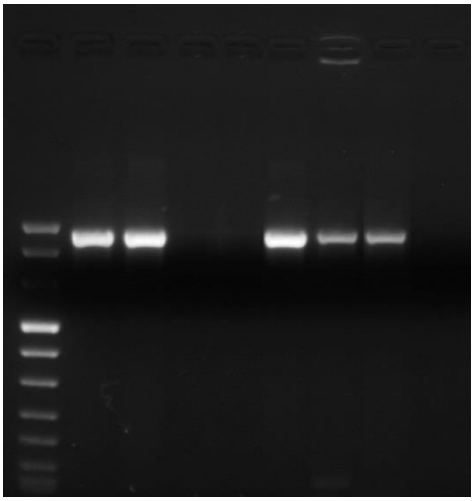

Figure 44: Amplicons of the PCR targeting SXT *IntI* gene:
lane 1, molecular weight marker
lane 2, positive control
lane 3, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 4, *E. coli* K-12 J53-S (recipient strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lanes 6 to 8, the obtained transconjugants (*E. coli* K-12 J53-S SXT+, *Salmonella* LT2 SXT+, *Salmonella* LT2 SXT+/SGI1-C+)
lane 9, negative control (no DNA).

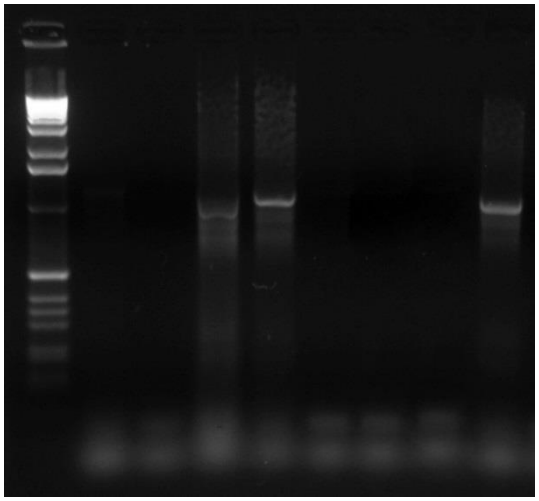

Figure 45: Amplicons of the PCR targeting SGI1 *Int* gene:
lane 1, molecular weight marker
lane 2, *E. coli* K-12 J53-S (recipient strain)
lane 3, *Salmonella* Typhimurium LT2 (recipient strain)
lane 4, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 5, positive control
lane 6, negative control (no DNA)
lanes 7 to 9, the obtained transconjugants (*E. coli* K-12 J53-S SXT+, *Salmonella* LT2 SXT+, *Salmonella* LT2 SXT+/SGI1-C+).

The *Salmonella* SXT+/SGI1+-*Coli* transconjugants showed an antibiotic resistance profile identical to that of the donor strain (StrSssTimChl resistance) and the additional resistance to Sodium Azide characteristic of the recipient strain (Table 16). The presence of SXT was confirmed by PCR (see Figure 44). The conjugative transfer of SXT occurred at frequencies of about 10^{-3} transconjugants per donor, so it is even higher than what was previously obtained in other mating when *E.coli* represented the recipient strain.

When using the *Salmonella* LT2 as recipient strain, SXT alone was transferred at a frequency varying between 10^{-5} and $8x10^{-7}$ transconjugants per donor. SXT induced also the SGI1 mobilization, determining the transfer of this island to the recipient strain (see Table 16 and Figure 45). In order to distinguish the different Salmonella serotypes and the presence/absence of the different parts of SGI1 in both donors and transconjugants, specific sets of primers were designed based on literature data, as shown in Figure 46.

Primer U7-L12 is located in the *thdf* gene upstream of the left direct repeat (DR-L), while primer C9-L2 is located downstream of the right direct repeat (DR-R) in the *int2* gene of a

retronphage (see Figure 46). This retronphage-like sequence is a 4.3 kb fragment containing three genes, which display homology to a previously described retronphage sequence in *E. coli* (Φ-R73). It is located between the DR-R and the *yidY* gene and probably derives from a phage that has inserted into the *Salmonella* genome and may have lost the majority of the phage-related genes leaving only the retron sequences (Boyd *et al*., 2000).

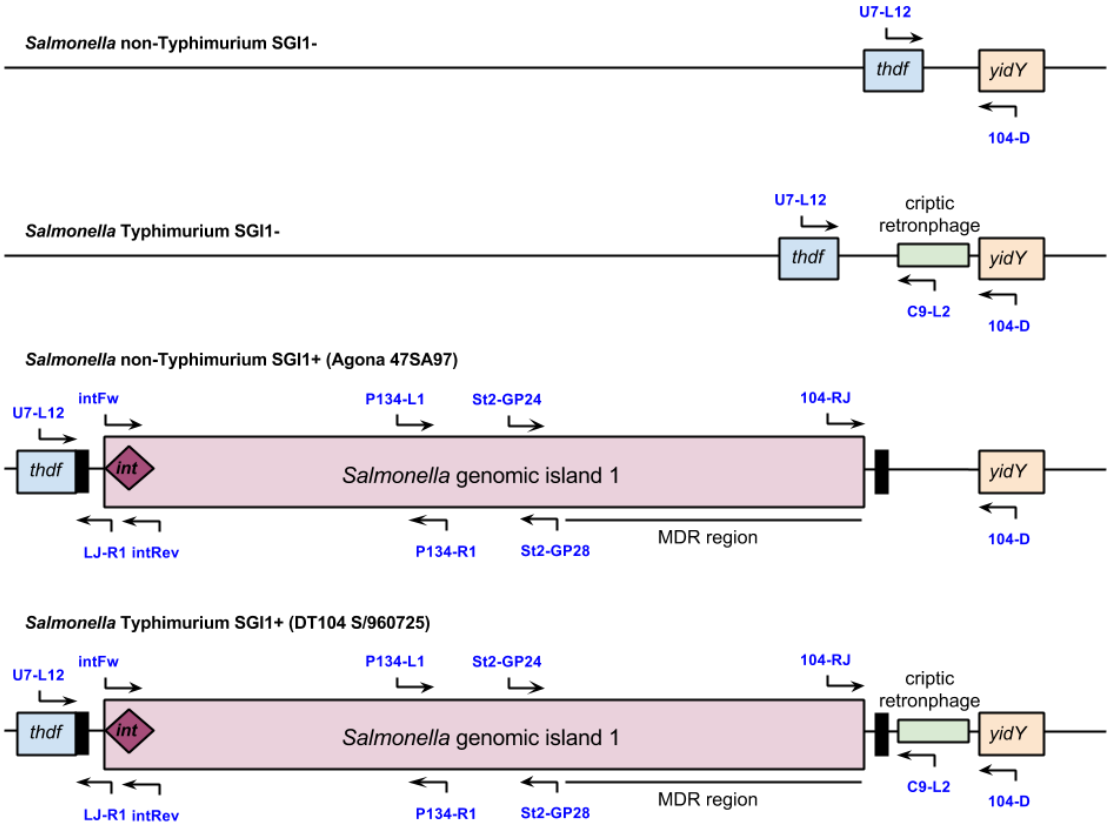

Figure 46: Schematic representation of the regions between thdf and yidY in the organisms listed. Name and location of primers referred to in the text are shown (adapted from Boyd et al., 2000).

Primer U7-L12 is located in the *thdf* gene upstream of the left direct repeat (DR-L), while primer C9-L2 is located downstream of the right direct repeat (DR-R) in the *int2* gene of a retronphage (see Figure 46). This retronphage-like sequence is a 4.3 kb fragment containing three genes, which display homology to a previously described retronphage sequence in *E. coli* (Φ-R73). It is located between the DR-R and the *yidY* gene and probably derives from a phage that has inserted into the *Salmonella* genome and may have lost the majority of the phage-related genes leaving only the retron sequences (Boyd *et al*., 2000).

Agona strains have SGI1 inserted at the 3' end of the *thdf* gene as in serovar Typhimurium strains, but they do not contain the cryptic retronphage in the *thdF-yidY* intergenic region (Figure 46). PCR using primers U7-L12 and C9-L2 can thus discriminate the different

Salmonella serovars (Boyd *et al*., 2001): in the *Salmonella* non-Typhimurium strains no amplificates are produced because of the absence of the retron sequence, in the *S. enterica* Typhimurium strains a product would be expected only if they don't harbour a SGI1 (Figure 47). In fact, if the SGI1 is present no PCR product would be obtained due to the larger size of the predicted amplicon (44 kb).

PCR to detect the SGI1 left junction (*thdf*) was carried out with the primer pair U7-L12 and LJ-R1, and PCR to detect the SGI1 right junction (*int2* of the cryptric retrophage or *yidY*) was carried out with the primer pair 104-RJ and C9-L2 or 104-RJ and 104-D (primers are detailed in Table 6 in Materials & Methods section). All transconjugants SGI1+ strains showed a product of the expected size for the left junction, independently from the serovars (Figure 48).

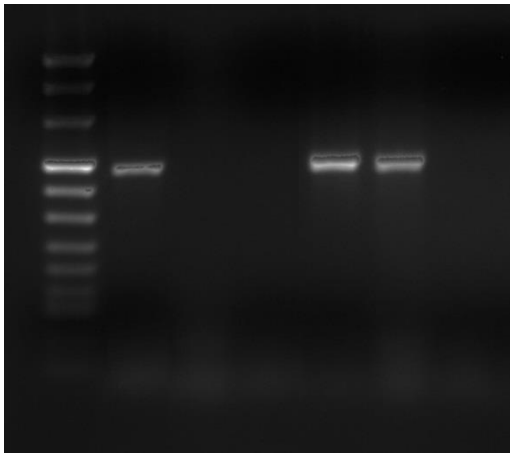

Figure 47: Amplicons of the PCR targeting the *thdf-int2* retron for *Salmonella* Typhimurium SGI1- identification (U7-L1/C9-L2):
lane 1, molecular weight marker
lane 2, positive control
lane 3, negative control (no DNA)
lane 4, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lane 6, *Salmonella* LT2 SXT+ transconjugants
lane 7, *Salmonella* LT2 SXT+/SGI1-C+ transconjugants.

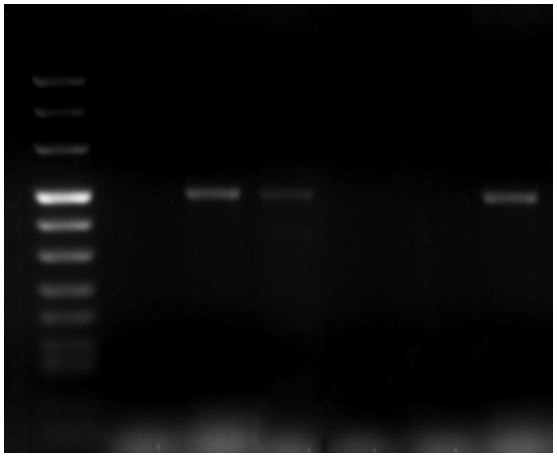

Figure 48: Amplicons of the PCR targeting the *thdf-int* for the detection of SGI1 left junction (U7-L1/LJ-R1)
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, positive control
lane 4, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lane 6, *Salmonella* LT2 SXT+ transconjugants
lane 7, *Salmonella* LT2 SXT+/SGI1-C+ transconjugants.

The serovar Typhimurium SGI1+ strains were positive for the right junction only using primers targeting the cryptic retronphage (Figure 49), while the Agona SGI1+ strains were positive for the PCR targeting *yidD* gene (Figure 46). These results confirmed the presence of both the left and right SGI1 junction regions in all the obtained transconjugants. (Boyd *et al*., 2001).

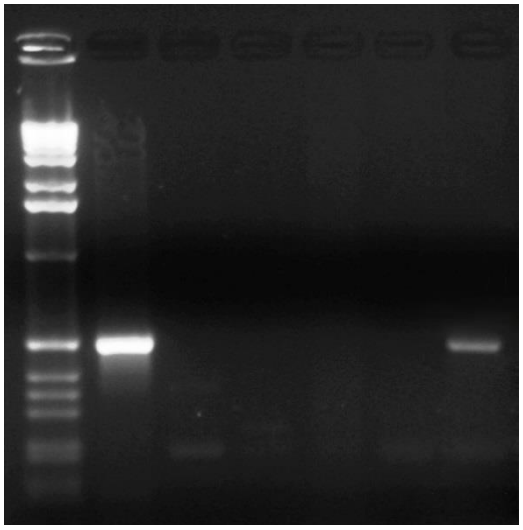

Figure 49: Amplicons obtained after PCR targeting the *int2* retron to detect the SGI1 right junction in *Salmonella* Typhimurium (104-RJ/C9-L2):
lane 1, molecular weight marker
lane 2, positive control
lane 3, negative control (no DNA)
lane 4, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lane 6, *Salmonella* LT2 SXT+ transconjugants
lane 7, *Salmonella* LT2 SXT+/SGI1-C+ transconjugants.

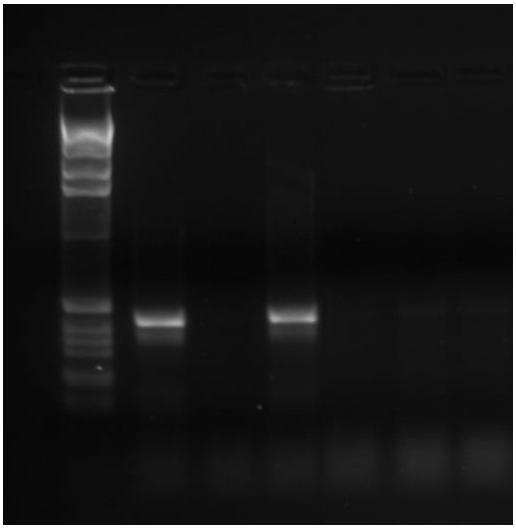

Figure 50: Amplicons obtained after PCR targeting the *yidY* to detect the SGI1 right junction in *Salmonella* non-Typhimurium (104-RJ/104-D):
lane 1, molecular weight marker
lane 2, positive control
lane 3, negative control (no DNA)
lane 4, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lane 6, *Salmonella* LT2 SXT+ transconjugants
lane 7, *Salmonella* LT2 SXT+/SGI1-C+ transconjugants.

To ensure that SGI1 was intact before and after the transfer process, both donors and transconjugants were subjected to additional PCR with primers representing some internal regions of the 43-kb SGI1 element (PCR St4 and St6, see Figure 46). The PCR results confirmed that SGI1 was transferred in its intact form to the transconjugants, because both the left and right extremities and the two internal fragments were observed after PCR (see Figures 51 and 52).

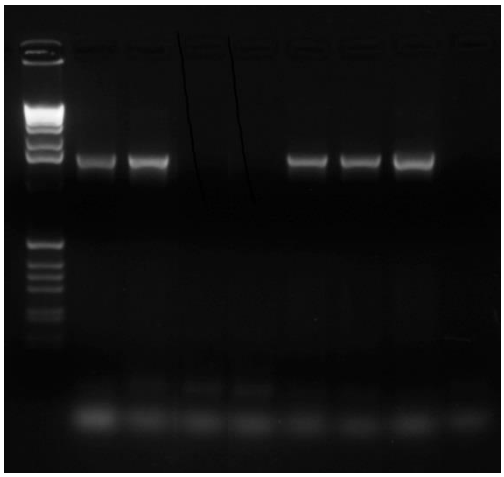

Figure 51: Amplicons obtained after PCR targeting the SGI1 region 16784–17839 (St4):
lane 1, molecular weight marker
lane 2, positive control
lane 3, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 4, *E. coli* K-12 J53-S (recipient strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lanes 6 to 8, the obtained transconjugants (*E. coli* K-12 J53-S SXT+, *Salmonella* LT2 SXT+, *Salmonella* LT2 SXT+/SGI1-C+)
lane 9, negative control (no DNA).

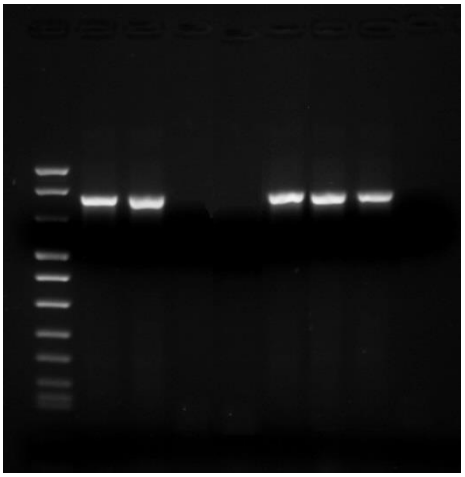

Figure 52: Amplicons obtained after PCR targeting the SGI1 region 24363–25201 (St6):
lane 1, molecular weight marker
lane 2, positive control
lane 3, *Salmonella* Agona SXT+/SGI1-C+ (donor strain)
lane 4, *E. coli* K-12 J53-S (recipient strain)
lane 5, *Salmonella* Typhimurium LT2 (recipient strain)
lanes 6 to 8, the obtained transconjugants (*E. coli* K-12 J53-S SXT+, *Salmonella* LT2 SXT+, *Salmonella* LT2 SXT+/SGI1-C+)

In summary, we have showed that SXT is capable of mobilizing the *Salmonella* genomic island 1, even if at low frequencies, driving its transfer from one *Salmonella* strain to another.

VPI-2 mobilization experiments

Pathogenicity islands from both *E. coli* and *V. cholerae* are non-self mobilizable, they do not encode any protein or conjugation systems needed for cell to cell mobility. The mechanism of transfer for most pathogenicity islands remains to be elucidated but likely involves hitchhiking with plasmids, conjugative transposons, Integrative and Conjugative Elements (ICEs), generalized transducing phages or uptake by transformation.

For example, it is known that the *Vibrio* pathogenicity island VPI-1 can be transferred between *V. cholerae* O1 serogroup strains via a transducing phage, CP-T1. Nothing is known about the elements that drives VPI-2 transfer, we just know that VPI-2 as well as two other PAIs of *V. cholerae* (VSP-I and VSP-II), can excise and form extrachromosomal circular intermediates (CIs), even when present in a truncated form. This ability is in fact maintained if the P4-like integrase is active and intact *attL* and *attR* attachment sites are present. The

excision from host chromosome and the formation of CIs are probably the first steps in HGT of these elements (Murphy and Boyd, 2008).

On other hand, some similarities between SGI1 and pathogenicity islands have been observed: they both contain large segments of DNA flanked by small direct repeats, they both have different G+C contents compared to the chromosomal DNA and they both harbour cryptic and functional genes encoding mobility factors (Boyd *et al*., 2001).

It will be thus interesting to determine which elements are involved in VPI-2 mobility and transfer in that in a previous study conducted in our lab, during the screening of a collection of marine non-pathogenic *Vibrio* strains isolated in the area of the Venetian Lagoon, it was observed that a number those environmental strains carried modified versions of the *V. cholerae* pathogenicity island VPI-2.

We investigated on the effective structure of VPI-2 in our strains, considering that in many cases it is present in a truncated version due the instability caused by the Mu phage that induces spontaneous deletions and insertions (Jermyn and Boyd, 2005). To do that we used different sets of primers (listed in Table 6 in the Materials & Methods section) that recognize specifically the different regions that characterize the VPI-2, as shown in Figure 53.

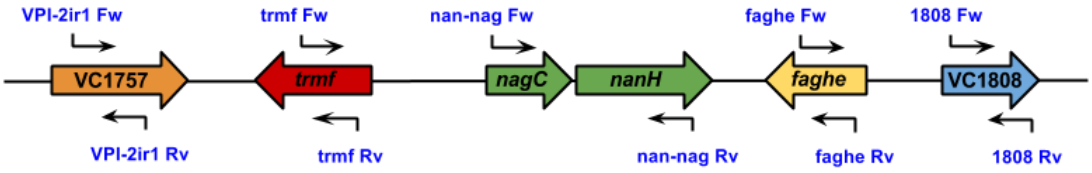

Figure 53: Canonical structure of VPI-2 in V. cholerae O1; arrows indicate the primers used in this study.

Primers VPI-2ir1Fw and VPI-2ir1Rv have as a target the 5' insertion site (IS5′) of the *V. cholerae* pathogenicity island VPI-2 into the chromosome, in particular region VC1757-VC1760. This region includes a fragment of the chromosome, the VPI-2 tRNA and a fragment of the VPI-2 integrase gene (Gennari *et al*., 2012). The presence of the cluster *nan-nag* was investigated with primers nan-nagFw and nan-nagRv; this region encodes a number of genes including *nagC* and *nanH* and is homologous to an equivalent gene cluster in *Haemophilus influenza*. PCR was also set up to detect two internal region of the VPI-2: the type 1 restriction modification system region, in particular the *trmf* gene (primers trmfFw and trmfRv) and the phage-like region (*faghe* gene) (fagheFw and fagheRv). Finally, primers 1808Fw and 1808Rv recognise the 3' terminal insertion site (IS3'a) of the VPI-2 into the chromosome, in the VC1808-VC1810 region.

We found out that strain *V. cholerae* AS1 SXT+ strain also carries a truncated version of VPI-2 shown in Figure 54, in particular its genomic island consists of the VC1757-VC1760 region (IS5'), region VC1791- VC1793 (phage-like region), and VC1808-VC1810 (IS3'). Both the type 1 restriction modification system and the cluster *nan-nag* were absent from this strain as confirmed by negative results obtained by the corresponding PCR assays. Alternatively, the not detected regions might have been modified in such a way that were no more recognizable by the designed primers.

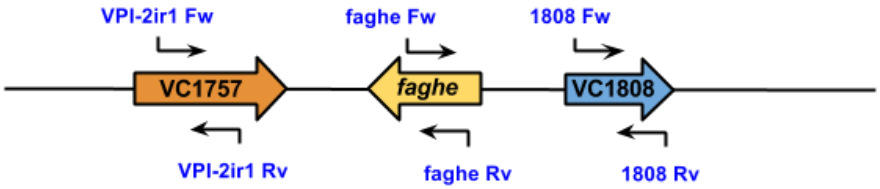

Figure 54: Effective truncated structure of the VPI-2 in the Vibrio cholerae AS1 SXT+ strain.

Considering that the VPI-2 region present in this strain was conserved at the 5' and 3' insertion sites, it should be assumed that this VPI-2 has the potential for excision from the genome to form an extrachromosomal CI, and that if an MGE is present it can probably drive the transfer of this CI towards other bacteria.

The *Vibrio cholerae* AS1 strain was then a good candidate for testing the possible transfer of VPI-2 promoted by SXT, because of the simultaneous presence of both elements.

Conjugations were set up using AS1 as donor and *E. coli* and *Salmonella* Typhimurium LT2 as recipient strains. We used *Enterobacteriaceae* bacteria sharing the same aquatic ecosystem with vibrios to prove the possibility for this genetic element carrying virulence genes to spread in the marine environment. Transconjugants containing the VPI-2 were obtained only when using *Salmonella* as recipient (Table 17).

DONOR STRAIN	**RECIPIENT STRAIN**	**transconjugant strain**	**relevant genotype and resistance profile**	**SXT/VPI-2 transfer frequency**
Vibrio cholerae AS1 SXT+, VPI-2 IS5'+, VPI-2 IS3'+, VPI-2 phage-like+	*Salmonella* Typhimurium LT2	*Salmonella* Typhimurium LT2 SXT+, VPI-2+	**SXT+**, StrSssTimChlRif, **VPI-2 IS5'+, VPI-2 IS3'+**	$5x10^{-4}$

Table 17: Donor and recipient strains, strain name of the obtained transconjugants, genotype characteristics and SXT/VPI-2 transfer frequency.

The SXT and the VPI-2 fragment were successfully co-transferred to the *Salmonella* strain, indicating that SXT is able to drive the mobilization also of this pathogenicity island. Even if in the donor three parts of the VPI-2 were present (IS5', phage-like region and IS3') as shown in Figure 58, in the obtained *Salmonella* transconjugants only the left and right terminal regions were transferred (Figure 55), the phage-like fragment was in fact absent, as shown in the corresponding electrophoresis images (Figures 56, 57 and 58). This could be due to the instability of the specific phage region or, again, because the region has been modified in such a way that was no more recognizable by the designed primers.

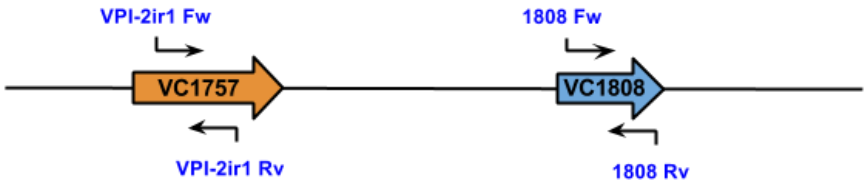

Figure 55: Truncated structure of the VPI-2 in the Salmonella Typhimurium SXT+ strain.

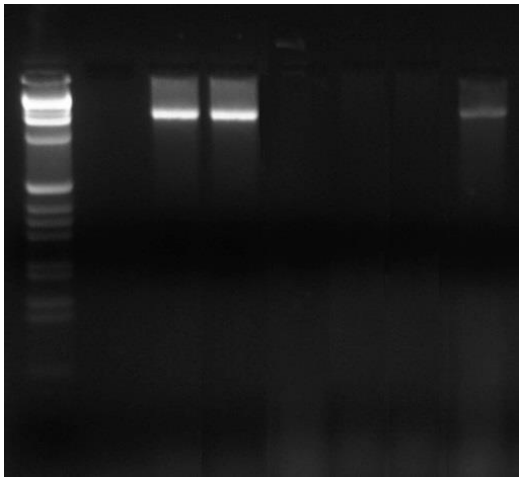

Figure 56: Amplicons obtained after PCR targeting the 5' insertion site (IS5′) of the VPI-2:
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, positive control
lane 4, *Vibrio cholerae* AS1 SXT+/VPI-2+ (donor strain)
lane 5, *E. coli* K-12 J53-S (recipient strain)
lane 6, *Salmonella* Typhimurium LT2 (recipient strain)
lane 7, *E. coli* SXT+ obtained transconjugants
lane 8, *Salmonella* LT2 SXT+/VPI-2+ obtained transconjugants.

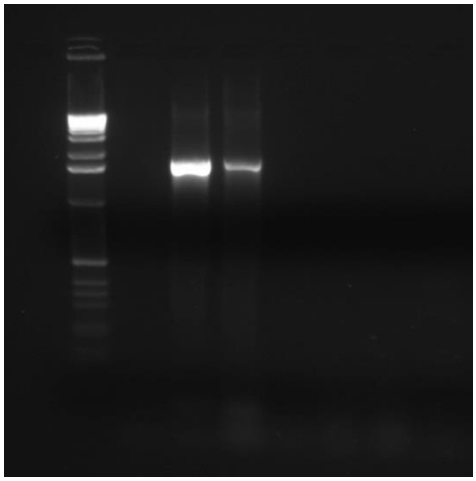

Figure 57: Amplicons obtained after PCR targeting the *faghe* gene:
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, positive control
lane 4, *Vibrio cholerae* AS1 SXT+/VPI-2+ (donor strain)
lane 5, *E. coli* K-12 J53-S (recipient strain)
lane 6, *Salmonella* Typhimurium LT2 (recipient strain)
lane 7, *E. coli* SXT+ obtained transconjugants
lane 8, *Salmonella* LT2 SXT+/VPI-2+ obtained transconjugants.

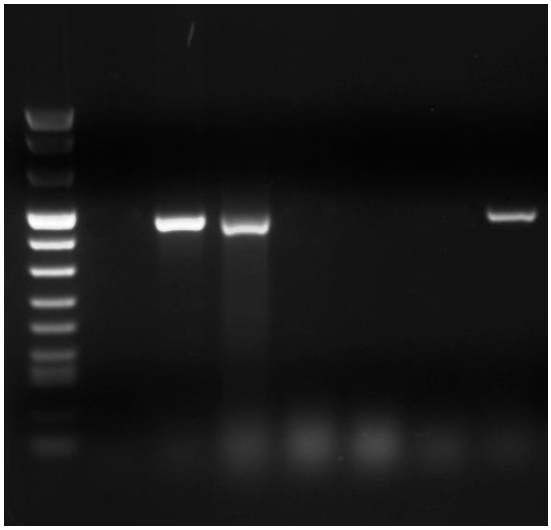

Figure 58: Amplicons obtained after PCR targeting the 3' insertion site (IS3′) of the VPI-2:
lane 1, molecular weight marker
lane 2, negative control (no DNA)
lane 3, positive control
lane 4, *Vibrio cholerae* AS1 SXT+/VPI-2+ (donor strain)
lane 5, *E. coli* K-12 J53-S (recipient strain)
lane 6, *Salmonella* Typhimurium LT2 (recipient strain)
lane 7, *E. coli* SXT+ obtained transconjugants
lane 8, *Salmonella* LT2 SXT+/VPI-2+ obtained transconjugants.

To conclude, we have showed that SXT is capable of mobilizing the VPI-2 from an environmental *Vibrio cholerae* to a *Salmonella* strain; as seen in literature, the island is deeply unstable, in fact also in this case only a part of it was transferred, probably due to the presence of the Mu phage that induces chromosomal rearrangements.

Discussion

The worldwide growth of aquaculture has been accompanied by a rapid increase in therapeutic and prophylactic usage of antimicrobials, including those important in human therapeutics. Approximately 80% of antimicrobials used in aquaculture enter the environment with their activity intact, here they select for bacteria whose resistance arises from mutations or, more importantly, from mobile genetic elements containing multiple resistance determinants transmissible to other bacteria (Cabello *et al*., 2013). The commonality of the mobilome (total mobile genetic elements in a genome) between aquatic and terrestrial bacteria, together with the presence of residual antimicrobials, biofilms and high concentrations of bacteriophages in the aquatic environment, can stimulate exchange of genetic information between aquatic and terrestrial bacteria also because seawaters can be contaminated with pathogens of human and animal origin. Several recently found genetic elements and resistance determinants for quinolones, tetracyclines, and β-lactamases are shared between aquatic bacteria, fish pathogens, and human pathogens, and appear to have originated in aquatic microorganisms.

The study here presented had its starting point in recent findings indicating the presence of a high number of antibiotic resistant and multi-resistant marine bacterial strains in the coastal area and fish farms in the Italian Adriatic Sea (Labella *et al*., 2013). In the same area, it has been detected environmental strains belonging to pathogenic and non-pathogenic species of the marine autochthonous flora and carrying virulence genes and pathogenicity islands. On the basis of these data and because it has been demonstrated that genetic determinants found in AR bacteria isolated from cases of infection are, in some cases, very similar to those detected in their environmental counterparts, we propose that this marine environment might constitute a reservoir of virulence and AR genes transmissible to autochthonous and allochthonous bacteria present in seawaters.
To demonstrate this hypothesis we focused on two issues:
1) To demonstrate the possibility of MGE exchange among bacteria present in the aquatic environment.
2) To test the possible mobilization, mediated by the SXT conjugative element, of non-mobile genetic elements carrying genes of medical interest.

Integrative conjugative elements (ICEs) are a major driving force of bacterial genome evolution allowing rapid acquisition of a variety of new traits and adaptive functions such as virulence, metabolic pathways and resistance to antimicrobial compounds, heavy metals or

bacteriophage infection (Burrus *et al*., 2002) For instance, ICEs of the SXT/R391 family largely contribute to the spread of antibiotic resistance genes in the seventh-pandemic lineage of *Vibrio cholerae*, the etiologic agent of cholera, which remains a major cause of mortality and morbidity on a global scale (Wozniac *et al*., 2009). The SXT/R391 ICEs are grouped together because they share a common set of 52 highly conserved genes, among which ~25 are important for their maintenance, dissemination by conjugation and regulation. We have confirmed that the SXT element can be found in environmental bacteria in the area of the Italian Adriatic Sea and that it **can be transferred from marine autochthonous bacteria** such as a *Vibrio*, **to allochthonous bacterial species** considered human pathogens, such as *E. coli* and *Salmonella*, that can be transitorily present in the marine environment. The SXT element found in donors and transconjugants was present **both in the circular extrachromosomal intermediate form and in the integrated form**, supporting the proposed transfer mechanism based on a site-specific excision from the host, the conjugal transfer and the integration into the recipient chromosome. The integration site of SXT elements is always the 5' end of *prfC* locus, a conserved gene coding for peptide chain release factor 3 (RF3). Speculations have been done to understand why this particular gene is the target of the integration and some hypotheses have been proposed. Genes encoding proteins involved in translation have been found to be integration hot spots for elements such as phages, plasmids and pathogenicity islands. RF3 has an important role in the regulation of translation and for this reason it might be an adequate integration site. It is in fact possible that the translation of the genes present in the SXT element may be influenced by the recombinant RF3 (Hochhut and Waldor, 1999).
It has been confirmed in this study that independently from the bacteria genus, SXT always integrates specifically in the *prfC* locus that is present in a wide range of bacterial genomes (Kawazu *et al*., 1995), providing thus a large host range for SXT dissemination.

The *in situ* HGT rates in aquatic environments have historically been studied using microcosms, systems modelling the coastal or estuarine ecosystems, in absence of macro and micro-biota. Their limit is that they do not reproduce the natural scale and thus some important variables affecting HGT might be missed. Moreover, the use of model donors and recipients selected in the lab determines a lack of variability and information of the real phenomenon. It is important to notice that, in nature, different MGEs may interact with each other and that in a free environment bacteria can be transferred across very distant areas. The geographic component should thus be considered when estimating the HGT frequencies in nature and the obtained results have to be interpreted taking into account all those elements (Aminov, 2011).

In spite of all these criticisms, **the transfer ability of SXT was observed in this study also in marine water microcosms** confirms that HGT is a natural phenomenon that can take place in aquatic environments. Our results in terms of MGE transfer frequencies are in line with those reported in literature: the rates of HGT in marine bacterial communities varied in fact from $2x10^{-6}$ to $2x10^{-4}$ transconjugants per recipient (Dahlberg *et al*., 1997), while we obtained values of about 10^{-5}.
Aquatic environment represents thus a favourable ecologic niche where HGT takes place between different bacterial genera. Our data provide useful insights into the basis for drug-resistance dissemination among bacteria in aquacultures and their possible clinical impact.

On the basis of the experiments performed in urine microcosms we can also propose bladder has a probable hot spot for HGT; as a consequence, the phenomenon may have a clinical relevance. The presence of low concentrations of cutaneous and intestinal flora in the urinary district is common. An AR bacterium might in fact transfer some resistance genes to a susceptible one and determine the bladder colonization by AR strains. Not only bladder, but also other human districts could be interested by HGT, especially those presenting a high bacterial concentration such as the mouth or the gut. More efforts should be dedicated to the comprehension of the mechanisms involved in this phenomenon in order to prevent the spread of AR traits in human districts.

Our latest experiments, confirming that SXT is able to mobilize two different **genomic islands (SGI1 and VPI-2)**, clearly show that this element could not only be transferred between different strains, but might also contribute to the diffusion of other virulence and AR genes. In most cases, the mechanisms of acquisition and exchange of GIs between bacteria are unknown. Some studies reporting the excision and circularization of the three pathogenicity islands VPI-2, VSP-I and VSP-II found in seventh pandemic *V. cholerae* isolates, gave new emphasis to this subject (Murphy and Boyd, 2008). It was already hypothesized that the re-emergence of *V. cholerae* El Tor isolates as the predominant cause of cholera may be the result of reduced fitness of the O139 strains due to the loss of the VPI-2 region genes. The neuraminidase gene (*nanH*), located in VPI-2 and involved in providing a receptor for the cholera toxin by hydrolyzing sialic acid, was retained among most pathogenic strains, while the loss of this region may have resulted in reduced fitness in O139 isolates when infecting the human host. Sialic acid is an amino sugar present in the human intestine and a possible important source of carbon and nitrogen; therefore, carriage of VPI-2 by the O1 serogroup isolates could give them a competitive advantage (Murphy and Boyd, 2008). Data from a study by Almagro-Moreno *et al*. indicate that environmental factors such

as UV light can affect the induction of excision and circularization of VPI-2, which is the first step required for the horizontal transfer of the region. The mobilization of this island seems thus to be inducted by environmental conditions, similar to what happens with ICEs (Almagro-Moreno *et al*., 2010).

The possibility for a non-pathogenic Vibrio strain of acquiring an entire or partial pathogenicity island is highly relevant, as this island is putatively involved in bacterial virulence due to the presence of several virulence factor genes. In fact, it has been demonstrated that VPI-2 is present in toxigenic *V. cholerae* isolates and is absent from non-pathogenic isolates, again supporting its role in pathogenicity.

The previously reported data (Gennari *et al*., 2012) and the data presented in this study confirm that the VPI-2 is highly unstable, in fact also our environmental isolates contained only truncated forms of the genetic elements as already reported in literature (Gennari *et al*., 2012).

Salmonella genomic island 1 (SGI1), is a genomic island containing an antibiotic resistance gene cluster identified in several *S. enterica* serovars. The presence of this island in *Salmonella* infecting different animal species and humans around the world indicates a large diffusion of this genomic island and led to the hypothesis of a high SGI1 horizontal transfer potential. In this study, SGI1 was successfully transferred by conjugative mobilization from a donor (*S.* Agona) to a recipient strain (*S.* Typhimurium) due to the presence in the donor strain of an SXT element. **To the best of our knowledge, this study represents the first description of a specific relationship between this MDR genomic island and SXT.** More than just its own dissemination, SXT element may also contribute to the spread of the antibiotic resistance genomic island SGI1 and its close derivatives among enteric pathogens and potentially more widely.

Collectively, our results demonstrate that **ICEs of the SXT/R391 family trigger the excision of non-mobile elements such as multidrug-resistance GI found in *Salmonella* and the VPI-2 found in *V. cholerae***, promoting their conjugative transfer to recipient cells. MDR *Salmonella* Typhimurium DT104 has been associated with higher rates of morbidity and mortality when compared to Salmonella strains lacking the SG1 island. In addition, a study analyzing the incidence of blood infections caused by *S*. Typhimurium DT104, demonstrated that the incidence is higher when infections were caused by MDR strains carrying the genomic island (Boyd *et al*., 2001). Our results indicate that GIs are not necessarily defective or decaying genetic elements, unable to propagate. Instead, most are likely quiescent elements waiting opportunities to hijack helper self-transmissible elements to be transferred. Those hypotheses underline the impact of MGEs on evolution and

adaptation of genomes on the basis of environmental conditions, and are particularly relevant in the current context of massive emergence of multidrug resistant pathogens worldwide.

Although the majority of *Vibrio* spp. analysed in our study are not pathogenic for humans, our study indicates that those environmental bacterial represent a reservoir for emerging AR and virulence genes that could spread also among human pathogens. The main consequence of the use of antibiotics is the concomitant development of resistant strains; this has prompted continuous efforts to exert control over antibiotic usage. Trimethoprim and streptomycin are prescribed in human therapy but they are also extensively used in aquaculture, it is thus possible that their sub-inhibitory concentrations in water may select for waterborne resistant strains SXT+ and enhance transfer of this naturally occurring resistance determinant to *Enterobacteriaceae*. This consideration emphasizes **the possible role of the aquatic environment in emergence of drug resistance and underlines the importance of monitoring this habitat.**

Other mobile genetic elements may be implied in the development of resistance in the *Vibrio* spp in these environments. It is known that sub-inhibitory antibiotics concentrations increase the frequency of HGT, both *in vitro* and *in vivo*, resembling to a positively-regulated mechanism of switch. This leads to a new concept of "hormesis" introduced by Aminov: low antibiotic concentrations may regulate a set of genes in target bacteria to increase their survival rate (for example having a stimulatory effect on the movement of MGEs), higher concentrations determine a stress response and extremely high quantities are lethal. Research on AR has been focused on bacteria pathogens isolated from clinical settings. However, the fact that HGT-acquired genes originated in environmental bacteria and that the first step in the transfer occurs in natural ecosystems emphasizes the need to analyse the phenomenon in non-clinical settings. **The evaluation of the MGE incidence might indirectly provide an idea of the gene exchange extent in the aquatic environment, while the identification of their source and dissemination mechanisms might contribute to a better management of antibiotic therapy, reduce the spread of known AR determinants and limit the possible emergence of new AR mechanisms derived from DNA recombination events. The acquisition of genetic material from bacterial species of clinical interest might facilitate the spread of virulence and/or AR genes and the delivery of those genes to humans, representing then a concern for public health and constituting a risk for human health.**

Final Conclusions

A microarray has been specifically designed and developed to contemporary search in marine samples almost 200 different genes involved in virulence and antibiotic resistance typical of marine bacteria and pathogens of human and veterinarian interest. This tool has allowed us to confirm the presence of virulence genes and pathogenicity islands in strains belonging to non-pathogenic marine bacteria isolated in the area of the Italian Adriatic Sea. In the same coastal area and fish farms, a significant number of antibiotic resistant and multi-resistant strains have been isolated, identified and characterized.

Many of the considered genes are located in mobile genetic elements and genomic islands unable of autonomous transfer. This fact might facilitate gene transfer and spread of genes of medical interest among members of the autochthonous marine flora and between this microflora and human pathogens transitory present in seawaters. The results obtained in this study confirm the possibility for *Vibrio* strains of transfer MGE with significant transfer frequencies to *E. coli* and *Salmonella* in culture medium, aquatic environment and human body sites such as bladder.

More interesting, it has been demonstrated in this study the high impact that the transfer of integrative conjugative elements might have in spreading virulence and AR genes in that they can also mobilize genetic elements carrying those genes and lacking transfer autonomy.

On the basis of the data here presented we consider that the coastal area and areas surrounding fish farms in the Italian studied region constitute a reservoir of virulence and antibiotic resistance traits that might represent a risk for human health. The reported results taken together emphasize the impact of MGEs on evolution and adaptation of genomes on the basis of environmental conditions and are relevant in the actual context of antibiotic resistance emergence highlighting the possible role of the aquatic environment in this phenomenon.

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