RESEARCH ARTICLE

Environmental *Vibrio* spp., isolated in Mozambique, contain a polymorphic group of integrative conjugative elements and class 1 integrons

Elisa Taviani[1], Daniela Ceccarelli[2], Nivalda Lazaro[3], Stefania Bani[2], Piero Cappuccinelli[4], Rita R. Colwell[5] & Mauro M. Colombo[2]

[1]Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD, USA; [2]Dipartimento di Biologia Cellulare e dello Sviluppo, Universitá di Roma La Sapienza, Rome, Italy; [3]Hygiene National Laboratory of Water and Food, Ministry of Health, Maputo, Mozambique; [4]Dipartimento di Scienze Biomediche, Facoltà di Medicina University of Sassari, Sassari, Italy; and [5]University of Maryland Institute for Advanced Computer Studies, College Park, Hyattsville, MD, USA

Correspondence: Mauro M. Colombo, Dipartimento di Biologia Cellulare e dello Sviluppo, Universitá di Roma La Sapienza, Via dei Sardi 70, 00185 Rome, Italy. Tel.: +39 0649917585; fax: +39 0649917594; e-mail: mauro.colombo@uniroma1.it

Received 8 August 2007; revised 17 December 2007; accepted 5 January 2008.
First published online March 2008.

DOI:10.1111/j.1574-6941.2008.00455.x

Editor: Kornelia Smalla

Keywords
ICE; integron; *Vibrio*; Mozambique.

Abstract

Circulation of mobile genetic elements linked to drug resistance spread was studied in *Vibrio* strains isolated from surface urban water (river and sea) and shellfish samples in 2002–2003 in Maputo, Mozambique. Class 1 integrons and integrating conjugative elements (ICE) were investigated by PCR and mating experiments in strains of major health interest: 10 *Vibrio cholerae*, six *Vibrio parahaemolyticus*, two *Vibrio alginolyticus* and one *Vibrio fluvialis*. Resistance to at least two antibiotics (predominantly β-lactams) was detected in all the strains, with additional resistances to sulfamethoxazole, spectinomycin, streptomycin and/or trimethoprim. Class 1 integrons contributed partially to the expression of drug resistance and were found in five isolates: four *V. cholerae* (*blaP1* cassette, one strain also contained the *dfrA15* cassette) and one *V. alginolyticus* (*aadA2* cassette). ICEs, apparently devoid of resistance genes, were found in eight *V. cholerae*, three *V. parahaemolyticus* and one *V. fluvialis* isolates. A wide variability was observed by molecular characterization of ICEs. Five ICEs were included in the SXT/R391 family and seven ICEs were not classified. Our results indicate that the SXT/R391 family and related ICEs comprise a large class of polymorphic genetic elements widely circulating in environmental *Vibrio* strains in Africa, beside those evidently linked to drug resistance in clinical isolates.

Introduction

The genus *Vibrio* includes many harmless species, natives of fresh, brackish and marine waters and is an important component of the aquatic microbial communities (Colwell & Huq, 1994), also able to persist in shellfish. Among these should also be mentioned some etiologic agents of enteric diseases and epidemics (Faruque *et al.*, 1998; Sechi *et al.*, 2000; Gil *et al.*, 2004): O1 and O139 serotypes of *Vibrio cholerae*, some *V. cholerae* non-O1 serotypes (Thungapathra *et al.*, 2002), *Vibrio parahaemolyticus* (Utsalo *et al.*, 1992), *Vibrio fluvialis* (Ahmed *et al.*, 2004) and *Vibrio alginolyticus* (Ripabelli *et al.*, 2003).

Integrons are genetic mobile elements, able to acquire antibiotic resistance genes in several bacterial species (Mazel, 2006). These elements are not autonomously mobile but can capture, integrate and express resistance gene cassettes in their variable region (VR) and can be transmitted via transposons and conjugative plasmids (Hall & Collis, 1995). Among other classes, class 1 integrons are organized in two conserved regions (CS): 5′CS containing the integrase gene, and 3′CS usually characterized by *qacEΔ1* and *sul1* resistance genes to quaternary ammonium compounds and sulfonamide, respectively (Rowe-Magnus & Mazel, 2001; Mazel, 2006). Class 1 integrons have been found in clinical (Dalsgaard *et al.*, 2000a, 2001; Ceccarelli *et al.*, 2006a, b) and environmental *V. cholerae* isolates (Park *et al.*, 2003; Biyela *et al.*, 2004); very few resistance integrons have been found in *Vibrio* spp. other than *V. cholerae* (Park *et al.*, 2003; Ahmed *et al.*, 2004; Ceccarelli *et al.*, 2006b).

Integrating conjugative elements (ICEs) are able to confer drug resistance to both clinical and environmental bacterial strains. They can be present in the bacterial chromosome and can excise, transfer and integrate again into a compatible new host chromosome by conjugation (Burrus & Waldor, 2004a). To date, many different ICEs have been described, the SXT/R391 family being the most diffused in the *Vibrio* genus.

The first such ICE, R391, was discovered in 1991 in a South African *Providencia rettgeri* environmental strain, isolated in 1967 and coding for kanamycin (*aph*) and mercury resistances (*mer* operon) (Peters *et al.*, 1991). SXT^{MO10} is an ICE originally found in the chromosome of *V. cholerae* O139 isolated in India in 1992 (Waldor *et al.*, 1996). This ICE confers resistance to florphenicol (*floR*), streptomycin (*strA* and *strB*), sulfamethoxazole (*sul2*) and trimethoprim (*dfrA18*) (Hochhut *et al.*, 2000, 2001). The resistance genes are embedded in a transposon-like element that interrupts the SXT-encoded *rumAB* operon (Hochhut *et al.*, 2001). When not disrupted, this operon encodes proteins that are homologs of the *Escherichia coli* UmuDC and perform a UV-protection function (Pembroke *et al.*, 2002).

SXT^{MO10} and R391 encode an integrase with the ability to integrate into a nonessential chromosomal gene (*prfC*) (Hochhut & Waldor, 1999), and share a highly conserved genetic backbone encoding for their regulation, excision/integration and conjugative transfer (Beaber *et al.*, 2002; Boltner *et al.*, 2002). Genes specific for each ICE of the SXT/R391 family are interspersed in the conserved sequence, and three Hotspots have been identified near the *tra* genes as targets for different insertions (Beaber *et al.*, 2002). Figure 1 shows how the molecular analysis of these regions allows discrimination between SXT and R391.

Several SXT-like elements have been described in *Vibrio*, characterized by various molecular rearrangements and their antibiotic resistance pattern. ICE*Vch*Hko1 and ICE*Vch*Ind1 are characterized by the loss of the antibiotic cluster inserted in the *rumAB* operon, and by the presence of *dfrA1* in the *s073/traF* Hotspot, respectively (Hochhut *et al.*, 2001).

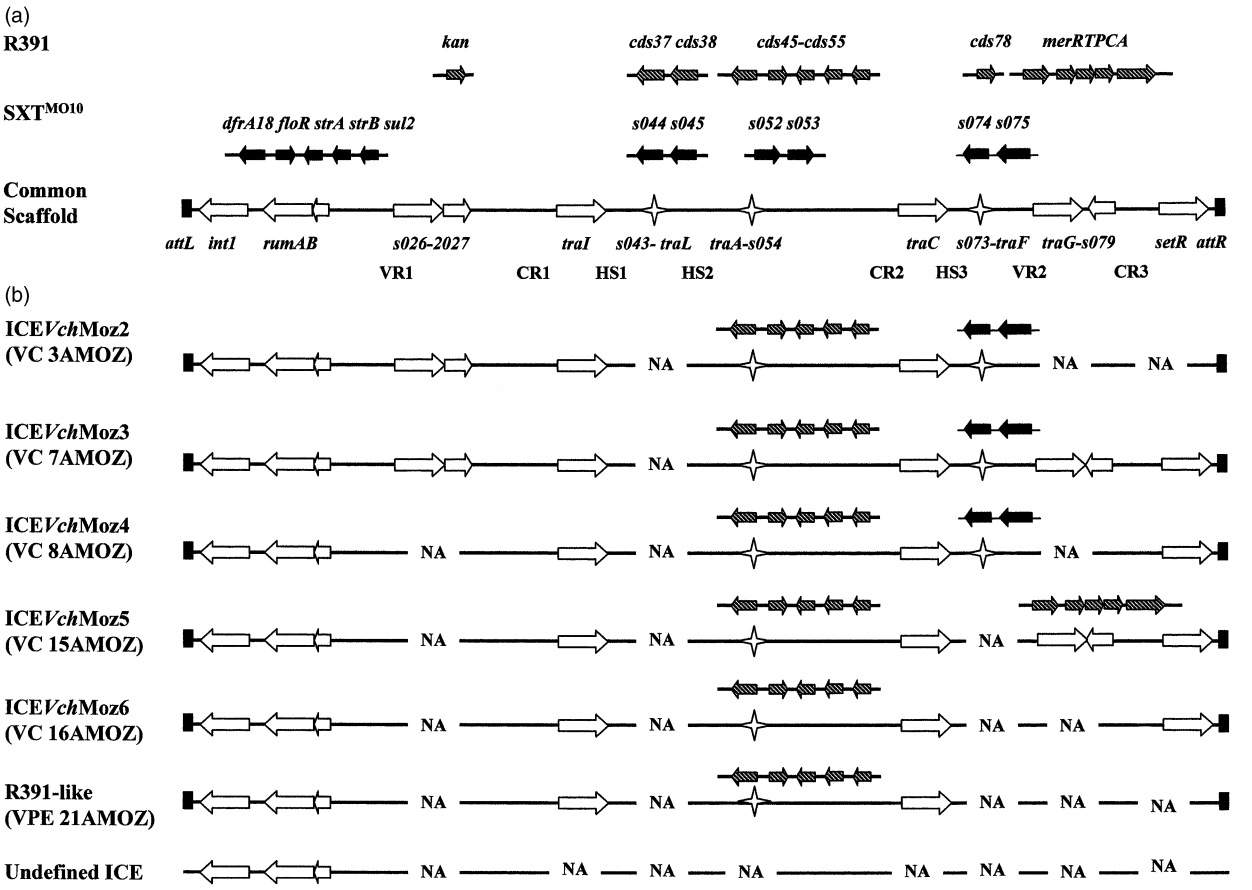

Fig. 1. Molecular structure of ICEs belonging to the SXT/R391 family and characterization of seven new ICEs. (a) Linear representation (not to scale) of the common scaffold of ICEs of SXT/R391 family. The black line represents the conserved genes in SXT and R391. Specific insertion for SXT^{MO10} (stripes) and R391 (dotted) are depicted above the common scaffold. (b) Schematic representation of the seven ICEs found in *Vibrio* environmental isolates described in this work. VR: Variable Region; CR: Conserved Region; HS, Hotspot; NA, not amplified.

New variants of SXT-related ICEs were described both in *V. fluvialis* clinical isolates (ICE*Vfl*Ind1) and in *V. cholerae* environmental isolates (ICE*Vch*Mex1) (Ahmed *et al.*, 2005; Burrus *et al.*, 2006b). A new nomenclature has been proposed to classify ICEs belonging to the SXT/R391 family (Burrus *et al.*, 2006a). ICEs are named with the prefix ICE followed by abbreviations for the species of origin and for the country of origin, and a number to distinguish different isolates from identical species and country.

Mozambique, an endemic area for cholera and site of recent severe outbreaks, is a region with climate, environmental and social conditions, and notably poor sanitation, favorable for the transmission of environmental pathogens to humans (Folgosa *et al.*, 2001; Collins, 2002; Lee *et al.*, 2006). Integrons and ICEs have already been found in clinical *V. cholerae* O1 isolates in Mozambique, where they greatly contributed to the drug resistance profiles (Ceccarelli *et al.*, 2006a). The aim of this study was to explore the circulation of integrons and ICEs in aquatic environmental *Vibrio* strains, isolated in the Maputo area, Mozambique, in 2002 and 2003.

Materials and methods

Bacterial isolation

The surveillance and sampling were performed along Maputo Bay and Infulene Valley (Table 1). The first area is both a popular recreational beach and a final destination for the Maputo area run off; the second area is very densely populated with intensive agriculture and is located 5 km east of Maputo city. The Infulene River is a water source for both agriculture and domestic use by the local population that lives under extremely poor sanitary conditions. It is also a collection site for industrial and domestic waste-water and drainage from the local waste treatment plant (ETAR). Five representative sites in each locality were selected to collect water samples and the positions of the sites established by global position system (GARMIN GPS III, accurate to 5 m). Shellfish were also collected from the Bairro dos Pescatores, the informal fish market located north of Maputo Bay.

These strains belong to a collection of isolates (among others, 231 *Aeromonas* spp., 46 *V. fluvialis*, 16 *V. cholerae* non O1, 15 *Vibrio mimicus*, eight *V. alginolyticus*, six *V. parahaemolyticus*) from more than 1500 different environmental samples (water and shellfish) collected in the Maputo area, during April 2002–June 2003 (Lazaro *et al.*, 2006). Only 19 isolates were sent to Italy for further molecular characterization. The species were identified by API 20E and 20 NE (bioMérieux, France): 10 *V. cholerae* non O1 (confirmed by PCR amplification of the specific outer membrane protein *omp*W gene, but negative to specific O1 and O139 antiserum) (Nandi *et al.*, 2000), six *V. parahaemolyticus*, two *V. alginolyticus* and one *V. fluvialis*.

All of the *V. cholerae* isolates were negative for *ctx*AB operon and *tcp*A virulence gene, and all but the 03FG *V. parahaemolyticus* isolate were *tl* positive and *tdh* and *trh* negative by PCR testing, as described previously (Colombo *et al.*, 1997; Bej *et al.*, 1999).

Vibrio cholerae O1 N16961 (provided by D. Mazel, Ins. Pasteur, Paris, France), *V. cholerae* MO10 O139 (provided by M. K. Waldor, Channing Laboratory, Brigham's and Women's Hospital and Harvard Medical School, Boston, MA), *E. coli* AB1157:R391 (provided by J. T. Pembrooke, University of Limerick, Limerick, Ireland) and *V. cholerae* O1 582 from our collection (Ceccarelli *et al.*, 2006b), were used as negative or positive controls in PCR analysis for detection of class 1 integrons and ICEs. *Escherichia coli* 803 (rifampicin-resistant) was used as recipient in mating experiments.

Antibiotic resistance and mating assays

The following concentrations of antibiotics were used for testing susceptibility: ampicillin ($30\,\mu g\,mL^{-1}$), chloramphenicol (2 and $20\,\mu g\,mL^{-1}$), erythromycin ($10\,\mu g\,mL^{-1}$),

Table 1. Description and localization of sampling sites included in this study

Sampling site	Site and sample description	GPS coordinates
Maputo Bay		
Clube Maritimo	Recreation and fishing area, sea water	S25 56.889, E32 37.039
Miramar	Mouth of the waste channel to the bay, waste water	S25 57.259, E32 36.638
Ponta Vermelha	Mouth of the waste channel to the bay, waste water	S25 59.046, E32 35.522
Catembe Bridge	Mouth of the waste channel to the bay, waste water	S25 58.730, E32 34.519
Matola River	Fishing school, sea water	S25 59.165, E32 27.410
Infulene River Valley		
Benfica	Infulene River irrigation channel, fresh water	S25 53.242, E32 33.507
Infulene River	Irrigation point source, fresh water	S25 54.751, E32 31.213
Infulene Bridge	Irrigation point source, fresh water	S25 55.159, E32 32.540
Drain	Rain drainage channel, rain drainage water	S25 55.255, E32 32.621
ETAR	Waste treatment plant, posttreatment water	S25 55.223, E32 32.352

kanamycin (30 μg mL^{-1}), nalidixic acid (40 μg mL^{-1}), penicillin (20 μg mL^{-1}), rifampicin (100 μg mL^{-1}), streptomycin (10 and 100 μg mL^{-1}), spectinomycin (20 μg mL^{-1}), sulfamethoxazole (160 and 600 μg mL^{-1}), tetracycline (10 μg mL^{-1}) and trimethoprim (10 μg mL^{-1}). The antibiotics were included in ISO sensitest (Oxoid) agar plates and the bacterial strains were spotted onto the plates as previously described (Ceccarelli *et al.*, 2006b).

The following concentrations of drugs were used for selection in mating experiments: rifampicin (100 μg mL^{-1}), sulfamethoxazole (160 μg mL^{-1}) and spectinomycin (20 μg mL^{-1}). Matings were performed by mixing donor strains (previously treated with mitomycin C 50 ng mL^{-1}, for 1 h at 37 °C) and recipient strains at a ratio of 1 : 1; 200 μL of the mix were washed twice and concentrated 20 times, spotted onto nutrient agar plates and incubated at 37 °C for 6 h. Mitomycin is known to increase the transfer frequency of SXT by inducing the expression of its transfer genes (Beaber *et al.*, 2004). After mating, bacteria were collected by washing the plate surface and serial dilutions where spread on appropriate drug selecting plates (V. Burrus, pers. commun.). The frequency of transfer was expressed as the number of exconjugants per donor in the mating mixture at the time of plating.

Molecular analysis

Bacterial DNA for PCR testing was extracted according to Ausubel *et al.* (1990). Class 1 integron cassette content was studied using the *In*-F and *In*-B (Levesque *et al.*, 1995) primer pair that amplifies the VR from the integrase gene to the 3′ CS, containing the inserted gene cassettes.

Detection of ICEs belonging or related to the SXT/R391 family was preliminarily performed by PCR, using specific primer pairs for int_{SXT} (integrase gene). Strains positive for int_{SXT} were then investigated for integration into *prfC* in the *V. cholerae* chromosome, by amplification of the *prfC*/SXT right junction of the chromosomal integration region (P3/P1 primer pair) (Hochhut & Waldor, 1999; Dalsgaard *et al.*, 2001). We designed a new specific backward primer on *V. parahaemolyticus prfC* gene (VPprfC-R, CCTGCTTGA TACTCCAGGACACG, nucleotides 1313–1336, GenBank accession no. BA000031) to be coupled with P3 forward primer on SXT right junction (Hochhut & Waldor, 1999), generating a 330 bp amplicon. To better define the integration region, we designed specific primers for PCR amplification of *prfC* intact gene, in both *V. cholerae* (PVC-F, TCC TGCACCTTGCTCTGCTCT, nucleotides 703526–703547, and PVC-B, ACCACGCTCTTTTTCCATTTCCAT, nucleotides 704053–704077, GenBank accession no. AE003852) and *V. parahaemolyticus* (PVP-F, GGCAATGGAAAAGAAG ACGCGA, nucleotides 19354–19376 and PVP-B, ATCAAA CTCAGGACATGCACCC, nucleotides 20231–20253, GenBank accession no. AAWQO1000037), generating, respectively, 550 and 898 bp amplicons.

All int_{SXT}-positive strains were further tested for the genetic arrangement of the *rumAB* operon and for the resistance genes typically associated with SXT^{MO10} or R391: *floR, strA* and *strB*, *sul2, dfrA18* and *dfrA1* and *kan* (Hochhut *et al.*, 2001; Iwanaga *et al.*, 2004; Bani *et al.*, 2007).

To better characterize the ICE molecular profile, we analyzed three regions within the functionally conserved scaffold shared by SXT^{MO10} and R391: traI-R/orf171 (*traI* gene, CR1), traC-B/traC-F (*traC* gene, CR2) and SetRp-R/SetRp-F (*setR* gene, CR3) (see Fig. 1a) (Bani *et al.*, 2007). Five additional regions allowing distinction between the SXT^{MO10} and R391 elements (Fig. 1a) were also investigated. We chose two VRs carrying inserted sequences belonging to R391: *s026/s027* bearing the *kan* gene (VR1) and *traG/s079* bearing the *mer* operon conferring mercury resistance (VR2), and three so called Hotspots, which distinguish between SXT^{MO10} and R391, with alternatively specific inserted sequences: *s043/traL* (Hotspot 1), *traA/s054* (Hotspot 2) and *s073/traF* (Hotspot 3) (Beaber *et al.*, 2002; Bani *et al.*, 2007).

All PCR reactions were carried out in 50 μL of reaction buffer containing 1 U of Taq polymerase as directed by the manufacturer (Promega).

Amplicons to be sequenced were either directly purified from the PCR reaction by Nucleospin Extract kit (Macherey-Nagel, Oensingen, Switzerland) or extracted from agarose gels by Rapid Gel Extraction System (Marligen BioSciences, Liamsville, MD).

Nucleotide sequences were determined by IDI-IRCCS (Nucleic Acid Facility, Rome, Italy) using the Sanger method and an ABI Prism 377–96 genetic analyzer. DNAMAN software was used to align bidirectional DNA sequences, subsequently blasted and analyzed against the GenBank database.

Results

Antibiotic resistance and conjugative ability

The resistance profile of the 19 isolates examined in this study is detailed in Table 2. β-Lactam resistance, followed by aminoglycosides, were observed as prevalent among the strains.

In addition, resistance to sulfamethoxazole, spectinomycin, streptomycin and/or trimethoprim was present among the strains. All were susceptible to chloramphenicol, nalidixic acid and rifampicin.

Because a multiple resistance profile is generally a strong evidence of the presence of mobile elements, we analyzed our strain collection for the presence of class 1 integrons and ICEs of the SXT/R391 family.

Table 2. Strains analyzed in this study, site and date of isolation, drug resistance profile, integron content and SXT integrase evidence

Species	Strain	Isolation date	Sample site	Drug resistance profile	Integron cassette	SXT integrase
V. cholerae	3AMOZ*	04/06/2002	Catembe Bridge	PEN, SPT, TMP	*bla*P1	+
V. cholerae	7AMOZ*	04/06/2002	Miramar	PEN, SPT	*bla*P1	+
V. cholerae	8AMOZ*	11/06/2002	ETAR	AMP, PEN, STR, SUL, TMP	*bla*P1, *dfr*A15	+
V. cholerae	11AMOZ	18/06/2002	Ponta Vermelha	PEN, SPT, SUL	*bla*P1	−
V. cholerae	12AMOZ	11/02/2003	Infulene Bridge	AMP, PEN		−
V. cholerae	13AMOZ*	11/02/2003	Infulene Bridge	SPT, TET		+
V. cholerae	14AMOZ*	18/02/2003	Ponta Vermelha	AMP, ERY, PEN, SPT, STR, SUL, TMP		+
V. cholerae	15AMOZ	11/03/2003	Miramar	AMP, PEN, SUL, TMP		+
V. cholerae	16AMOZ*†	11/03/2003	Catembe Bridge	PEN, SPT, SUL		+
V. cholerae	22AMOZ	25/02/2003	Shellfish	AMP, PEN, SPT		+
V. parahaemolyticus	1AMOZ	16/04/2002	Clube Maritimo	AMP, PEN		−
V. parahaemolyticus	03FG	11/02/2003	Infulene Bridge	AMP, PEN		+
V. parahaemolyticus	11Fon	18/02/2003	Clube Maritimo	AMP, PEN		−
V. parahaemolyticus	21AMOZ	25/02/2003	Shellfish	AMP, PEN		+
V. parahaemolyticus	23AMOZ	25/02/2003	Shellfish	AMP, KAN, PEN, SPT, STR		−
V. parahaemolyticus	53AMOZ*	18/03/2003	Shellfish	AMP, PEN, SPT, SUL		+
V. alginolyticus	12FP	18/02/2003	Miramar	AMP, ERY, PEN, SPT, STR, SUL, TMP	*aad*A2	−
V. alginolyticus	41NF	03/11/2003	Clube Maritimo	AMP, PEN, SPT		−
V. fluvialis	15FG	18/02/2003	Matola River	AMP, PEN		+

AMP, ampicillin; ERY, erythromycin; KAN, kanamycin; PEN, penicillin; STR, streptomycin; SPT, spectinomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

*Submitted to mating experiments.

†Positive for *sul2* gene.

Distribution of class 1 integrons

PCR amplification of integron VR allowed detection of a common 1200 bp amplicon in *V. cholerae* 3AMOZ, 7AMOZ, 8AMOZ and 11AMOZ. An additional 750 bp amplicon in 8AMOZ and a single 950-bp amplicon in *V. alginolyticus* 12FP were also found. The 1200-bp amplicon from isolate 3AMOZ, revealed 99% identity to the *blaP1* gene, conferring resistance to β-lactams (GenBank accession no. AF221899). The same *blaP1* integron, identified by the 1200-bp amplicon, was found in strains 7AMOZ, 8AMOZ and 11AMOZ. The 750-bp amplicon from 8AMOZ was 99% identical to the *dfrA15* gene cassette conferring resistance to trimethoprim (GenBank accession no. AF221900). The 950-bp *V. alginolyticus* amplicon encoded a cassette 99% identical to the *aadA2* aminoglycoside adenylyltransferase gene, which encodes resistance to spectinomycin (GenBank accession no. AF221903), already described in *V. cholerae* O1 isolates (Dalsgaard *et al*., 2001).

ICE detection and molecular characterization

Twelve of the 19 isolates were positive for int_{SXT} integrase gene when tested with the int1-F and int1-B primer pair in PCR amplification (Table 2). None of the *V. alginolyticus* isolates were positive. Identity of the amplicons was confirmed by nested PCR with the nint-F/nint-B internal primer pair. Amplicons obtained from the isolates 3AMOZ and 21AMOZ were selected as representatives for sequencing, and both showed 96% and 94% of identity to *P. rettgeri* R391 (GenBank accession no. AY090559.1) (Boltner *et al*., 2002) and *V. cholerae* SXT^{MO10} (GenBank accession no. AF055428.1) (Beaber *et al*., 2002) integrase *int* gene, respectively.

We then assessed, in the 12 integrase-positive strains, the presence of the resistance genes typically associated with SXT^{MO10} or R391: *sul2*, *strA*, *strB*, *dfrA18*, *floR*, *dfrA1*, and *kan*. All were negative except the isolate 16AMOZ containing the *sul2* resistance gene.

Because most of these genes are clustered in the *rumAB* operon in SXT^{MO10}, to confirm this result we amplified this region. The amplification revealed a 750-bp amplicon in all 12 ICEs, suggesting the integrity of *rumAB* operon and the absence of any insertion. Sequence of the amplicon obtained from 7AMOZ showed 95% identity with *rumAB* operon of *P. rettgeri* R391 (GenBank accession no. AY090559) (Boltner *et al*., 2002) and *V. cholerae* ICE*Vch*Mex1 (GenBank accession no. DQ180350.1) (Burrus *et al*., 2006b).

To classify these ICEs, in addition to the int_{SXT} gene, we investigated the right junction of the integration site in the chromosomal *prfC* gene. Only five out of 12 *Vibrio* isolates generated the positive 350-bp amplicon characterizing the integration into this chromosomal gene (Table 3). These strains were negative when tested for the intact *prfC* gene by PCR. On the contrary, an intact *prfC* was found in *V. cholerae* 13AMOZ, 14AMOZ, 22AMOZ, *V. parahaemolyticus* 21AMOZ and 53AMOZ, and *V. fluvialis* 15FG

Table 3. ICE molecular characterization by PCR amplification of conserved and variable regions*

Isolates	ICE	Variable region 1	Hotspots 2†	Hotspots 3‡	Variable region 2	Conserved common regions *traI*	*traC*	*setR*	*prfC*
V. cholerae 3AMOZ	ICE*Vch*Moz2	653§	491¶	988§	–	697	657	–	350
V. cholerae 7AMOZ	ICE*Vch*Moz3	653	491	988	601§	697	657	888	350
V. cholerae 8AMOZ	ICE*Vch*Moz4	–	491	988	–	697	657	888	350
V. cholerae 15AMOZ	ICE*Vch*Moz5	–	491	–	515¶	697	657	888	350
V. cholerae 16AMOZ	ICE*Vch*Moz6	–	491	–	–	697	657	888	350
V. parahaemolyticus 21AMOZ	R391-like	–	491	–	–	697	657	–	–

*Variable Regions and Hotspots are listed according to the ICE genetic map (see Fig. 1). Figures are referred to related amplicon sizes expressed in bp. Results of s045F/traLR and cds38F/traLR primer pairs, corresponding to Hotspot 1, were negative for all strains.

†Pairing of traAF primer with s052R, able to amplify SXT corresponding insert, no signal detected.

‡Pairing of traFR primer with s075F, able to amplify R391 corresponding insert, no signal detected.

§Amplicon corresponding to SXT profile.

¶Amplicon corresponding to R391 profile.

(interestingly, its *prfC* sequence was amplified by PVC-F/PVCF-B *V. cholerae*-specific primers), indicating different undetected chromosomal integration sites. For the strain *V. parahaemolyticus* 03FG, it was not possible to amplify either an intact *prfC* or the right junction *prfC*/ICE; this might be due to a molecular rearrangement of *prfC* in this strain.

A group of seven representative strains, showing appropriate selectable markers and the presence of ICEs (see Table 2) were further subjected to mating procedures under mitomycin induction. Resistance to spectinomycin and sulfamethoxazole were appropriately used as selective markers. Six strains were able to conjugate: *V. cholerae* 3AMOZ, 7AMOZ, 8AMOZ, 16AMOZ, *V. cholerae* 13AMOZ and 14AMOZ. The frequency of transfer (number of exconjugants per donor cell) was *c.* 10^{-6} for all donor strains, resembling the frequency of transfer of SXT^{MO10}. All the exconjugants were confirmed by PCR for int_{SXT}; exconjugants of *V. cholerae* 16AMOZ were also positive for *sul2*.

We then analyzed the conserved structure, common to the SXT/R391 family elements. Four *V. cholerae* isolates were positive for the three conserved genes (*traI*, *traC* and *setR*) (Table 3). One *V. cholerae* and one *V. parahaemolyticus* strain were positive for two conserved regions (Table 3, Fig. 1b), and six isolates were negative for all of them.

As previously shown by Beaber *et al.* (2002), SXT^{MO10} and R391 bear insertions of additional DNA sequences in three specific Hotspots within the conserved backbone, and two additional VRs that confer specific properties to each element. These specific sequences were tested to discriminate between the two elements (Fig. 1).

Amplification of Hotspot 1 was negative in all 12 strains with both primer combinations, specific for SXT^{MO10} and R391, respectively. Amplification of Hotspot 2 showed the R391-specific profile in six isolates, including the five *V. cholerae* integrating into *prfC*. Analysis of Hotspot 3 showed the presence of the typical SXT^{MO10} insertion in three *V. cholerae* isolates (Table 3, Fig. 1b).

Analysis of VR 1 revealed the typical SXT^{MO10} profile in the isolates 3AMOZ and 7AMOZ, while VR 2 showed an intact site (belonging to the SXT^{MO10} profile) in the isolate 7AMOZ and the presence of the mercury resistance operon (belonging to the R391 profile) only in the isolate 15AMOZ. No signal at all was obtained in the other isolates for both VRs.

Extensive differences in the molecular profiles emerged from the results, indicating the presence of SXT-like, R391-like and SXT-R391 hybrid profiles. The molecular profile of these ICEs is shown in Table 3 and Fig. 1b.

Isolate *V. parahaemolyticus* 21AMOZ showed a unique molecular profile similar to R391, but no evidence of *prfC* integration (Table 3).

The ICEs described here could be distinguished in two groups. The first group belongs to the SXT/R391 family, according to the presence of int_{SXT} integrase gene and to the integration into *prfC*; therefore its components were named ICE*Vch*Moz2 for *V. cholerae* 3AMOZ, ICE*Vch*Moz3 for *V. cholerae* 7AMOZ, ICE*Vch*Moz4 for *V. cholerae* 8AMOZ ICE*Vch*Moz5 for *V. cholerae* 15AMOZ and ICE*Vch*Moz6 for *V. cholerae* 16AMOZ (Fig. 1b), according to Burrus' new nomenclature, adopted here (Burrus *et al.*, 2006a).

The second group includes six isolates not listed in Table 3 (*V. cholerae* 13AMOZ, *V. cholerae* 14AMOZ, *V. cholerae* 22AMOZ, *V. parahaemolyticus* 03FG, *V. parahaemolyticus* 53AMOZ and *V. fluvialis* 15FG) positive for int_{SXT}, an intact *rumAB*; 13AMOZ and 14AMOZ were able to transfer. Because of no evidence of *prfC* integration and no proof of the presence of any conserved or variable region under study, these ICEs were considered undefined and thus subject to further studies.

Discussion

We found the presence of genetic mobile elements in *V. cholerae* non-O1 and non-O139, *V. parahaemolyticus*,

V. alginolyticus and *V. fluvialis* strains. This study is based on the cultivable fraction of *Vibrio* population only; therefore, it might not represent the real picture of mobile elements among the aquatic flora because of the presence of VBNC.

The antibiotic resistance profiles of these strains revealed a general resistance to β-lactams and a broad resistance to aminoglycosides. This is not surprising because both are naturally produced antibiotics, able to select the emergence of resistant determinants in environmental bacterial strains (Rosser & Young, 1999). Furthermore, resistance to β-lactams was found in *c.* 40% of the *V. cholerae* O1 clinical strains isolated during the 1998 cholera epidemic in Maputo (Folgosa *et al.*, 2001), indicating a wide circulation and prevalence of such resistance in the area under study.

The spread among the environmental isolates of resistance to synthetic drugs like sulfamethoxazole and trimethoprim may be explained by the exposure to wastes discharged into the environment from industrial and clinical sources, these two antibiotics being extensively used in the therapy of many bacterial infections.

More than 100 different gene cassettes have been described within integrons (Mazel, 2006), but *V. cholerae* strains seem to be characterized by a limited pattern of gene cassettes both in clinical and environmental isolates (Dalsgaard *et al.*, 2000b; Thungapathra *et al.*, 2002; Ceccarelli *et al.*, 2006a).

Four *V. cholerae* strains isolated in June 2003, from both sea- and waste-water samples, were characterized by the presence of class 1 integrons containing the *blaP1* resistance cassette. One strain also contained the *dfrA15* cassette in a separate integron: this is congruent with the finding that these cassettes are predominant in clinical and environmental *V. cholerae* O1 and non-O1 isolates in Africa, Thailand and India (Dalsgaard *et al.*, 2000a, b; Thungapathra *et al.*, 2002; Ceccarelli *et al.*, 2006a, b). An *aadA2* integron cassette was detected in *V. alginolyticus*, isolated in February 2003 from waste-water in the bay. To our knowledge, this is the first report of the presence of class 1 integrons in *V. alginolyticus* isolates. This cassette has been previously described only in *V. cholerae* O1 isolates during a 1998 epidemic in South Africa (Dalsgaard *et al.*, 2001). Based on these results, class 1 integrons, although a major mechanism for the acquisition of antibiotic resistance, accounted only partially for the antibiotic resistance observed in the isolates under study, opposing the extensively reported circulation of resistance associated with class 1 integrons in clinical isolates in all the austral African area (Dalsgaard *et al.*, 2000b, 2001; Ceccarelli *et al.*, 2006a, b).

All the ICEs were characterized by the absence of the SXT^{MO10} typical resistance cluster generally inserted into the *rumAB* operon, evidenced by an intact *rumAB* operon. Furthermore, neither the *dfrA1* gene insertion into Hotspot 3 (observed in ICE*Vch*Ind1), nor the *kan* gene and *mer* operon found in VRs 1 and 2 in R391 were detected, with the exception of ICE*Vch*Moz5 characterized by the *mer* operon, even if we cannot exclude the presence of different resistant determinants located elsewhere. ICE variants devoid of resistant genes have already been described (Hochhut *et al.*, 2001; Burrus *et al.*, 2006b; Ceccarelli *et al.*, 2006a; Bani *et al.*, 2007). Their circulation and maintenance confirm the possible presence of cryptic genetic information encoding for favorable factors beside drug resistance.

Some ICEs bear genes for nitrogen fixation and for symbiotic growth with plant roots (Burrus & Waldor, 2004a); they participate in the mobilization of virulence factors carrying pathogenicity islands involved in the expression of virulence genes (Schubert *et al.*, 2004) or code for genes involved in DNA repair (Munoz-Najar & Vijayakumar, 1999). Thus, the ubiquitous presence of an intact *rumAB* cluster encoding for proteins implicated in the repair of UV-damaged DNA, and the transfer induction of SXT/R391 by UV exposure (McGrath *et al.*, 2006), might reflect a selective advantage for the aquatic microbial communities exposed to UV radiation. It might also hypothesize that environmental strains would conserve ICEs devoid of resistance genes (keeping a functional *rumAB*), compared with clinical strains not exposed to UV but to antibiotics.

Evidence of conjugative ability was observed in six out of seven strains tested. Unfortunately, it was difficult to check this ability in the other ICE-containing strains due to the absence of effective resistance markers to be followed. This seems to be a shared feature in environmental ICEs, as observed in ICE*Vch*Mex1, which is transmitted via conjugation but not associated with antibiotic resistance genes (Burrus *et al.*, 2006b).

Analysis of ICE insertion into the chromosome revealed integration into *prfC* gene only in five of the *V. cholerae* isolates. This group of five ICEs, assigned to the SXT/R391 family, is characterized by sequences that belong both to SXT^{MO10} and R391. The presence of such hybrid forms matches with the notion that they may arise by recombination events of the two ICEs that can coexist in the same cell (Marrero & Waldor, 2005). This often leads to the formation of tandem arrays that seem to promote the formation of recombinant ICEs (Burrus & Waldor, 2004b). However, our hybrid ICEs might also be evidence supporting the hypothesis that SXT^{MO10} and R391 are rearrangements of the same progenitor that acquired different inserted sequences.

Interestingly, this group of ICEs was found in *V. cholerae* non-O1 strains isolated in 2002–2003, from different waste water sampling sites, suggesting their persistence and diffusion among the isolates and not just a sporadic accidental presence in a local context. This idea is reinforced by the SXT^{MO10} population conferring drug resistance found in clinical *V. cholerae* O1 isolates from the 1997 to 1998 cholera epidemic (Ceccarelli *et al.*, 2006a), and indicates a wide

circulation of variable SXT related elements in *V. cholerae* from Mozambique.

A second interesting group is composed of seven ICEs, found in three *V. cholerae*, three *V. parahaemolyticus* and one *V. fluvialis* strains isolated in February and March 2003, both from shellfish and waste waters. These ICEs showed no evidence of *prfC* integration. This should be due to the insertion into another chromosomal locus, because the *prfC* gene appears to be intact. The observed polymorphism was further evidenced by the fact that all these isolates yielded no amplification for VRs, Hotspots, and, more interestingly, for conserved regions (with the exception of the ICE of *V. parahaemolyticus* 21AMOZ that showed a molecular profile similar to R391). Therefore, this group characterized by an integrase, an intact *rumAB* operon (two out of seven tested and able to transfer) is similar to, but does not match the definition of SXT/R391 family members given by Burrus *et al.* (2006b).

The six ICEs showing no evidence of both Hotspots and VRs might correspond to the same element, but this requires further molecular characterization. An analogous profile was reported for ICE*Vch*Ang1, ICE*Vch*Ang2 and ICE*Vpa*Ang1, devoid of *rumAB* operon, found in clinical and environmental isolates of *V. cholerae* and *V. parahaemolyticus* in Angola (Ceccarelli *et al.*, 2006b). These unidentified genetic elements found in different *Vibrio* spp. may have a function yet to be determined. SXT^{MO10} and related ICEs should then not be considered as just means of resistance transmission among *V. cholerae* clinical strains, but a potential vector for genetic information, widely distributed among bacterial strains of different origin.

The results reported here reinforce the hypothesis suggested by several authors that ICEs are widely distributed among environmental *Gammaproteobacteria* and represent a large class of polymorphic genetic elements (Juiz-Rio *et al.*, 2005; Burrus *et al.*, 2006b). Investigation of ICE origin and evolution might be more fruitful, approaching the aquatic environmental microbial communities including *Vibrio* as a natural component, where clinical *Vibrio* strains also circulate.

The wide ICE distribution in different African geographic areas, in extended periods, both in clinical and environmental isolates of different bacterial species, suggests that ICEs might be autochthonous in Africa, and not recently introduced by *V. cholerae* epidemic strains from other continents.

Acknowledgements

We are grateful to M. Sidat (Department of Community Health, Faculty of Medicine, UEM, Maputo) for preliminary environmental survey and R.A. Pereira (Faculty Medicine, UEM, Maputo) for project management in Mozambique; L. Neves for allowing preliminary molecular identification of *V. cholerae* at the Center of Biotechnologies, UEM, Maputo; E. Suffredini (ISS, Italy) for *Vibrio* spp. identification, to M. Spagnoletti and R. Pino for technical help. We are also grateful to V. Burrus for critical reading and valuable advice. This work was supported by General Directorate of Cooperation and Development, MAE and MIUR Italy. D. Ceccarelli was supported by Cenci Bolognetti – Institute Pasteur Foundation, Italy.

Author contribution

E.T. and D.C. contributed equally to the work.

References

Ahmed AM, Nakagawae T, Arakawa E, Ramamurthy T, Shinoda S & Shimamoto T (2004) New aminoglycoside acetyltransferase gene, *aac(3)-Id*, in a class 1 integron from a multiresistant strain of *Vibrio fluvialis* isolated from an infant aged 6 months. *J Antimicrob Chemother* **53**: 947–951.

Ahmed AM, Shinoda S & Shimamoto T (2005) A variant type of *Vibrio cholerae* SXT element in a multidrug-resistant strain of *Vibrio fluvialis*. *FEMS Microbiol Lett* **242**: 241–247.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K (1990) *Current Protocols in Molecular Biology, Vol. I*. Green Publishing Associates and Wiley, New York. p. 2.4.1.

Bani S, Mastromarino PN, Ceccarelli D, Van AL, Salvia AM, Viet QTN, Hai DH, Bacciu D, Cappuccinelli P & Colombo MM (2007) Molecular characterization of ICE*Vch*Vie0 and its disappearance in *Vibrio cholerae* O1 strains isolated in 2003 in Vietnam. *FEMS Microbiol Lett* **266**: 42–48.

Beaber JW, Burrus V, Hochhut B & Waldor MK (2002) Comparison of SXT and R391, two conjugative integrating elements: definition of a genetic backbone for the mobilization of resistance determinants. *Cell Mol Life Sci* **59**: 2056–2070.

Beaber JW, Hochhut B & Waldor MK (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**: 72–74.

Bej AK, Patterson DP, Brasher CW, Vickery MC, Jones DD & Kaysner CA (1999) Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl, tdh* and *trh*. *J Microbiol Methods* **36**: 215–225.

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

Boltner D, MacMahon C, Pembroke JT, Strike P & Osborn AM (2002) R391: a conjugative integrating mosaic comprised of phage, plasmid, and transposon elements. *J Bacteriol* **184**: 5158–5169.

Burrus V & Waldor MK (2004a) Shaping bacterial genomes with integrative and conjugative elements. *Res Microbiol* **155**: 376–386.

Burrus V & Waldor MK (2004b) Formation of SXT tandem arrays and SXT-R391 hybrids. *J Bacteriol* **186**: 2636–2645.

Burrus V, Marrero J & Waldor MK (2006a) The current ICE age: biology and evolution of SXT-related integrating conjugative elements. *Plasmid* **55**: 173–183.

Burrus V, Quezada-Calvillo R, Marrero J & Waldor MK (2006b) SXT-related integrating conjugative element in New World *Vibrio cholerae*. *Appl Environ Microbiol* **72**: 3054–3057.

Ceccarelli D, Bani S, Cappuccinelli P & Colombo MM (2006a) Prevalence of *aada1* and *dfra15* class 1 integron cassettes and SXT circulation in *V. cholerae* O1 isolates from Africa. *J Antimicrob Chemother* **58**: 1095–1097.

Ceccarelli D, Salvia AM, Sami J, Cappuccinelli P & Colombo MM (2006b) New Cluster of plasmid-located class 1 integrons in *Vibrio cholerae* O1 and a *dfrA15* cassette-containing integron in *Vibrio parahaemolyticus* isolated in Angola. *Antimicrob Agents Chemother* **50**: 2493–2499.

Collins AE (2002) Health ecology and environmental management in Mozambique. *Health Place* **8**: 263–272.

Colombo MM, Mastrandrea S, Leite F, Santona A, Uzzau S, Rappelli P, Pisano M, Rubino S & Cappuccinelli P (1997) Tracking of clinical and environmental *Vibrio cholerae* O1 strains by combined analysis of the presence of toxin cassette, plasmid content and ERIC PCR. *FEMS Immunol Med Microbiol* **19**: 33–45.

Colwell RR & Huq A (1994) *Disease in Evolution: Global Changes and Emergence of Infectious Diseases*, *Vol. 740* (Wilson ME, Levins R & Spielman A, eds), pp. 44–54. Annals of the New York Academy of Sciences, New York.

Dalsgaard A, Forslund A, Serichantalergs O & Sandvang D (2000a) Distribution and content of class 1 integrons in different *Vibrio cholerae* O-serotype strains isolated in Thailand. *Antimicrob Agents Chemother* **44**: 1315–1321.

Dalsgaard A, Forslund A, Petersen A, Brown DJ, Dias F, Monteiro S, Molbak K, Aaby P, Rodrigues A & Sandstrom A (2000b) Class 1 integron-borne, multiple-antibiotic resistance encoded by a 150-kilobase conjugative plasmid in epidemic *Vibrio cholerae* O1 strains isolated in Guinea-Bissau. *J Clin Microbiol* **38**: 3774–3779.

Dalsgaard A, Forslund A, Sandvang D, Arntzen L & Keddy K (2001) *Vibrio cholerae* O1 outbreak isolates in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the STX element and the *aadA2* gene located on Class 1 Integrons. *J Antimicrob Chem* **48**: 827–838.

Faruque SM, Albert MJ & Mekalanos JJ (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* **62**: 1301–1314.

Folgosa E, Mastrandea S, Cappuccinelli P, Uzzau S, Rappelli P, Brian MJ & Colombo MM (2001) Molecular identification of pathogenicity genes and ERIC types in *Vibrio cholerae* O1 epidemic strains from Mozambique. *Epidemiol Infect* **127**: 17–25.

Gil AI, Louis VR, Rivera IN *et al.* (2004) Occurrence and distribution of *Vibrio cholerae* in the coastal environment of Peru. *Environ Microbiol* **6**: 699–706.

Hall RM & Collis CM (1995) Mobile gene cassettes and integrons: capture and spread of genes by site specific recombination. *Mol Microbiol* **15**: 593–600.

Hochhut B & Waldor MK (1999) Site-specific integration of the conjugal *Vibrio cholerae* SXTelement into prfC. *Mol Microbiol* **32**: 99–110.

Hochhut B, Marrero J & Waldor MK (2000) Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in *Vibrio cholerae* O139. *J Bacteriol* **182**: 2043–2047.

Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R & Waldor MK (2001) Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob Agents Chemother* **45**: 2991–3000.

Iwanaga M, Toma C, Miyazato T, Insisiengmay S, Nakasone N & Ehara M (2004) Antibiotic resistance conferred by a class 1 integron and SXT constin in *Vibrio cholerae* O1 strains isolated in Laos. *Antimicrob Agents Chemother* **48**: 2364–2369.

Juiz-Rio S, Osorio CR, de Lorenzo V & Lemos ML (2005) Subtractive hybridization reveals a high genetic diversity in the fish pathogen *Photobacterium damselae* subsp. *piscicida*: evidence of a SXT-like element. *Microbiol Mol Biol Rev* **151**: 2659–2669.

Lazaro N, Taviani E, Sidat M & Colombo MM (2006) Monitorização da presença de *Vibrio cholerae* potencialmente epidémico no meio ambiente da provincia de Maputo. *A cólera no ambiente: um problema global que afecta Moçambique*, *Vol. 8* (I Quaderni della Cooperazione Italiana ed), pp. 29–43. Maputo, Mozambique. http://www.italcoopmoz.com.

Lee JH, Han KH, Choi SY *et al.* (2006) Multilocus sequence typing (MLST) analysis of *Vibrio cholerae* O1 El Tor isolates from Mozambique that harbor the classical CTX prophage. *J Med Microbiol* **55**: 165–170.

Levesque C, Piche L, Larose C & Roy PH (1995) PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother* **39**: 185–191.

Marrero J & Waldor MK (2005) Interactions between inner membrane proteins in donor and recipient cells limit conjugal DNA transfer. *Dev Cell* **8**: 963–970.

Mazel D (2006) Integrons: agents of bacterial evolution. *Nat Rev Microbiol* **4**: 608–620.

McGrath BM, O'Halloran JA, Piterina AV & Pembroke JT (2006) Molecular tools to detect the IncJ elements: a family of integrating, antibiotic resistant mobile genetic elements. *J Microbiol Methods* **66**: 32–42.

Munoz-Najar U & Vijayakumar MN (1999) An operon that confers UV resistance by evoking the SOS mutagenic response in streptococcal conjugative transposon Tn*5252*. *J Bacteriol* **181**: 2782–2788.

Nandi B, Nandy RK, Mukhopadhyay S, Nair GB, Shimada T & Ghose AC (2000) Rapid method for species specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein W. *J Clin Micro* **38**: 4145–4151.

Park JC, Lee JC, Oh JY, Jeong YW, Cho JW, Joo HS, Lee WK & Lee WB (2003) Antibiotic selective pressure for the maintenance of antibiotic resistant genes in coliform bacteria isolated from the aquatic environment. *Water Sci Technol* **47**: 249–253.

Pembroke JT, MacMahon C & McGrath B (2002) The role of conjugative transposons in the *Enterobacteriaceae*. *Cell Mol Life Sci* **59**: 2055–2064.

Peters SE, Hobman JL, Strike P & Ritchie DA (1991) Novel mercury resistance determinants carried by IncJ plasmids pMERPH and R391. *Mol Gen Genet* **228**: 294–299.

Ripabelli G, Sammarco ML, McLauchlin J & Fanelli I (2003) Molecular characterization and antimicrobial resistance of *Vibrio vulnificus* and *Vibrio alginolyticus* isolated from mussels (*Mytilus galloprovincialis*). *Syst Appl Microbiol* **26**: 119–126.

Rosser SJ & Young HK (1999) Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *J Antimicrob Chemother* **44**: 11–18.

Rowe-Magnus DA & Mazel D (2001) Integrons: natural tools for bacterial genome evolution. *Curr Op Microbiol* **4**: 565–569.

Schubert S, Dufke S, Sorsa J & Heesemann J (2004) A novel integrative and conjugative element (ICE) of *Escherichia coli*: the putative progenitor of the *Yersinia* high-pathogenicity island. *Mol Microbiol* **51**: 837–848.

Sechi LA, Duprè I, Deriu A, Fadda G & Zanetti S (2000) Distribution of *Vibrio cholerae* virulence genes among different *Vibrio species* isolated in Sardinia, Italy. *J Appl Microbiol* **88**: 475–481.

Thungapathra M, Amita, Sinha KK, Chaudhuri SR, Garg P, Ramamurthy T, Nair GB & Ghosh A (2002) Occurrence of antibiotic resistance gene cassettes *aac(6′)-Ib, dfrA5, dfrA12*, and *ereA2* in class 1 integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* **46**: 2948–2955.

Utsalo SJ, Eko FO & Antia-Obong EO (1992) Features of cholera and *Vibrio parahaemolyticus* diarrhea endemicity in Calabar, Nigeria. *Eur J Epidemiol* **8**: 856–860.

Waldor MK, Tschape H & Mekalanos JJ (1996) A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. *J Bacteriol* **178**: 4157–4165.