

# A Highly Promiscuous Integron, Plasmids, Extended Spectrum Beta Lactamases and Efflux Pumps as Factors Governing Multidrug Resistance in a Highly Drug Resistant *Vibrio fluvialis* Isolate BD146 from Kolkata, India

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**Abstract** In an earlier study from this laboratory, *Vibrio fluvialis* BD146, a clinical isolate from Kolkata, India, 2002, was found to be resistant to all the fourteen antibiotics tested. It harboured a high copy number plasmid pBD146 and a low copy number plasmid. In the present study, a more detailed analysis was carried out to unravel different resistance mechanisms in this isolate. Sequencing showed that variable region of class 1 integron located on low copy number plasmid harbored *arr3-cmlA-bla<sub>OXA10</sub>-aadA1* gene cassettes. Analysis for extended spectrum beta lactamases (ESBLs) revealed that BD146 was ESBL positive. Efflux pumps were involved in the drug resistance phenotype for chloramphenicol, kanamycin, streptomycin and tetracycline. Sequence analysis of pBD146 revealed the presence of genes encoding *BDint* an integrase with a unique sequence having little similarity to other known integrases, toxin–antitoxin (*parE/parD*), a replicase, trimethoprim resistance (*dhfrVI*) and quinolone resistance (*qnrVC5*). Presence of *cmlA*, putative novel integrase and toxin–antitoxin system in *V. fluvialis* has been documented for the first time in this report. pBD146 showed 99% sequence similarity with pVN84 from *V. cholerae* O1 of

Vietnam, 2004 and a plasmid from *V. parahaemolyticus* v110 of Hong Kong, 2010. Conjugation experiments proved the ability of pBD146 and the low copy number plasmid, to get transferred to another host imparting their antibiotic resistance traits to the transconjugants. Therefore, present study has indicated that plasmids played an important role for dissemination of drug resistance.

**Keywords** Plasmid · *Vibrio fluvialis* · Integrase · Multi drug resistance · Toxin/antitoxin system · Integron

## Introduction

*Vibrios* have been reported to cause diarrhea in humans [1–5]. Though antibiotics are used as secondary line of treatment for diarrhea, rise of resistance to these drugs has seriously jeopardized the successful treatment. Thus, it becomes imperative to decipher the mechanisms responsible for acquisition and dissemination of drug resistance in bacteria for development of new treatment options and management of diseases.

Horizontal gene transfer (HGT) that leads to rapid dissemination of genes is a major factor responsible for evolution of multi drug resistance (MDR) [6]. HGT is mediated by mobile genetic elements such as integrons, transposons and plasmids [6]. Integrons are gene capture platforms that integrate the exogenous gene cassettes by site-specific recombination converting them into their functional forms [7]. Till date, five classes of integrons have been well defined based on ~ 40–58% identities in their integrase sequences but class 1 integrons are well characterized for their role in spread of MDR among bacteria [8]. Integrons are composed of three key elements, an integrase; a promoter and a primary recombination site

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*attI* [7]. All the exogenous gene cassettes harbour imperfect repeats called 59-base elements (*attC*) at their 3' end. Thus, *attI* × *attC* recombination facilitates integration of gene cassettes while their excision is carried out by *attC* × *attC* recombination [7]. Class 1 integrons consist of two conserved segments (CS). The 5' CS contains the integrase gene (*intI*) and 3' CS comprises of *qacEΔ1* encoding resistance to quaternary ammonium compounds and *sulI* encoding resistance to sulphonamides. These conserved segments are separated by a variable region that usually comprises of one or more foreign gene cassettes [7].

A previous study from this laboratory had shown the role of multiple plasmids and integrons in imparting MDR to a clinical isolate of *V. fluvialis* BD146, 2002, that carried a high copy number plasmid of 7.5 kb (pBD146) and a low copy number plasmid [3]. In the present study, analysis of class 1 integrons, extended spectrum beta lactamases and role of efflux pumps in drug resistance was carried out. Detailed analysis of pBD146 sequence revealed the presence of a novel integrase, *Vibrio cholerae* repeat (VCR) regions associated with *dfxVI* and *qnrVC5* genes, and the toxin–antitoxin module. BLAST analysis of BD146 indicated the possibility of HGT of this plasmid between three *Vibrio* species from three different geographical locations in Southeast Asia.

## Materials and Methods

### Bacterial Isolates and DNA Isolation

*Vibrio fluvialis* isolates were obtained from patients with acute cholera-like diarrhea, admitted to the Infectious Diseases Hospital, Kolkata, India, between 1998 and 2006 [3, 5, 9]. Genomic DNA and plasmid DNA were isolated as described previously [3, 10]. The patients provided their written consent for participating in the study and in case of children, written consent was obtained from their parents. The consent procedure was approved by the Institutional Ethical Clearance Committee of NICED. The study was also approved by the Institutional Biosafety Committee of Institute of Advanced Research (IAR), Gandhinagar and the Review Committee on Genetic Manipulation governed by the guidelines laid down by the Department of Biotechnology, Government of India.

### Polymerase Chain Reaction (PCR) and Reverse Transcription PCR (RT-PCR)

An ORF (nt 154–1062) corresponding to an integrase named BDint, was predicted in pBD146. To check its presence in other clinical isolates and transconjugants, PCR was carried out using BDint-specific primers Vcint-F/

Vcint-R [3] and genomic DNA (200 ng) or plasmid DNA (10 ng) as templates. The conditions for PCR were an initial denaturation at 95 °C for 4 min, followed by 25 cycles each of denaturation at 95 °C, 0.5 min, annealing (55 °C, 1 min) and extension (72 °C, 1 min) with final polymerization at 72 °C for 7 min. Recombinant Taq DNA polymerase (Thermo-Scientific) was used. For the analysis of class 1 integron in transconjugants, PCR was carried out with the primers specific for 5' CS, 3' CS and variable region of class 1 integrons as described earlier [3].

RNA was isolated by RNeasy kit (Qiagen) as per manufacturer's protocol involving lysis with lysozyme and proteinase K digestion. The RNA preparations were subsequently treated with DNaseI (Thermo-Scientific) to remove the residual genomic DNA contamination. Reverse transcription was carried out at 50 °C for 30 min using one step RT-PCR kit (Qiagen) and Vcint-F/Vcint-R primers followed by PCR to amplify cDNA. Conditions for PCR involved initial denaturation at 95 °C for 15 min, followed by 25 cycles with conditions as described above.

### Antibiotic Susceptibility Test and Minimum Inhibitory Concentration (MIC) Assays

*Vibrio fluvialis* BD146, *E. coli* XL1-Blue and their transconjugants were tested for susceptibility to AMP, Ampicillin (10 µg); CHL, Chloramphenicol (30 µg); CIP, Ciprofloxacin (5 µg); COT, Co-Trimoxazole (1.25 µg trimethoprim/23.75 µg sulfamethoxazole); GEN, Gentamicin (10 µg); KAN, Kanamycin (30 µg); NAL, Nalidixic Acid (30 µg); NEO, Neomycin (30 µg); NOR, Norfloxacin (10 µg); STR, Streptomycin (10 µg); SUL, Sulfisoxazole (300 µg); TET, Tetracycline (30 µg); TRI, Trimethoprim (5 µg) and RIF, Rifampicin (5 µg) using commercial disks (HiMedia). When no interpretive criteria for *V. cholerae* were available based on the CLSI guidelines [11], breakpoints for enterobacteriaceae were applied. MIC for various antibiotics were determined using the Ezy MIC<sup>TM</sup> strips (HiMedia) according to manufacturer's instruction and interpreted using the CLSI criteria. *E. coli* ATCC 25922 was used as control.

### Efflux Pump Assay

To ascertain the role of efflux pumps in imparting resistance to drugs, synergy test was carried out as described earlier [12–14]. The test was performed using one drug for each class of antibiotics to which BD146 was resistant. The efflux pump inhibitor carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) was added on MHA at 4 mg L<sup>-1</sup> concentration. Susceptibility testing for antibiotics by MIC strip was performed as described in earlier section, both in the presence and absence of CCCP. Lowering in MIC of

the isolates in the presence of CCCP indicated the role of efflux pumps in reducing the concentration of that drug inside the cell by throwing it out.

### Bacterial Conjugation

BD146 was analysed for its ability to transfer resistance traits to a recipient strain by conjugation [5, 15]. BD146 donor harboured resistance to ampicillin ( $\text{MIC} > 256 \mu\text{g mL}^{-1}$ ) and trimethoprim ( $\text{MIC} > 32 \mu\text{g mL}^{-1}$ ) and intermediate resistance to tetracycline ( $\text{MIC} \leq 16 \mu\text{g mL}^{-1}$ ). The recipient *E. coli* XL1-Blue was sensitive to ampicillin ( $\text{MIC} = 4 \mu\text{g mL}^{-1}$ ) and trimethoprim ( $\text{MIC} = 0.25 \mu\text{g mL}^{-1}$ ) and highly resistant to tetracycline ( $\text{MIC} > 256 \mu\text{g mL}^{-1}$ ). The recipient and the donor were mixed (2:1) on a sterile  $0.45 \mu\text{m}$  nitrocellulose membrane (PALL Life Sciences) and incubated overnight for mating on LB agar at  $37^\circ\text{C}$ . The transconjugants were selected on LB agar plates containing two antibiotic combinations; ampicillin ( $50 \mu\text{g mL}^{-1}$ ) and tetracycline ( $120 \mu\text{g mL}^{-1}$ ) or trimethoprim ( $20 \mu\text{g mL}^{-1}$ ) and tetracycline ( $120 \mu\text{g mL}^{-1}$ ). The transconjugants from both the selections were analysed for their plasmid profiles, presence of *BDint*, presence of integron and antibiotic susceptibility profiles.

### Analysis of DNA Sequences

The ORF finder tool (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was used to predict all the possible ORFs in pBD146 sequence. These ORFs were analysed by BLAST search. I-TASSER server was used for 3D protein structure prediction [16–18]. Multiple sequence alignments were carried out using CLUSTAL Omega (1.2.1) ([http://www.expasy.org/genomics/sequence\\_alignment](http://www.expasy.org/genomics/sequence_alignment)).

## Results

### Analysis of Class 1 Integron of BD146

BD146 was found to be resistant to all the 14 antibiotics tested [3]. Integron analysis had shown that BD146 consisted of class 1 integron associated with low copy number plasmid. ORF analysis of the 4.0 kb variable region amplified from integron (KY883670) revealed the presence of genes for; rifampin ADP-ribosylating transferase, a hypothetical protein, chloramphenicol efflux pump MFS transporter, extended spectrum beta-lactamase OXA-10 and aminoglycoside 3' adenylyl transferase. BLAST analysis of 4.0 kb integron sequence showed 99% identity for 100% query coverage with segments of plasmid A from *E. coli* H3 (CP010168.1), segments of plasmid pNDM15-1078

from *E. coli* N15-01078 (CP012902.1), segments of plasmid pNDM-116-17 from *V. cholerae* 116-17a (LN831185.1), class 1 integron from *Pseudomonas aeruginosa* pae G18 (EU886979.1) and part of *Enterobacter aerogenes* HN0711 plasmid pHN-NDM0711. This clearly established the promiscuous nature of this integron associated with multiple genera and multiple species of organisms located at different geographical locations around the world.

### Extended Spectrum Beta Lactamases in BD146

As the class 1 integron carried *bla*<sub>OXA10</sub> gene, ESBL and ampC beta lactamases activity was analysed using EzyMIC strip for the cefotaxime (CTX)/cefotaxime and clavulanic acid (CTX+), ceftazidime (CAZ)/ceftazidime and clavulanic acid (CAZ+) and the mixture (MIX) of ceftazidime, cefotaxime, cefepime and cloxacillin and MIX+ with clavulanic acid and tazobactam. The MIC ratio for CAZ+/CAZ and CTX+/CTX was 42.67 and 64 respectively while the MIC ratio for MIX+/MIX was 64. These results revealed that the isolate possessed high ESBL and ampC beta lactamase activity.

### Role of Efflux Pumps in Imparting Drug Resistance in BD146

To ascertain the synergy between the resistance genes and the efflux pump activity, synergy test was carried out at least three times to detect the efflux pump activity as described in materials and methods. The MIC of ampicillin, chloramphenicol, ciprofloxacin, kanamycin, streptomycin, tetracycline and trimethoprim were tested in the presence and absence of efflux pump inhibitor CCCP. Fold decrease in MIC of BD146 for a particular drug in the presence of CCCP indicated the involvement of efflux pump activity in imparting resistance to that drug. Synergy tests revealed that efflux pumps were involved for the drug resistance phenotype (1.33–2 fold decrease in MIC value) for chloramphenicol, kanamycin, streptomycin and tetracycline, while they did not contribute in imparting drug resistance for ampicillin, ciprofloxacin and trimethoprim as no changes were observed in MIC values (Table 1).

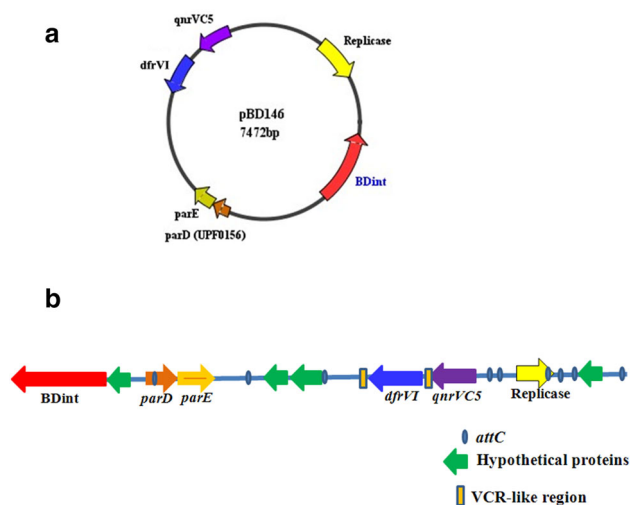
### Sequence Analysis of pBD146 Indicated the Presence of Various Genes

Sequence of pBD146 (EU574928) revealed that it carried genes encoding integrase (*intI*), replicase (*repA*), trimethoprim resistance (*dhfrVI*), quinolone resistance (*qnrVC5*), toxin–antitoxin (*parE/parD*) and some hypothetical proteins (Fig. 1a). Though the presence of the first three genes was described in an earlier report [3],

**Table 1** Synergy test for efflux pump activity in *V. fluvialis* BD146 isolate

Antibiotics	Antibiotics with CCCP (4 mg L <sup>-1</sup> )	BD146 (µg mL <sup>-1</sup> )	Fold change in MIC
Ampicillin	–	> 256	0
	+	> 256	
Chloramphenicol	–	12	<b>2</b>
	+	6	
Ciprofloxacin	–	6	0
	+	6	
Trimethoprim	–	> 32	0
	+	> 32	
Tetracycline	–	16	<b>1.33</b>
	+	12	
Kanamycin	–	12	<b>1.5</b>
	+	8	
Streptomycin	–	64	<b>1.33</b>
	+	48	

Bold values indicate the antibiotics for which the efflux pumps were active in BD146



**Fig. 1** Schematic representation of plasmid pBD146. **a** Protein coding regions present on the plasmid pBD146; **b** Organisation of different genes such as integrase BDint (nt 154–1062), *parD/parE* genes (nt 2304–2546; 2539–2823), hypothetical proteins (nt 1055–1300; 3214–3429; 3557–3949; 7192–7428), *dfrVI* (nt 4106–4579), *qnrVC5* (nt 4742–5173) and a replicase (nt 6032–6922) on pBD146 with respect to 59-base elements and two VCR-like regions. The ORF finder tool was used to predict the ORFs in pBD146

identification of *qnrVC5* and *parE/parD* toxin–antitoxin genes became possible due to newer entries in GenBank leading to refinement in the BLAST search. It is possible that the hypothetical proteins would also be assigned functions in due course of time with expanding GenBank entries. Interestingly, these hypothetical proteins seemed to have their origin chiefly in the members of *Vibrionaceae* family barring one protein which was derived from gamma proteobacterium and has been described later in this section. For the hypothetical protein corresponding to ORF

3214–3429 nt, 87 and 84% identity was observed with the hypothetical protein VCHE16\_3780 (EKG80837) from *Vibrio cholerae* HE-16 and another hypothetical protein from *V. parahaemolyticus* (WP\_023622789) respectively. The hypothetical protein (ORF 7192–7428 nt) showed 86 and 85% identity with the hypothetical proteins from *V. tasmaniensis* (AKN38121, AKN38323, AKN39122) and *V. cyclitrophicus* (WP\_016786352) respectively. The protein (ORF 1055–1300 nt) showed 96% identity with the hypothetical proteins from *V. parahaemolyticus* (KKX78394, KKX90213) and *V. fluvialis* (KQH89087). The ORF corresponding to 3557–3949 nt had 99 and 62% identity with the hypothetical proteins from *V. cholerae* HE-16 (EKG80837) and gamma proteobacterium BDW918 (EIF41853) respectively.

The ORFs (nt 2539–2823 and nt 2304–2546) in pBD146 sequence corresponded to ParE (toxin) and ParD (antitoxin to ParE) respectively. ParE/ParD are addiction modules involved in plasmid stabilization. This is the first report of a putative toxin–antitoxin system in a plasmid from *V. fluvialis*.

Additionally, BLAST analysis of pBD146 revealed that some part (integrase, toxin–antitoxin system and hypothetical proteins) of this plasmid was present in *V. tasmaniensis* isolates (KP795520; KP795494; KP795636; KP795574 and KP795658) of 2014 from USA and *V. parahaemolyticus* clinical isolates from Canada (2001–2006) [LRTI01000063.1(2001); LRF01000033.1 (2002); LRTB01000025.1(2003); LRTF01000062.1(2003); LRTF01000018.1 (2003); LRTC01000022.1(2003); LRTD01000012.1 (2003); LOHO01000004.1(2004); LOBT01000042.1 (2004); LRSU01000008(2006); LRSU01000014.1 (2006)]. This fragment of pBD146 was even named as unlocalized plasmid pBD146 (the name given in this laboratory) in the *V. parahaemolyticus* isolates from Canada.

Analysis of pBD146 sequence revealed the presence of *attC* sites associated with various gene cassettes as shown in Fig. 1B. The *dfrVI* and *qnrVC5* genes were encompassed by *Vibrio cholerae* repeat regions (VCRs), the *attC*-like regions found on the superintegron of *V. cholerae*, indicating the superintegron origin of these cassettes. BLAST analysis of *qnrVC5* and *dfrVI* genes together revealed that this cassette of two genes was 99% similar to their counterparts in *V. cholerae* and *V. parahaemolyticus* mentioned below and 86% similar to a transposon from *V. cholerae* MCV09 (HM015627) and a part of SXT element from *E. coli* J53 (FJ968160). Even the VCR regions were similar suggesting that these two genes have been moving together between different organisms either as part of integrons, plasmids or SXT elements [19–22].

### BLAST Analysis of pBD146 Indicated Horizontal Gene Transfer Between Three *Vibrio* Species

The sequence of pBD146 showed 99% identity with a plasmid pVN84 of *V. cholerae* O1 El Tor (2004) from Vietnam (AB200915) and another plasmid of *V. parahaemolyticus* v110 (2010) from Hong Kong (KC540630). While the isolates BD146 and VN84 were clinical in origin, v110 was environmental in origin [23]. This suggested horizontal transfer of a plasmid between three different *vibrio* species at three different locations of Southeast Asia over a period of eight years. Alternately, this plasmid could have been present in these species for a much longer time but detected recently.

### pBD146 Horizontally Transferred to *Escherichia coli* Through Conjugation

To examine the capability of pBD146 for horizontal gene transfer, conjugation was carried out between BD146 and *E. coli* XL1-Blue. Transconjugants selected on ampicillin and tetracycline ( $\text{amp}_{50} + \text{tet}_{120}$ ) and those selected on trimethoprim and tetracycline ( $\text{tri}_{20} + \text{tet}_{120}$ ) were

obtained with conjugation efficiency of  $8 \times 10^{-5}$  and  $4.1 \times 10^{-5}$  respectively. Both these transconjugants showed different plasmid profiles (Supplementary Fig. 1A). Transconjugants  $\text{amp}_{50} + \text{tet}_{120}$  showed resistance to ampicillin, tetracycline, rifampicin and nalidixic acid while transconjugants  $\text{tri}_{20} + \text{tet}_{120}$  harbored all the above resistance traits in addition to trimethoprim (Table 2). Only the  $\text{tri}_{20} + \text{tet}_{120}$  transconjugants showed the 657 bp amplicon of BDint (Supplementary Fig. 1B). These results revealed that pBD146 could transfer to another bacterium hitchhiking with another conjugative plasmid that harbored ampicillin and rifampicin resistance. PCR analysis of these transconjugants for 5' CS, 3' CS and variable regions of class 1 integrons revealed that the integron was present in both type of transconjugants and it harboured the 4.0 kb variable region having *arr3-cmlA-bla<sub>OXA10</sub>-aadA1* gene cassette.

### BDint is a Putative Novel Integrase

BDint belonged to DNA\_BRE\_C superfamily consisting of DNA breaking rejoining enzymes including tyrosine recombinase. BDint showed 86, 85, 81 and 75% identity with integrases from *V. alginolyticus*, *V. parahaemolyticus*, *V. cyclitrophicus* and *Photobacterium* sp. respectively in a BLASTp analysis. The nucleotide sequence of BDint (KT182072) showed 99% identity with its counterparts in *V. cholerae* pVN84 and *V. parahaemolyticus* pv110 and 77–82% identity with the integrases from *V. tasmaniensis* (AKN40441; AKN39714; AKN36978). PCR showed the presence of a 657 bp BDint amplicon in twelve out of eighteen *V. fluvialis* isolates from 1998 to 2006 (Supplementary Fig. 1C). Three of these 100% identical sequences were submitted to GenBank (KT182073, KT182074 and KT182075). A 657 bp amplicon produced in reverse transcription confirmed the presence of mRNA for BDint in BD146. A negative control without reverse transcription step did not show any band corresponding to the expressed

**Table 2** Antibiotic susceptibility profile of *Vibrio fluvialis* BD146 and its transconjugants

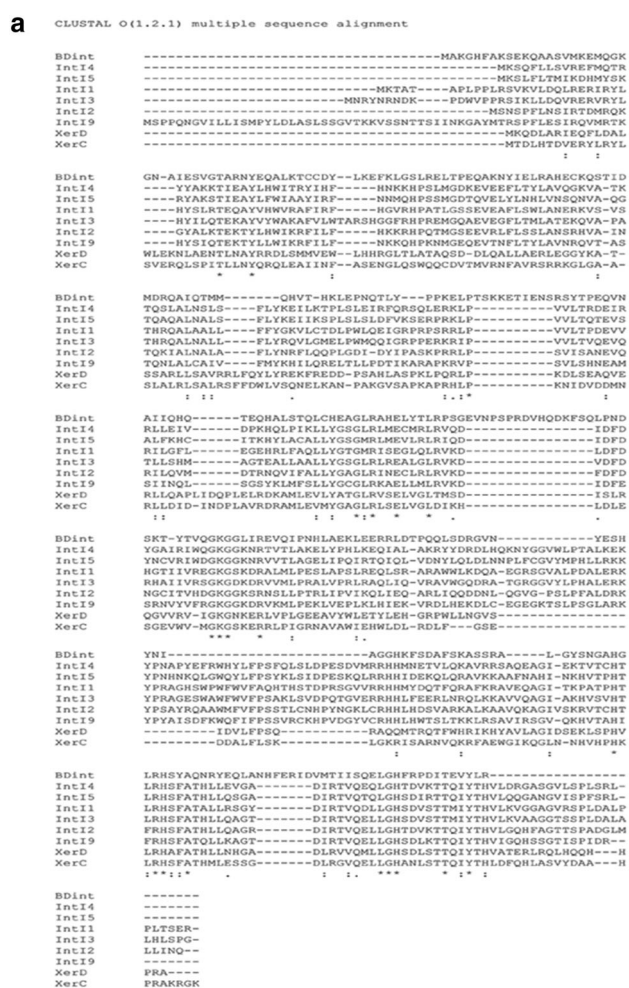
	<i>Vibrio fluvialis</i> BD146	Transconjugants $\text{TRI}_{20} + \text{TET}_{120}$	Transconjugants $\text{AMP}_{50} + \text{TET}_{120}$	<i>E. coli</i> XL1- Blue
Resistant	AMP, COT, CIP, SUL, NAL, TRI, RIF, CHL, GEN, STR, NOR, KAN, NEO, TET	AMP, TET, TRI, RIF, NAL	AMP, TET, RIF, NAL	TET, NAL

Intermediate resistance and complete resistance were together considered as a resistance trait

AMP, Ampicillin (10  $\mu\text{g}$ ); CHL, Chloramphenicol (30  $\mu\text{g}$ ); CIP, Ciprofloxacin (5  $\mu\text{g}$ ); COT, Co-Trimoxazole (1.25  $\mu\text{g}$  trimethoprim/23.75  $\mu\text{g}$  sulfamethoxazole); GEN, Gentamicin (10  $\mu\text{g}$ ); KAN, Kanamycin (30  $\mu\text{g}$ ); NAL, Nalidixic Acid (30  $\mu\text{g}$ ); NEO, Neomycin (30  $\mu\text{g}$ ); NOR, Norfloxacin (10  $\mu\text{g}$ ); STR, Streptomycin (10  $\mu\text{g}$ ); SUL, Sulfisoxazole (300  $\mu\text{g}$ ); TET, Tetracycline (30  $\mu\text{g}$ ); TRI, Trimethoprim (5  $\mu\text{g}$ ); RIF, Rifampicin (5  $\mu\text{g}$ )

$\text{TRI}_{20}$ , Trimethoprim (20  $\mu\text{g}$   $\text{mL}^{-1}$ );  $\text{TET}_{120}$ , Tetracycline (120  $\mu\text{g}$   $\text{mL}^{-1}$ );  $\text{AMP}_{50}$ , Ampicillin (50  $\mu\text{g}$   $\text{mL}^{-1}$ )

Experiments were performed at least three times



**Fig. 2** Comparison of BDint with other known integrases. Multiple sequence alignment of BDint protein sequence with other classes of integrase/recombinase sequences using Clustal O (1. 2.1) program **(a)**; and their phylogenetic analysis **(b)**

gene ensuring the absence of DNA contamination in RNA templates.

Comparison of BDint with other tyrosine recombinases such as IntI1-IntI5, IntI9, XerC and XerD using BLASTp indicated that BDint had very low similarity (19–29%) with them indicating that it was sufficiently different from all these integrases (Supplementary Table 1). This result was further confirmed by CLUSTAL analysis of BDint (Fig. 2). Alignment of all these recombinase sequences revealed that the unique additional domain (row 6 in Fig. 2a) present in the integrases related to integrons was absent in BDint establishing it to be a different entity [24].

The amino acid tail at the C-terminus of these recombinases was also absent in BDint. Additionally, BDint carried certain stretches of amino acids TSKKETIENS (row 3); EVNPSPRDVHQDKFSQ (row 4); NHFERID (row 7); that were absent in all other recombinases. Therefore, sequence analysis and phylogenetic tree (Fig. 2b) established it to be a novel integrase.

The above observations were further confirmed by I-TASSER analysis (Supplementary Fig. 2). BDint showed the presence of additional helices that were distinctly missing in other integrases. In this analysis, some of the proteins structurally close to BDint included XerD recombinase from *E. coli* (1aOpA), XerA from archaea (4a8eA), Cre-loxP synaptic structure from *Enterobacteria* phage P1(3c29H), human topoisomerase I DNA complex (1ej9A) and site-specific recombinase *IntI4* (2a3vB). The molecular function for BDint was predicted to be topoisomerase activity and biological process was predicted to be DNA recombination/integration (Supplementary Table 2).

Therefore, all these results mentioned above suggested that BDint and its siblings from *V. cholerae* and *V. parahaemolyticus* were novel integrases.

## Discussion

An earlier study from this laboratory had described an MDR isolate of *V. fluvialis* BD146 (2002) from Kolkata, equipped with multiple plasmids and integrons to combat multiple antibiotics. One integron carried the putative exporter protein, while the other integron resident on a low copy number plasmid carried genes responsible for rifampicin, ampicillin, gentamicin and kanamycin resistance [3]. Apart from the low copy number plasmid, another 7.5 kb plasmid, pBD146, was also present in this bacterium carrying trimethoprim resistance gene. In other studies from the laboratory, *qnrVC5*, *aac(6')-Ib-cr* and mutations in GyrA (S<sub>83</sub> → I) and ParC (S<sub>85</sub> → L) were found to be involved in quinolone resistance of BD146 [5, 25]. In the present study, the remaining drug resistance phenotypes were characterized in BD146.

The *cmlA* gene cassette resident on class 1 integron was for the first time reported from *V. fluvialis*. BLAST analysis of this integron showed 99% identity with segments of pNDM plasmids from *E. coli* N15-01078 (CP012902.1), *V. cholerae* 116-17a (LN831185.1), and *Enterobacter aerogenes* HN0711 (KU764665.1). ESBL and ampC beta lactamase analysis of this isolate revealed that it had high ESBL and ampC beta lactamase activity. Presence of ESBL activity has also been reported in *V. fluvialis* isolates from India [26, 27]. Efflux pump activity was also involved in determining the resistance phenotype for

**Table 3** Correlation of resistance to different antibiotics with corresponding genes in clinical isolate of *V. fluvialis* BD146

Antibiotic	Factor/genes responsible for resistance phenotype in <i>V. fluvialis</i> BD146
Fluroquinolone	Mutation in Topoisomerase, <i>qnrVC5</i> , <i>aac-6'-Ib-cr</i>
Aminoglycoside	<i>aadA1</i> (Aminoglycoside-3'-adenyltransferase) and efflux pump
Beta-lactam	<i>blaOXA-10</i> (extended spectrum $\beta$ -lactamase OXA)
Rifampicin	<i>arr-3</i> (rifampicin ADP-ribosylating transferase)
Chloramphenicol	<i>cmlA</i> (MFS efflux pump)
Sulphonamide	<i>sul1</i> gene on Class 1 integron
Trimethoprim	<i>dfrVI</i> (dihydrofolate reductase)
Tetracycline	Efflux pump

chloramphenicol, kanamycin, streptomycin and tetracycline. There are very few studies that described the role of efflux pumps in imparting fluroquinolone resistance in *V. fluvialis* [9, 28] and synergy test has not been used till date in *V. fluvialis*.

BLAST analysis showed a high genetic relatedness between the three plasmids (pBD146, pVN84 and pV110) from clinical and environmental isolates of different species of *Vibrios* suggesting that these plasmids were exchanged and maintained in these *Vibrio* sp. from 2002 to 2010. This also indicated the possibility of this transfer via an intermediate species or descent of this plasmid from a common ancestor.

The presence of *attC* and VCR regions encompassing some of the genes of pBD146 were indicative of their probable origin from superintegrons. In an earlier report, presence of *qnrVC2* on class 1 integron of *V. cholerae* plasmid pVN84 had been reported [22] but the structure of a class 1 integron was not apparent from this plasmid sequence. Similarly, pBD146 did not appear to carry an integron though the recombination sites associated with some of the gene cassettes did indicate their provenance in a superintegron.

pBD146 was able to get transferred to *E. coli* during conjugation with the help of another conjugative plasmid/low copy number plasmid that carried multiple drug resistance genes. The propensity of pBD146 to transfer horizontally between bacteria has also been shown in the earlier studies from this laboratory where pBD146 and similar plasmids moved from one host to another through transformation/conjugation, with concomitant expression of antibiotic resistance traits in their corresponding transformants/transconjugants [3, 5].

BLASTp, CLUSTAL and I-TASSER analysis of BDint with other integrases revealed that it was sufficiently different from other classes of integrases to be considered a novel integrase. BLAST analysis of *parE* and *parD* genes revealed that these genes constituted the first toxin-antitoxin module reported from *V. fluvialis*. The toxin-antitoxin modules have been attributed multiple functions such as plasmid stability, stabilization of DNA segments, protection against invading plasmids and phages and gene

regulation [29]. Most interestingly, recent submissions in GenBank from mid 2015 to April 2016 have shown the presence of fragment of pBD146 harbouring the integrase, toxin-antitoxin module and a hypothetical protein in *V. parahaemolyticus* (2001–2006, Canada) and *V. tasmaniensis* (2014, USA). Therefore, there appears to be a very wide dispersal of these modules across the globe at least from 2001 to 2014. The results presented here for pBD146 show the genesis of a mosaic of genes derived from sources such as superintegrons and transposons. This plasmid/its modules have been maintained in different *Vibrio* spp. through the years 2001–2014, as a carrier of many traits capable of persistence in different bacteria and dissemination of drug resistance.

To summarize, in the present study multiple plasmids and integrons along with other genetic factors such as efflux pump contributed to the drug resistance phenotype of this clinical isolate (Table 3).

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#### Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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