Genomic plasticity associated with antimicrobial resistance in *Vibrio cholerae*

Jyoti Verma[a,1], Satyabrata Bag[a,1,2], Bipasa Saha[a], Pawan Kumar[a], Tarini Shankar Ghosh[a,3], Mayanka Dayal[a], Tarosi Senapati[a], Seema Mehra[a], Prasanta Dey[a,4], Anbumani Desigamani[a], Dhirendra Kumar[a], Preety Rana[a], Bhoj Kumar[b], Tushar K. Maiti[b], Naresh C. Sharma[c], Rupak K. Bhadra[d], Ankur Mutreja[a,e], G. Balakrish Nair[f,5], Thandavarayan Ramamurthy[a], and Bhabatosh Das[a,g,5]

[a]Molecular Genetics Laboratory, Centre for Human Microbial Ecology, Translational Health Science and Technology Institute, Faridabad 121001, India; [b]Regional Centre for Biotechnology, Faridabad 121001, India; [c]Maharishi Valmiki Infectious Diseases Hospital, Kingsway Camp, Delhi 110009, India; [d]Infectious Diseases and Immunology Division, Council of Scientific & Industrial Research (CSIR)–Indian Institute of Chemical Biology, Kolkata 700032, India; [e]Department of Medicine, Addenbrookes Hospital, University of Cambridge, CB20QQ Cambridge, United Kingdom; [f]Microbiome Laboratory, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala 695014, India; and [g]Kasturba Medical College, Manipal Academy of Higher Education, Manipal, Karnataka 576104, India

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The Bay of Bengal is known as the epicenter for seeding several devastating cholera outbreaks across the globe. *Vibrio cholerae*, the etiological agent of cholera, has extraordinary competency to acquire exogenous DNA by horizontal gene transfer (HGT) and adapt them into its genome for structuring metabolic processes, developing drug resistance, and colonizing the human intestine. Antimicrobial resistance (AMR) in *V. cholerae* has become a global concern. However, little is known about the identity of the resistance traits, source of AMR genes, acquisition process, and stability of the genetic elements linked with resistance genes in *V. cholerae*. Here we present details of AMR profiles of 443 *V. cholerae* strains isolated from the stool samples of diarrheal patients from two regions of India. We sequenced the whole genome of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *V. cholerae* to identify AMR genes and genomic elements that harbor the resistance traits. Our genomic findings were further confirmed by proteome analysis. We also engineered the genome of *V. cholerae* to monitor the importance of the autonomously replicating plasmid and core genome in the resistance profile. Our findings provided insights into the genomes of recent cholera isolates and identified several acquired traits including plasmids, transposons, integrative conjugative elements (ICEs), pathogenicity islands (PIs), prophages, and gene cassettes that confer fitness to the pathogen. The knowledge generated from this study would help in better understanding of *V. cholerae* evolution and management of cholera disease by providing clinical guidance on preferred treatment regimens.

cholera | antimicrobial resistance | mobile genetic elements | genome | proteome

Cholera is an acute secretory diarrheal disease caused by the *Vibrio cholerae*, a Gram-negative comma-shaped bacterium that infects humans through contaminated water or food. It is still a major public health burden in many developing countries, including India (1). The pathogen has extraordinary competency to acquire exogenous DNA through horizontal gene transfer (HGT) and adapt them into its genome for structuring metabolic processes, developing drug resistance, colonizing the human intestine, and producing cholera toxin (2, 3).

Although administration of oral or i.v. rehydration solution containing glucose, sodium chloride, potassium chloride, and trisodium citrate is the major choice for the treatment of cholera, antibiotics are also used to reduce stool volume and duration of diarrhea (4). Antimicrobial resistance (AMR) in *V. cholerae* is becoming increasingly common across the globe (5). Emergence of antibiotic resistance in bacteria is a natural phenomenon, wherein the indiscriminate usage of antibiotics in healthcare, livestock, and agriculture endorses the resistant variants to flourish in the ecosystem (6, 7). Several mechanisms such as efflux pumps, reduced permeability, alternative metabolic pathways, target modifications, enzymatic inactivation of antimicrobials, and so forth can confer antibiotic resistance in bacterial species (8). Many of the antibiotic resistance genes are physically linked with mobile genetic elements (MGEs) and disseminate to closely or distantly related bacterial species by lateral and vertical gene transfer (9, 10). The genes that encode resistance function and the genetic elements that carry the resistance genes widely vary depending upon the type of pathogen and their geographic locations (11). In India, despite the alarming increase in the prevalence of resistant pathogens, only limited information is available about the current scenario of AMR in *V. cholerae* isolates and the genetic identity of resistance traits (6).

In this study, we have analyzed the antibiotic susceptibility of 443 *V. cholerae* strains isolated during 2008 to 2015 from two

EVOLUTION

Significance

Emergence of multidrug-resistant (MDR) pathogens and decreasing effectiveness of antibiotics pose a global threat to public health. Horizontally acquired genetic elements are the major players in the antibiotic resistance crisis. The importance of horizontal gene transfer (HGT) in *V. cholerae* evolution has been well-accepted since the 1980s, when it was reported that intestinal colonization and regulation of cholera toxin production are due to horizontally acquired functions. We show that *V. cholerae* is evolving continuously by gaining fitness traits through HGT. Our results show that more than 99% of recent *V. cholerae* isolates are MDR and their genome is enriched with acquired genetic elements. We further find that the expression pattern of resistance genes does not change whether or not antibiotic is present in a growth medium.

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Data deposition: The whole-genome sequences of all four isolates reported in this paper have been deposited in the National Center for Biotechnology Information (NCBI) BioProject database (accession nos. PRJNA523098, PRJNA523099, PRJNA523107, PRJNA523119).

[1]J.V. and S.B. contributed equally to this work.

[2]Present address: Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610.

[3]Present address: APC Microbiome Ireland, University College Cork, Cork T12 K8AF, Ireland.

[4]Present address: School of Pharmacy, Sungkyunkwan University, 16419 Suwon, Republic of Korea.

[5]To whom correspondence may be addressed. Email: gbnair_2000@yahoo.com or bhabatosh@thsti.res.in.

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centers in India, one in a diarrheal disease-endemic area, Kolkata (east India), and the other in a diarrheal disease-nonendemic area, Delhi (north India). We investigated the genome sequences of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *V. cholerae* isolates to identify AMR genes and the MGEs that are physically linked with resistance genes. We report here the presence of $bla_{\text{NDM-1}}$, which encodes New Delhi metallo-beta-lactamase-1 in the chromosome of *V. cholerae* isolated from the stool samples of diarrheal patients. Earlier $bla_{\text{NDM-1}}$ was isolated only from septicemia patients. We further provide insights about the molecular identity of different AMR traits and the MGEs that carry resistance-associated genes in the genome of *V. cholerae*. In addition, we report the presence of multiple resistance genes against the same antibiotics in individual isolates. We engineered the genome of XDR *V. cholerae* strains to explore the contribution of autonomously replicating extrachromosomal genetic elements to antimicrobial resistance. Finally, we investigated the whole-cell proteome of XDR *V. cholerae* isolate to explore the expression of resistance genes in the presence and absence of antibiotics. Our findings provide insights into the genomes of drug-resistant cholera pathogens, functionality of resistance genes, expression of AMR genes, and mobile nature of genetic elements linked with resistance- and fitness-encoding functions.

Results

Antimicrobial Resistance Profile of *V. cholerae* Isolated During 2008 to 2015. Natural isolates of *V. cholerae* can be intrinsically resistant to a few antibiotics. Most of the resistance traits in *V. cholerae* are acquired via spontaneous mutations in the target genes or by HGT. For a better understanding of acquired resistance traits in O1 and serogroups of *V. cholerae* isolates other than O1 and O139 (called nonO1-nonO139), we selected 22 antibiotics belonging to nine different classes that interact and interrupt cellular pathways involved in cell-wall biosynthesis, DNA, RNA, and protein syntheses, and metabolic processes. For all of the isolates, the number of antibiotics against which the resistance was observed is reported in Dataset S1. Overall, the resistance diversity of *V. cholerae* O1 is higher compared with nonO1-nonO139 isolates (Fig. 1). Based on the resistance profile, *V. cholerae* isolates could be clustered into three groups: (*i*) sensitive, (*ii*) multidrug-resistant (>2 but <10), and (*iii*) extensively drug-resistant (>10) isolates. Almost 99% of *V. cholerae* isolates ($n = 438$) are resistant against ≥2 antibiotics, 17.2% isolates ($n = 76$) are resistant against ≥10 antibiotics, and 7.5% isolates ($n = 33$) are resistant against ≥14 antibiotics. The highest resistance was detected against sulfamethaxozole (99.8%, $n = 442$), the antibiotic that inactivates bacterial dihydropteroate synthase (Fig. 2). In addition, resistance to nalidixic acid ($n = 429$), trimethoprim ($n = 421$), and streptomycin ($n = 409$) are also very high in *V. cholerae* isolated from both centers (Fig. 2). Among all of the selected antibiotics, resistance to neomycin was observed to be lowest (4.0%, $n = 18$) (Fig. 2). Resistance diversity (RD) of *V. cholerae* strains isolated from Kolkata (average RD 7.36; SD 3.1) is significantly higher compared with those from Delhi (average RD 6.07; SD 2.32). However, it is important to note that all of the *V. cholerae* strains ($n = 317$) of Kolkata origin were isolated during 2014 and 2015, while Delhi origin isolates ($n = 126$) were collected during 2008 to 2013. The years of isolation may also influence the resistance diversity of the isolates.

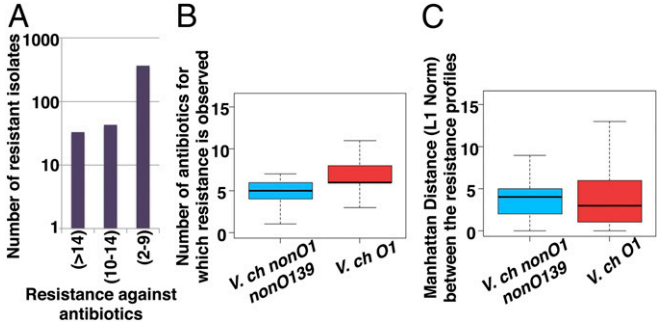

Fig. 1. (*A*) Antimicrobial resistance diversity in *V. cholerae* isolates. (*B*) Number of antibiotics against which resistance was detected. The *V. cholerae* strains were isolated during 2008 to 2015. (*C*) The resistance diversities showed significant variations between isolates belonging to different serotypes. Lines extending vertically from the boxes in *B* and *C* showing variability outside the upper and lower quartiles.

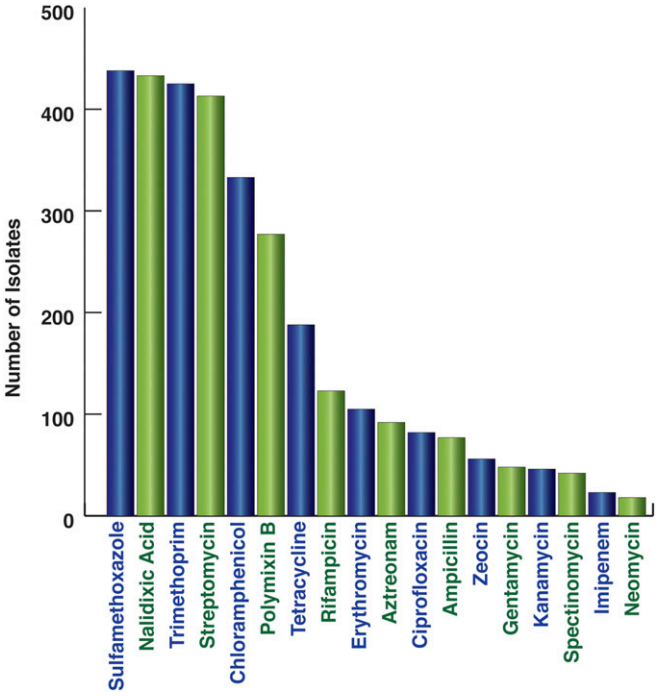

Fig. 2. Resistance profile of *V. cholerae* against different antibiotics. Bar graph showing the number of isolates in which resistance was detected against different antibiotics. The highest number of isolates showed resistance to sulfamethoxazole. Minimum resistance was detected against neomycin.

Differential Detection of Antibiotic Resistance. The variation in the resistance diversity of *V. cholerae* O1 ($n = 412$) and nonO1-nonO139 ($n = 31$) strains isolated during 2008 to 2015 was also determined (Fig. 3). While resistance to streptomycin, trimethoprim, nalidixic acid, tetracycline, and chloramphenicol are significantly higher in O1 isolates, resistance to polymixin B, rifampicin, and erythromycin were observed to be higher in nonO1-nonO139 isolates. The trend of resistance over the years to various antibiotics was observed to have specific patterns for some antibiotics (Fig. 4). While the resistance to tetracycline progressively decreased from 2008 to 2014, an increasing resistance trend was observed for imipenem and spectinomycin (Fig. 4). Since most of the *V. cholerae* strains were isolated from Kolkata during 2014 ($n = 148$) and 2015 ($n = 168$), we compared the resistance trends during this period. The resistance to spectinomycin, rifampicin, and ciprofloxacin increased in 2015, while resistance to chloramphenicol and polymixin B decreased (Fig. 4). The resistance against imipenem and neomycin was very low compared with other antibiotics. Overall, resistance against most of the antibiotics, except polymixin B and tetracycline, increased significantly post 2011 (*SI Appendix*, Fig. S1).

Genomics of *V. cholerae*-Resistant Isolates. We have sequenced and analyzed the whole genomes of four *V. cholerae* isolates belonging to O1, O139, and nonO1-nonO139 serotypes that showed different resistance profiles (*SI Appendix*, Fig. S2). All *V. cholerae* isolates harbored two nonhomologous, circular chromosomes (Chr1 and Chr2) containing close to 4,000 ORFs. Nearly 5% of the genomes

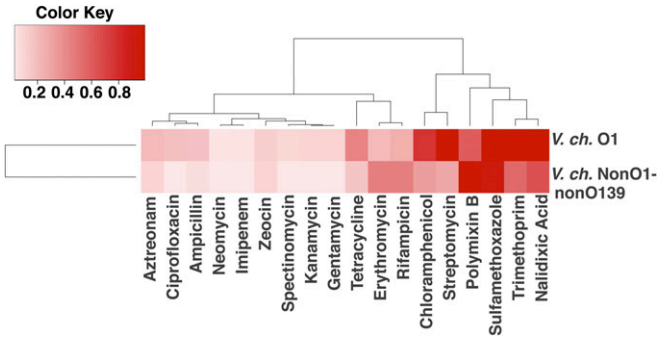

Fig. 3. Differential resistance pattern between *V. cholerae* O1 and *V. cholerae* nonO1-nonO139 clinical isolates. Resistance is high in O1 isolates against tetracycline, chloramphenicol, streptomycin, and trimethoprim. NonO1-nonO139 isolates showed higher resistance to polymixin B compared with O1 isolates.

of resistant *V. cholerae* isolates sequenced in the present study contained different MGEs including pathogenicity islands (PIs), metabolic islands, prophages, plasmids, and transposons that have been acquired by HGT from closely or distantly related bacterial species (Table 1). Integration of most of these MGEs in *V. cholerae* chromosomes is reversible, and can be excised and propagated to other *V. cholerae* cells. Comparative genomics of four isolates revealed close similarity between IDH08148 and IDH0046 (Table 2). Both VCE232 and IDH06781 belong to the nonO1-nonO139 serogroup, but the homology of VCE232 genome sequences is higher with the genome sequences of the O1 (IDH08148) and O139 (IDH0046) serogroups. However, the VCE232 genome has no plasmid, but the genome of IDH08148 harbors one autonomously replicating plasmid.

Most of the resistance can be due to the absence of susceptible target (intrinsic) or acquired functions through HGT. For a better picture of AMR traits in *V. cholerae* strains isolated from India, we did whole-genome sequencing and analysis of the genomes of two XDR and two MDR *V. cholerae* isolates belonging to the O96, O1, O139, and O4 serogroups, respectively (*SI Appendix*, Fig. S2). The whole-genome sequences of all of the isolates were made by pyrosequencing (454 GS FLX+), and the sequences of all of the isolates were deposited in the National Center for Biotechnology Information (NCBI) database. Relevant sequencing information and strain characteristics are reported in Table 1. All of the drug-resistant *V. cholerae* have a plastic bipartite genome equipped with several MGEs that encode antibiotic resistance, toxins, virulence factors, and metabolic enzymes (*SI Appendix*, Fig. S2). Our analysis of the genome sequences of all of the four resistant isolates identified more than 40 AMR genes that encode resistance against β-lactams, aminoglycosides, macrolides, tetracyclines, fluoroquinolones, bleomycin, and bicyclomycin antimicrobials (Fig. 5). The highest numbers of resistance genes were detected against aminoglycosides. Similarly, we have identified multiple β-lactamase genes representing four classes (A, B, C, and D) of the Ambler classification scheme in the genome of each isolate. The genomes of XDR *V. cholerae* isolates IDH06781 and IDH08148 harbor eight and seven different β-lactamase–encoding genes, respectively (Fig. 5). Similar resistance genes were also present in the genomes of ESKAPE pathogens (*SI Appendix*, Table S1). By analyzing the immediate genetic vicinity of the $bla_{\text{NDM-1}}$ gene of IDH06781 and IDH08148, we observed that the bleomycin resistance-encoding *sh ble* gene is physically linked with the $bla_{\text{NDM-1}}$ gene. Further analysis of the genomic scaffolds of these isolates revealed that the *sh ble* and $bla_{\text{NDM-1}}$ genes are coexpressed under the control of the same promoter, located upstream of the $bla_{\text{NDM-1}}$ gene and at the extremity of the insertion sequence ISAba125 (Fig. 6). Phenotypically, both isolates showed resistance against imipenem and zeocin, and the traits are functional in heterologous genetic backgrounds, including *Escherichia coli* (*SI Appendix*, Table S2). Plasmid-curing experiments provided convincing evidence that the ISAba125 element linked with the *sh ble* and $bla_{\text{NDM-1}}$ genes are located on the chromosome of the *V. cholerae* IDH06781 isolate (*SI Appendix*, Table S3). Previously, $bla_{\text{NDM-1}}$–positive nonO1-nonO139 *V. cholerae* was identified from a septicemia patient (12). This study reports the chromosomal integration of $bla_{\text{NDM-1}}$ in a *V. cholerae* genome isolated from diarrheal patients. It is important to note that $bla_{\text{NDM-1}}$–encoded metallo-beta-lactamase confers resistance against various beta-lactam antibiotics, including imipenem. The genomes of IDH0046 and VCE232 do not encode any β-lactamase–encoding genes. Other than β-lactamase, IDH06781, IDH08148, and IDH0046 genomes harbor multiple genes (*aph*, *aac*, and *ant*) that confer resistance against several aminoglycoside antibiotics (Fig. 5).

In addition, genomes of all of the isolates are enriched with genes for aminoglycoside protection protein, tetracycline efflux pumps, and tetracycline-modifying functions (Dataset S2). Further analysis of resistance genes and their translated proteins using publicly available Antibiotic Resistance Genes Database, Comprehensive Antibiotic Resistance Database (CARD), and The Protein Data Bank databases revealed the presence of similar AMR genes in the genomes of other enteric pathogens, including *Klebsiella pneumoniae*, enteropathogenic *E. coli*, *Salmonella enterica*, *Shigella* sp., and so forth.

Functional Evaluation of Resistance Traits. Several in silico studies have revealed a plethora of commensals, symbionts, and opportunistic pathogens inhabiting the human gastrointestinal tract housing hundreds of AMR genes in their genomes (10, 13). The functions of AMR genes were predicted by comparing sequence homology to the genes that have been cataloged as AMR genes in the databases. Therefore, to rule out possible false-positive annotation, we validated the functions of most of the resistance genes that contribute to the resistance phenotype of the IDH06781 isolate. We amplified 16 different genes from the genome of XDR isolate IDH06781, cloned them into expression vectors pBD62 or pBAD24, and determined their resistance function in heterologous genetic backgrounds (*SI Appendix*, Table S2). Most of the *bla* genes selected for functional evaluation confirmed resistance against reported minimum inhibitory concentration of the respective antibiotic in heterologous host *E. coli* (*SI Appendix*, Table S2). We also observed that $bla_{\text{NDM-1}}$ inactivates all of the tested β-lactam antibiotics, except aztreonam. As expected, the activity of the $bla_{\text{NDM-1}}$–encoded enzyme was completely inhibited in the presence of the metal ion chelator EDTA (*SI Appendix*, Table S2).

Antibiotic Resistance Genes Are Linked with Mobile Genetic Elements. The genomic elements contributing to the carriage and dissemination of resistance traits and emergence of MDR and XDR

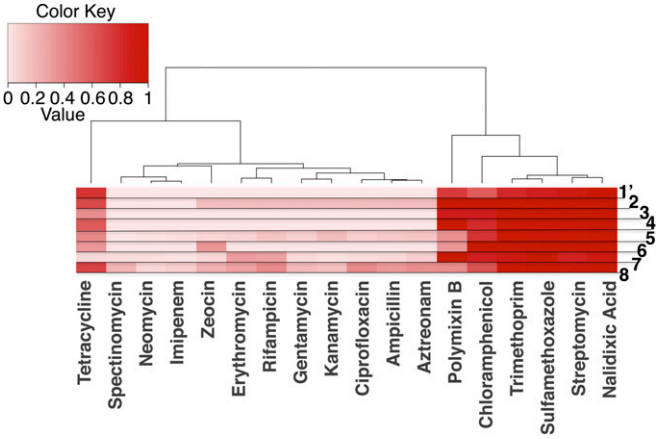

Fig. 4. Yearwise resistance pattern of *V. cholerae* strains isolated during 2008 to 2015. Resistance against polymixin B is reduced over the year (except 2014). Tetracycline resistance was also low until 2014. 1′, 2008; 2, 2009; 3, 2010; 4, 2011; 5, 2012; 6, 2013; 7, 2014; 8, 2015.

Table 1. Relevant whole-genome sequencing information of different *V. cholerae* isolates

Characteristic	IDH06781	VCE232	IDH08148	IDH0046
Year of isolation	2014	1980	2015	2001
Serogroup	O96	O4	O1 Ogawa	O139
Resistance phenotype	R22	R3	R19	R7
Sequence size, Mb	924	796	874	954
No. of reads	535,245	637,875	1,237,893	1,337,254
Avg. read lengths, bp	767	355	707	714
No. of scaffolds	105	65	43	32
GC content, %	47.2	47.5	47.5	47.5
N50	92,624	160,909	235,586	636,425
Longest scaffold size, bp	452,388	318,788	572,897	796,078
Total no. of ORFs	4,294	3,636	3,608	3,624
ORFs encoding virulence, toxins, and AMR	117	89	89	82
Phage, Tn, plasmids	14	31	23	21

pathogens are mostly mobile in nature. Many antibiotic resistance genes are physically linked with plasmids, integrative conjugative elements (ICEs), and transposons. Comprehensive genomic studies of all of the four isolates revealed several acquired genetic elements in their genomes (Fig. 6). The physical linkage between antibiotic resistance genes and plasmids, ICEs, and transposons was observed for both the XDR isolates IDH06781 and IDH08148 (Fig. 6). XDR isolate IDH06781 harbors two large plasmids, pVC1 (84.6 kb) and pVC2 (53.1 kb). pVC1 does not carry any AMR genes, and most of the predicted ORFs are hypothetical. For the plasmid pVC1, we could not find any DNA sequence similarity (>3%) to the available DNA sequences in the NCBI database. Several AMR genes, including *bla*, *ant(3′)*, and *aac(3′)*, are physically linked with pVC2. pVC1 and pVC2 have lower GC content (~40%) compared with the host chromosome. Both plasmids encode several mobility functions (*tra*) for their movement. In the plasmid-curing experiments, pVC2 was found to be essential for rifampicin, ciprofloxacin, tetracycline, neomycin, and aztreonam resistance phenotypes in the IDH06781 isolate (*SI Appendix*, Table S3). Curing of pVC1 had no effect on the resistance phenotype of IDH06781 under laboratory growth conditions (*SI Appendix*, Fig. S3). Similarly, IDH08148 also harbored a large plasmid (~94 kb) and encodes β-lactamases, chloramphenicol acetyltransferase, aminoglycoside 3′-phosphotransferase, aminoglycoside N(3′) acetyltransferase, and bleomycin resistance protein. Except for VCE232, all of the three isolates harbor self-transmissible ICEs at the *prfC* locus of Chr1. In addition, genomes of both the XDR isolates harbored transposons (Tn3) and insertion sequences (IS6, IS30) and are linked with AMR genes, including bla_{NDM-1}, *sh ble*, *aac(3′)*, and *ant(3′)*. Although all of the four isolates carry prophages like CTXϕ in their genome, there is no physical linkage between the phage genome and AMR genes.

Whole-Cell Proteome Analysis of XDR Isolate IDH06781 in the Presence and Absence of Imipenem. Since the IDH06781 genome harbors the maximum numbers of AMR genes, we used this isolate to investigate the whole-cell proteome profile in the presence and absence of the broad-spectrum β-lactam antibiotic imipenem. A total of 2,270 proteins were identified using iTRAQ analysis. Differential expression of 270 proteins was observed in the presence and absence of imipenem. Maximum repression (13.6-fold) was detected for a glucose metabolic enzyme phosphoglucomutase that helps interconversion of glucose 1-phosphate and glucose 6-phosphate. Expression levels (~2-fold) of cytochrome *c* oxidase and some uncharacterized proteins were elevated in the presence of imipenem. We were able to detect expression of most of the AMR proteins, including serine and metallo-β-lactamases, aminoglycoside acetyltransferase, aminoglycoside 3′-adenyltransferase, and several other MDR proteins in the absence and presence of imipenem (Fig. 7). We did not find any significant difference in the expression profile of AMR genes in the presence and absence of imipenem. More importantly, we could detect high-level expression of three different β-lactamases even in the absence of antibiotics. This finding may suggest that β-lactamases contribute to additional cellular functions other than only antimicrobial resistance.

Discussion

Antibiotic resistance is a serious global problem, and threatens the efficacy of nearly all antibiotics commonly used to cure or prevent microbial infections (11). Emergence of AMR is a natural phenomenon, and the resistance crisis has been expedited due to the intensive usage of antibiotics in health sectors and agriculture and the release of antibiotic-containing industrial waste into the environment (14, 15). The antibiotic pressure allows the selection of resistant variants and their proliferation and spread to other biospheres. The susceptible population sharing the same ecological niche with the resistant variants gets eliminated in the presence of antibiotics. Resistance to multiple drugs was first detected among enteric pathogens in the early 1960s (16). In the last few decades, AMR *V. cholerae* has also evolved rapidly and spread across the globe (17). Even during cholera outbreak periods, chemoprophylaxis is not preferred, to avoid the emergence of resistant pathogens. However, in clinical settings, rehydration therapy and antibiotics are in use mainly to reduce the duration of disease and volume of stools. During the 1940s to 1960s, streptomycin, chloramphenicol, and tetracycline were effectively used in the treatment of cholera (18). Sulfamethoxazole-trimethoprim, azithromycin, and ciprofloxacin were also used in the treatment of cholera during the 1970s (19). Several classes of β-lactam antibiotics along with β-lactamase inhibitors are also widely prescribed for acute gastritis. Over the years, resistance in *V. cholerae* against all these antibiotics turns out to be very high, and the resistance pattern directly correlates with the usages of antibiotics (17).

Recent genomic studies on *V. cholerae* isolates had major emphasis on the phylogenetic relationship between different lineages and routes of transmission of cholera pathogens (17, 20). However, little is known about the resistance profile, traits that confer resistance in *V. cholerae*, and mode of dissemination of resistance traits between bacterial species. To understand the emergence of resistant *V. cholerae*, it is important to identify the mechanisms of resistance, genetic nature of resistance traits, and

Table 2. Total number of ORFs (%) showing DNA sequence similarity >90% between different *V. cholerae* isolates

Isolate	IDH06781	VCE232	IDH08148	IDH0046
IDH06781	100	51.23	52.18	52.16
VCE232	59.75	100	88.23	87.53
IDH08148	61.02	88.40	100	93.82
IDH0046	60.60	87.75	93.21	100

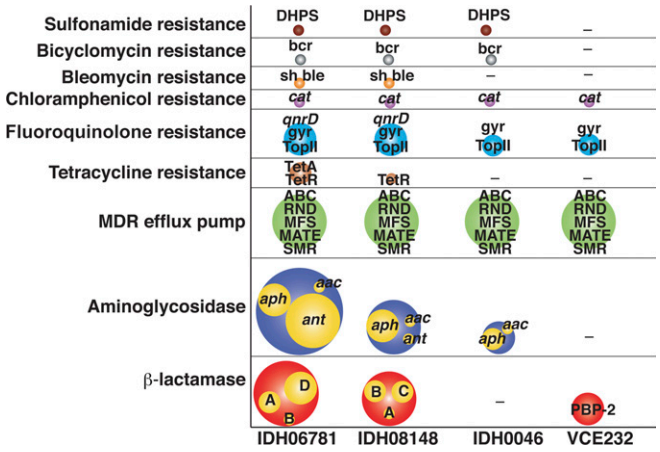

Fig. 5. Abundance of different antimicrobial resistance genes in the whole genome-sequenced *V. cholerae* isolates. Size of the bubbles corresponds to the abundance of resistance genes in the genomes of the four isolates. Subclasses of the resistance genes are also mentioned within the bubble. The picture was drawn to scale. Details of the resistance genes are provided in *SI Appendix*.

elements that contribute to the rapid dissemination of resistance. Enteric pathogens are intrinsically resistant to very few antimicrobials; indeed, almost all of the resistance traits have been acquired. The important factors that determine the probability for bacterial populations to develop resistance against antibiotics are (*i*) the frequency of exposure to antibiotics, (*ii*) the rate of accumulation of point mutations in the target gene by spontaneous mutation, and (*iii*) the competency of acquiring resistance functions from other organisms by HGT. *V. cholerae* is a natural inhabitant of the aquatic environment, and the chances of exposure to antibiotics are very high. In addition, the bacterium has remarkable genetic plasticity that allows *V. cholerae* to respond to a wide variety of environmental stresses, including antimicrobials (21). The bacterium is naturally competent and capable of acquiring DNA from the environment by all of the three major routes of HGT, namely transformation, conjugation, and transduction. Analysis of the genomes of all of the four MDR and XDR *V. cholerae* isolates revealed several genes involved in somatic antigen biosynthesis, regulatory functions, nutrient and metabolite transport, chemotaxis, DNA mobility, pathogenicity, antibiotic and heavy-metal resistance is linked with MGEs (Dataset S2). More than one dozen MGEs, including PIs, metabolic islands, prophages, ICEs, transposons, insertion sequences, and autonomously replicating and integrating plasmids are identified in both XDR isolates IDH06781 and IDH08148. Most of these acquired genetic elements encode tyrosine recombinase/integrase for their mobility, and carry specific genomic signatures that diverge from the core genome. In the XDR *V. cholerae* IDH06781, nearly 5% of the genomic content is part of a flexible gene pool. Currently, it is not clear the exact contribution of pVC1 to the physiology/fitness of IDH06781, since almost all of the ORFs present in the plasmid are hypothetical in nature and the curing of the plasmid from the XDR genome has no visible effect on resistance phenotypes.

It is important to mention that the resistance traits are very similar across the pathogens, and large numbers of AMR traits are physically linked with MGEs. Whole-genome sequence analysis of the XDR *V. cholerae* isolates revealed the presence of multiple resistance traits against each class of antimicrobial scaffold (Fig. 5). β-Lactams are the most widely used broad-spectrum antibiotics, and resistance to β-lactams is a serious threat for infectious disease management, surgery, and organ transplantation. Until now, more than 1,000 β-lactamases have been reported (www.lahey.org/studies). The present study showed the presence of several β-lactamase–encoding genes in both XDR isolates (Fig. 5). Our functional analysis showed that the multiple resistance genes present in the genome of XDR isolates are functionally active and reflect the continuous evolution of bacterial species. Like β-lactamases, multiple traits conferring resistance against aminoglycoside antibiotics are also detected in the sequenced genomes of both the XDR pathogens (Fig. 5). This correlates well with the overall increase in aminoglycoside usage in clinical facilities in India for many years (22). Finally, our whole-cell proteome analysis of IDH06781 revealed that the multiple resistance genes present in the genomes of XDR isolates are not only functionally active but that most of them are constitutively expressed even in the absence of antibiotics. This indicates the possibility of alternative action of the resistance traits in the physiology of bacterial cells.

Conclusion

V. cholerae is an ancient human pathogen with extraordinary genomic plasticity and capability to adapt to a changing environment. In the last 10 years, more than 370 reports have been published on the antimicrobial resistance of *V. cholerae*, but none have comprehensive analysis of the prevalence of AMR traits, diversity and abundance of resistance traits, and functionality of predicted resistance genes and their expression in the presence and absence of antibiotics. Our comprehensive analysis of 443 clinical *V. cholerae* isolates showed that the cholera pathogen is continuously evolving to counterbalance the antimicrobial effects of clinically important antibiotics. More importantly, the resistance genes are physically linked with MGEs, and could potentially propagate to other bacterial species through HGTs. To combat the serious threat of rising AMR in enteric pathogens and to prevent the decline in effectiveness of antibiotics of public health importance, it is imperative to develop strategies for robust surveillance, restriction on improper antibiotic usage, and identification of routes that are facilitating the rapid dissemination of antibiotic resistance in pathogenic and nonpathogenic bacterial cells.

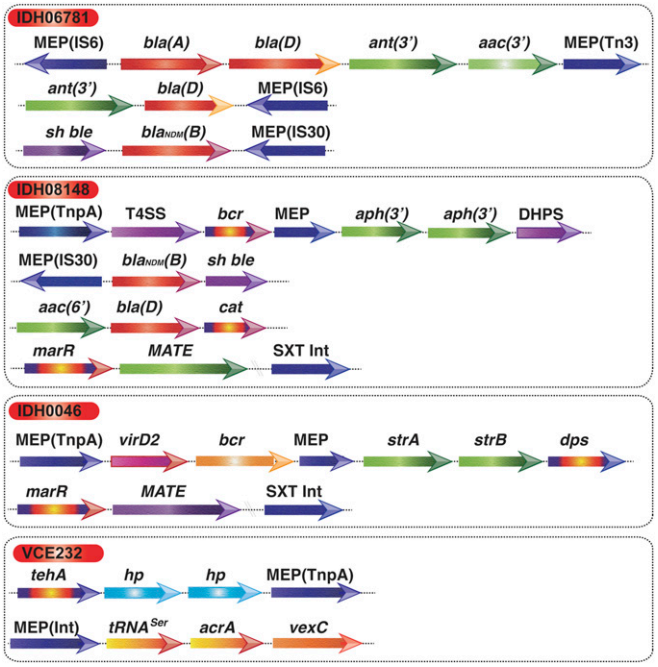

Fig. 6. Mobile genetic elements linked with the antibiotic resistance genes in the genome of *V. cholerae*. Mobile element proteins like transposase/integrase/site-specific recombinase are used as signatures of MGEs. For β-lactamase, genes A and D denote serine-β-lactamase, whereas B denotes metallo-β-lactamase. Details of the resistance genes are provided in *SI Appendix*.

EVOLUTION

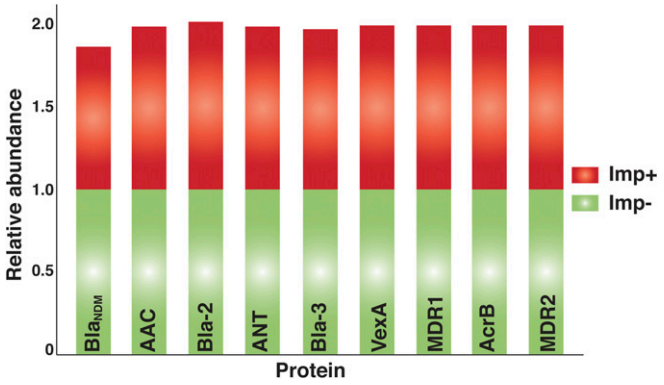

Fig. 7. Whole-cell proteome analysis revealed expression of different antimicrobial resistance genes in the presence and absence of antibiotic. The *V. cholerae* IDH06781 strain was cultivated in the presence and absence of imipenem. Whole-cell proteins were labeled using iTRAQ and detected by TripleTOF 5600 mass spectrometer.

Methods

Bacterial Strains and Plasmids. *V. cholerae* were isolated on a thiosulfate-citrate-bile salts-sucrose agar (Eiken) plate from the stool samples of acute diarrheal patients. AMR profiles and relevant characteristics of the isolates are provided in Dataset S1. Antibiotic susceptibility testing was performed using BBL Sensi-Disc (BD), E-strip (bioMérieux), and broth dilution methods (*SI Appendix*). Sensitivity to each antibiotic was determined by evaluating the annular radius of inhibition of growth around each disk in accordance with the Clinical and Laboratory Standards Institute (CLSI-2016). As standard strains, *E. coli* ATCC 25922 and *V. cholerae* O395 were used for all of the antibiotics.

Next-Generation DNA Sequencing. Genomic DNA was prepared by using the cetyltrimethylammonium bromide method followed by RNase treatment. Approximately 500 ng/μL DNA with no visible contamination of RNA or DNA degradation was used for whole-genome sequencing (Roche). Details of the sequencing are provided in *SI Appendix*.

Genome Assembly, Annotation, and Functional Analysis. A sequence assembly tool, GS de novo genome assembler (Roche), was used to assemble quality filtered sequencing reads. More than 90% identity and a minimum 40-nt overlap were assigned to assemble the sequencing reads. Contigs and scaffolds were validated using the MegaBLAST program in the NCBI database. Relevant information for all of the four genomes is provided in Table 1. The translated protein sequences of the AMR genes were cross-compared with the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/).

Cloning and Functional Validation of AMR Genes. All of the antibiotic resistance genes with putative enzymatic functions were amplified from the genome of the IDH06781 isolate and cloned under the control of the P_{BAD} promoter in pBD62 or pBAD24 expression vectors (*SI Appendix*, Table S2). Primer sequences are available in *SI Appendix*, Table S4. Vectors were introduced into *E. coli* by transformation. Resistance function was confirmed in the presence of the desired antibiotics in the selection plate.

Plasmid Curing. Plasmid curing in XDR *V. cholerae* was done by growing *V. cholerae* cells in elevated growth temperature at 42 °C. Details of plasmid curing are provided in *SI Appendix, Methods* and Fig. S3.

Whole-Cell Proteome Analysis. Total protein extraction and trypsin digestion were done for whole-cell proteome analysis of XDR isolate IDH06781. Both control and antibiotic-treated *V. cholerae* cells were used for proteome analysis.

Mass spectrometry analysis was performed using an LC system (Eksigent; 2D) coupled with a TripleTOF 5600 mass spectrometer (AB Sciex). Peptides from each sample were labeled with isobaric tags (iTRAQ) in a four-plex set (AB Sciex). MS/MS spectra were obtained in an information-dependent acquisition mode with a TOF/MS survey scan (350 to 1,250 *m/z*). MS data were analyzed by Protein Pilot 4.5 (AB Sciex) using the Paragon algorithm. Proteins with a ratio >1.5 were considered to be differentially expressed. In all cases, $P < 0.05$ (*t* test) was considered significant in protein quantification.

Statistical Analysis. Cross-comparison of resistance trends across different groups was performed with Mann–Whitney *U* tests using the wilcox.test function implemented in R programming package v3.0.0 (https://cran.r-project.org/bin/windows/base/old/3.0.0/). Details are provided in *SI Appendix*.

Nucleotide Sequence Accession Number. Whole genome sequences are available in the NCBI database under BioProject IDs PRJNA523098, PRJNA523099, PRJNA523107, PRJNA523119 (23–26).

Ethical Clearance. Stool samples were collected from diarrheal patients admitted either to Maharishi Valmiki Infectious Diseases (MVID) Hospital, Delhi (north India) or Infectious Diseases Hospital, Kolkata (east India) after obtaining informed consent. Relevant approval was given by the institutional ethical committees of MVID Hospital, Delhi (no. 1120/MS/MVIDH/2015) and National Institute of Cholera and Enteric Diseases, Kolkata. All of the samples were deidentified before use in this study.

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