World Journal of Medical Genetics

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DOI: 10.5496/wjmg.v4.i2.27

World J Med Genet 2014 May 27; 4(2): 27-33
ISSN 2220-3184 (online)

REVIEW

Acquisition and dissemination mechanisms of CTXΦ in *Vibrio cholerae*: New paradigm for *dif* residents

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Supported by Research in the Laboratory of Das B and Nair GB is funded in part by Department of Science Technology, No. SB/FT/LS-309/2012; Government of India (GOI) and the Department of Biotechnology, No. BT/MB/THSTI/HMC-SFC/2011; Research in the Laboratory of Bhadra RK is partly financially supported by Council of Scientific and Industrial Research, GOI and Indian Council of Medical Research, GOI
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Received: December 26, 2013 Revised: April 10, 2014
Accepted: May 13, 2014
Published online: May 27, 2014

Abstract

Vibrio cholerae (*V. cholerae*) genome is equipped with a number of integrative mobile genetic element (IMGE) like prophages, plasmids, transposons or genomic islands, which provides fitness factors that help the pathogen to survive in changing environmental conditions. Metagenomic analyses of clinical and environmental *V. cholerae* isolates revealed that dimer resolution sites (*dif*) harbor several structurally and functionally distinct IMGEs. All IMGEs present in the *dif* region exploit chromosomally encoded tyrosine recombinases, XerC and XerD, for integration. Integration takes place due to site-specific recombination between two specific DNA sequences; chromosomal sequence is called *attB* and IMGEs sequence is called *attP*. Different IMGEs present in the *attP* region have different *attP* structure but all of them are recognized by XerC and XerD enzymes and mediate either reversible or irreversible integration. Cholera toxin phage (CTXΦ), a lysogenic filamentous phage carrying the cholera toxin genes *ctxAB*, deserves special attention because it provides *V. cholerae* the crucial toxin and is always present in the *dif* region of all epidemic cholera isolates. Therefore, understanding the mechanisms of integration and dissemination of CTXΦ, genetic and ecological factors which support CTXΦ integration as well as production of virion from chromosomally integrated phage genome and interactions of CTXΦ with other genetic elements present in the genomes of *V. cholerae* is important for learning more about the biology of cholera pathogen.

Key words: *Vibrio cholerae*; Cholera toxin phage; VGJΦ; Plasmids, Integrative mobile genetic element; XerC; XerD; Dimer resolution sites; *attP*; *attB*

Core tip: Integrative mobile genetic element (IMGE) like prophage, plasmid, transposon or genomic island plays crucial roles in the evolution of bacterial pathogens. The *Vibrio cholerae* (*V. cholerae*) genome harbors several such IMGEs, which provides virulence, antibiotic resistance and other fitness traits to cholera pathogen and directly contributes in its evolution. Cholera toxin encoding phage (CTXΦ) is a well characterized IMGE, found integrated in the dimer resolution sites of all epidemic *V. cholerae* strains and exploits host encoded XerC and XerD recombinases for its lysogenic conversion. In this review we discussed about integration and dissemination of CTXΦ and related IMGEs of *V. cholerae*.

Das B, Nair GB, Bhadra RK. Acquisition and dissemination mechanisms of CTXΦ in *Vibrio cholerae*: New paradigm for *dif* residents. *World J Med Genet* 2014; 4(2): 27-33 Available from: URL: http://www.wjgnet.com/2220-3184/full/v4/i2/27.htm DOI:

http://dx.doi.org/10.5496/wjmg.v4.i2.27

INTRODUCTION

Bacterial genomes are highly dynamic and equipped with large number of integrative mobile genetic element (IMGE) like, prophages, plasmids, transposons or genomic islands (GIs), which allow bacteria to respond rapidly to changing environmental conditions and help them to survive in hostile environments[1]. The movement of genetic traits attributed to horizontal gene transfer systems is mediated by variety of homologous and non-homologous recombination processes that integrate, excise, and translocate genes into specific sites by DNA recombinases. The polynucleotidyl transferase that catalyzes the cutting and joining of phosphodiester bonds between DNA molecules necessary to move a segment of DNA is termed recombinase. DNA recombinases are generally needed to act at specific DNA sequences. Most IMGEs encodes specific dedicated recombinase for their movement[2]. Among several IMGEs, phages are the best-characterized genetic elements, which play major role in real-time evolution of toxigenic bacterial pathogens[3]. Most pathogenic bacterial cells acquired their virulence traits either from phages[3] or other IMGEs like, plasmids[4], transposons[5] or GIs[6]. Understanding the biology that supports emergence of pathogenic bacteria would be worthy to reduce disease burden and development of therapeutic agents. *Vibrio cholerae* (*V. cholerae*), the etiological agent of the acute secretory diarrheal disease cholera, represents a paradigm for toxin gene acquisition from cholera toxin phage (CTXΦ) and evolution from non-pathogenic strains to toxigenic cholera pathogen[3].

Both toxigenic and non-toxigenic *V. cholerae* strains are autochthonous inhabitant of estuaries and are introduced into the human intestine through contaminated water or food[7]. Only toxigenic *V. cholerae* isolates belonging to the serogroup O1 or O139 cause epidemic or pandemic cholera. Toxigenic *V. cholerae* harbor one or multiple copies of integrated CTXΦ either in large or in small or in both chromosomes. CTXΦ exploits host encoded tyrosine recombinases XerC and XerD to integrate into the chromosome dimer resolution site, called dimer resolution sites (*dif*), present in close proximity of the replication termination region of either chromosome of *V. cholerae*[8].

In this review, we provided a brief view about the genome of cholera pathogen, the major IMGEs reported in the genome of cholera pathogen and molecular insights into integration mechanisms of CTXΦ in the *V. cholerae* chromosomes. The main emphasis is on the mechanistic part of CTXΦ integration, how (+)ssDNA genome of CTXΦ is recognized by the XerC and XerD enzymes and proceed for unusual irreversible integration. We also discussed about other IMGEs that follow CTXΦ like integration. Finally, we discussed how cooperative interactions between CTXΦ and other IMGEs could lead to dissemination of CTXΦ among closely or distantly related bacterial cells.

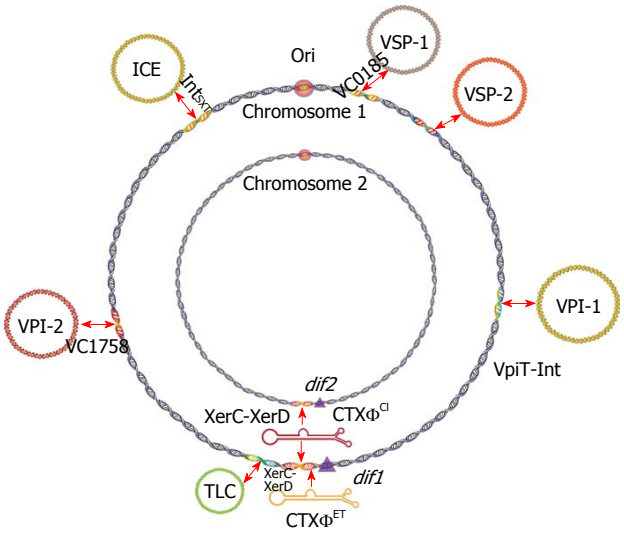

Figure 1 Schematic representation of the integrative mobile genetic elements present in the chromosome 1 (large) or 2 (small) of *Vibrio cholerae*. Except CTXΦ, all other IMGEs have unique *attB* site either in large or small chromosome. IMGEs present in the *dif* region exploit host-encoded recombinases for integration while the genome of other IMGEs encode their own recombinases for integration (see detail in the text). Except CTXΦ, integration of rest of the IMGEs relies on their dsDNA genome and the integration is reversible. IMGE: Integrative mobile genetic element; CTXΦ: Cholera toxin phage; ICE: Integrative conjugative elements; VPI: *Vibrio* pathogenicity island; VSP: *Vibrio* seventh pandemic; TLC: Plasmids; *dif*: Dimer resolution sites.

CTXΦ AND OTHER IMGEs IN *V. CHOLERAE* CHROMOSOMES

IMGEs are segments of DNA that encode or exploit host encoded recombinase(s) for intra- and inter-chromosomal movement within or between closely or distantly related bacterial cells. Comparative analysis of *V. cholerae* genomes revealed presence of IMGEs in both the chromosomes of toxigenic and non-toxigenic strains[9]. Three major classes of IMGEs are reported in cholera pathogens: prophages, GIs and integrative conjugative elements (ICEs) as shown in Figure 1. Both pathogenic determinants and antimicrobial resistance traits of cholera pathogen are encoded by these IMGEs[3].

As mentioned, CTXΦ and several other phages always integrate in the *dif* site present one in each of the two circular chromosomes of *V. cholerae*[10,11]. The *dif* sites present in the large and small chromosomes are called *dif1* and *dif2*, respectively. *dif* consists of 28-bp DNA sequences, 11-bp binding sites for XerC and XerD separated by a 6-bp central region[12]. Strand exchange occurs immediately after binding sites of XerC and XerD. CTXΦ, RS1 and TLC element integrates as a single copy or in multiple tandemly arrayed copies in *dif1*[10,13]. Other IMGEs were found integrated at the *dif* sites as a single copy[9]. Prophages and other genomic elements present in the *dif* regions do not encode any recombinase but exploit host encoded XerC and XerD enzymes for their

inter- and intra-chromosomal movement.

Other than prophages, almost all the current 7th pandemic *V. cholerae* isolates harbor four other GIs, namely, *Vibrio* pathogenicity islands-1 (VPI-1) and VPI-2 and *Vibrio* seventh pandemic islands-1 (VSP-1) and VSP-2[14] as shown in Figure 1. All four GIs encode putative tyrosine recombinases for their integration and excision. Although no experiment has yet been conducted to demonstrate the integration of GIs in the *V. cholerae* chromosomes, their excision has been established by conventional genetic analysis[15]. Among the four pathogenicity islands, VPI-1 is crucial since it participates at different levels in the CTXΦ associated cholera development: (1) VPI-1 encodes toxin co-regulated type-IV pilus that acts as a receptor for CTXΦ as well as it is a major colonization factor of *V. cholerae* and is essential for the disease development; and (2) it encodes the transcriptional factor ToxT that positively regulates the expression of cholera toxin (CT) genes *ctxAB*[16,17]. VPI-1 is a 41-kb DNA segment physically linked to a tmRNA gene (*ssrA*), flanked by two nearly identical repeat sequences[18]. It is found preferentially in the toxigenic strains. VPI-1 carries two putative tyrosine recombinases, called Int_{vpi} and VpiT[15]. Sequence analyses of these two putative recombinases indicate that they are quite different from each other. While Int_{vpi} contains the conserved R1-H-R2-Y signature motif of the tyrosine recombinases, this motif is not clear in VpiT. VPI-1 appeared to be mobile since an extrachromosomal circular form was detected in *V. cholerae* cells. This is further supported by the fact that it is absent in the genomes of non-pathogenic *V. cholerae* strains[15].

Presence or absence of VSPs could serve as distinct genetic signatures to differentiate the previous (6th) and the current (7th) pandemic *V. cholerae* isolates. VSP-1, a 16-kb DNA segment, harbors 13 open reading frame (ORF)s found in the sequenced seventh pandemic *V. cholerae* strain N16961 (Heidelberg *et al*[18] 2000). A XerCD like putative tyrosine recombinase (Int_{VSP-1}) is present in the VSP-1 might participate in the integration and dissemination of VSP-1 island. Compared to other GIs, VSP-1 is highly conserved among O1 El Tor isolates. Recent study has demonstrated that the VSP-1 encoded enzyme DncV preferentially synthesizes hybrid c-AMP-GMP molecule, which directly contribute to the fitness of cholera pathogen[19].

ICEs are usually large DNA fragments that can integrate in specific positions of bacterial chromosomes using their own tyrosine recombinase and able to excise and disseminate in closely or distantly related bacterial species by conjugation. Diverse ICEs have been detected in several gram-positive and gram-negative bacteria[20]. sulfamethoxazole and trimethoprim resistance traits (SXT), a 99.5 kb ICE, was first discovered in the chromosome of *V. cholerae* O139 MO10 strain from Southern India and it encodes resistances to several antibiotics like sulfamethoxazole (*sul2*), trimethoprim (*dfrA18* or *dfrA1*) chloramphenicol (*floR*), streptomycin (*strA* and *strB*), tetracycline (*tetA* and *tetR*), *etc*[21]. Wozniak *et al*[22] have sequenced and analyzed several SXT like elements present in diverse bacterial species including *V. cholerae* and their analysis indicated that similar organization and conservation of the core genes of these elements.

CTXΦ AND ITS INTEGRATION IN THE CHROMOSOMES OF *V. CHOLERAE*

All epidemic *V. cholerae* isolates carry CTX prophage in their large and/or small chromosomes. CT, the toxin responsible for profuse diarrhoeal disease cholera, is encoded by the genome of CTXΦ. CTX prophages characterized from different toxigenic *V. cholerae* strains are fairly different at the DNA sequence level but their genomic organization and function of each of the protein encoded by the phage genome are identical. CTXΦ has a approximately 7000-nt ss (+) DNA genome arranged in structurally and functionally distinct two modular structures, RS2 (repeat sequence 2) and core (Figure 2). RS2 comprises three genes designated as *rstR, rstA* and *rstB*. RstA, initiator of rolling circle replication, is essential for phage replication[23,24]. It carries a conserved Y-X-X-X-Y motif for initiation and termination of phage replication at *ori* region. RstB, a single stranded DNA binding protein, plays a crucial role in phage integration[8]. It was proposed that RstB maintains proper (+) *attP* structure of CTXΦ that is recognized by XerC-XerD and prevents access of cytoplasmic single stranded DNA binding proteins interference in phage integration[25]. RstR acts as a transcriptional modulator and repressed transcription of *rstA* and *rstB* from P_{rstA}, the only phage promoter required for CTXΦ replication and integration[26]. Core region comprises of seven genes responsible for phage morphogenesis and toxin production. Five genes, namely, *psh*, *cep*, $gIII^{CTX}$, *ace* and *zot* encode proteins essential for phage morphogenesis and phage assembly. The Psh, Cep, OrfU ($pIII^{CTX}$) and Ace proteins are phage structural proteins, which encapsulate single stranded phage genome whereas Zot protein play central role in phage assembly[3]. Interestingly, *ctxA* and *ctxB* genes coding for CT are not required for phage morphogenesis but is essential for disease development as discussed above.

Replicative genome of CTXΦ is detrimental to *V. cholerae* growth[27]. All toxigenic *V. cholerae* cells harbor integrated CTXΦ in large and/or small chromosomes. Although CTXΦ does not encode any recombinase for its integration, the replicative genome of CTXΦ carries two XerC-XerD binding sites in inverted orientations, called *attP1* and *attP2*[28]. In *attP1,* XerC and XerD binding sites are separated by 12-bp overlap region while in *attP2* the length of the overlap region is 5-6 bp. Both *attPs* are connected by a 90-bp DNA sequence. In the (+)ssDNA phage genome the 150-bp region encompassing *attP1* and *attP2* formed a hairpin structure by intra-strand complementary base pairing, which creates a phage integration site *attP*(+) (Figure 2).

The integration of CTXΦ is mediated by a complex DNA-protein reaction within a nucleoprotein complex consisting of one pair each of host encoded XerC and

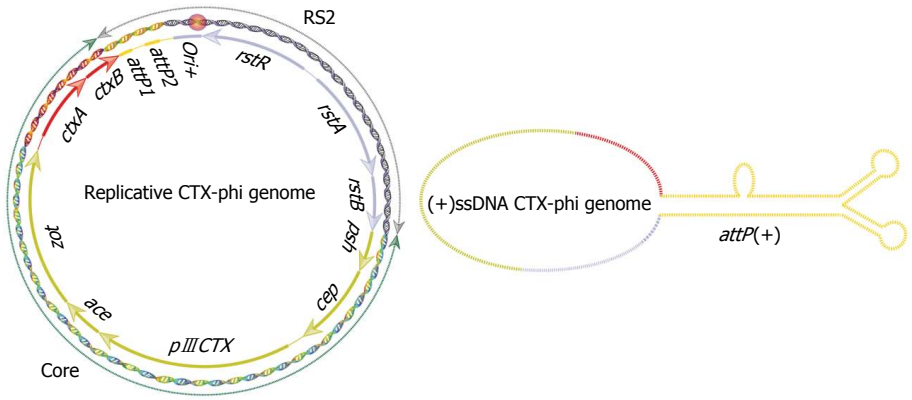

Figure 2 Replicative and integrative genomes of cholera toxin phage. Replicative genome of CTXΦ arranged in structurally and functionally distinct two modular structures RS2 and core. RS2 encoded proteins are essential for phage replication, integration and transcriptional regulation of phage genes. Core encoded proteins are essential for phage morphogenesis and virion production. Folded (+)ssDNA phage genome is essential for phage integration. Functional XerCD binding site, *attP*(+), is formed by complementary base pairing between *attP1* and *attP2* of (+)ssDNA of phage genome. We have used similar color codes for replicative and integrative phage genomes for easy understanding of genetic attributes of *attP*(+) region. CTXΦ: Cholera toxin phage; RS2: Repeat sequence 2.

XerD recombinases, *attP*(+) and *dif* site. Within this nucleoprotein complex, first the XerC recombinase catalyzes the cleavage of the phosphodiester bond between last base of its binding site and first base of overlap region and creates transient XerC-DNA covalent phosphotyrosyl linkages with its binding site and leave free 5' hydroxyl extremities on the overlap side. After cleavage, few bases from the 5' end of both overlap regions melt from their complementary strand and attack the XerC-DNA phosphotyrosyl bond of their recombining partner. For the formation of covalent phosphodiester bond between overlap region base and XerC binding site of *attP* and *dif,* stabilization of exchanged strand by Watson-Crick or wobble base-pairing interactions is essential[29]. Strand stabilization between exchanged strands determines the tropism of phage integration. Although both Xer proteins are essential for integration reaction, CTXΦ integration needs only the XerC catalytic activity for one pair of strand exchange and final integration[28]. As a consequence, *dif* compatibility of CTXΦ solely determines by the homology between the overlap region bases next to XerC binding sites. The pseudo-Holliday junction form after XerC mediated strand exchanges is probably resolved during host chromosome replication. Once integrated, *attP*(+) is again converted to *attP1* and *attP2*, none of these sequences are suitable for Xer-mediated reactions. This process makes CTXΦ integration irreversible. The only possibility to form the *attP*(+) hairpins from integrated phage genome is by cruciform, *i.e.*, two opposite hairpins could extrude through intra-strand complementary base pairing. This phenomenon is very rare and *in vivo* the cruciform structure is very unstable. *V. cholerae* cells carry several ssDNA nucleases, which could easily destabilize *attP*(+). Probably, for this reason CTXΦ excision is not detected under standard laboratory condition. Recently, CTXΦ excision from toxigenic *V. cholerae* isolates has been reported[30]. Although, the mechanisms of excision and detail genotype of the reported *V. cholerae* strain have not been investigated, it might be due to lack of some ssDNA nucleases, which stabilize the loop structure and form *attP*(+) for XerC-XerD mediated excision. Other possibilities might be some other IMGEs co-integrate with CTXΦ and provides functional XerC-XerD binding sites, hence clearance of CTXΦ.

CTXΦ VARIANTS: CONVENTIONAL *VS* CURRENT GROUPING

Conventionally, sequence of *rstR* gene is used for classification of CTXΦs. Based on the sequence similarity of *rstR*, CTXΦ has been grouped into four categories: CTXΦ^{ET}, CTXΦ^{Cl}, CTXΦ^{Cla} and CTXΦ^{Env}. Name of each of the phage classes has been chosen according to the host cells in which they were most frequently isolated. CTXΦ isolated from current pandemic isolates harbored a mosaic genome, few ORFs are identical to CTXΦ^{ET} and few are similar to CTXΦ^{Cl}. For example, CTX prophages present in the genome of current African (B33) and Asian (MJ-1236) isolates carry *ctxB* allele of CTXΦ^{Cl} type whereas *rstR* genes in the RS2 region are identical to those of CTXΦ^{ET} phage[31]. Nevertheless, recent Haitian *V. cholerae* isolates carries *ctxB* allele[32] neither identical to *ctxB*^{Cl} or *ctxB*^{ET}.

On the other side, CTXΦ can be classified into three broad classes based on their *dif* compatibility (Table 1). CTXΦ isolated from 6th pandemic *V. cholerae* isolates could integrate at both *dif1* and *dif2* sites. Bases immediate to XerC cleavage site of *attP*(+) could form Watson-Crick or Wobble base pair interaction with the similar bases of overlap region of both *dif1* and *dif2*[29]. In contrast, most well characterized CTXΦ from 7th pandemic El Tor cholera isolates could form such interaction only with the overlap region of *dif1* but not with *dif2*. Thus, the integration CTXΦ is specific for *dif1*. Recent *V. cholerae* isolates from Africa and India harbour CTXΦ in either chromosomes indicating their *attP* is similar to *attP* of CTXΦ isolated from 6th pandemic *V. cholerae*. In contrast, some environmental isolates carry *dif* sequenc-

Table 1 Cholera toxin phage variants and their dimer resolution sites sites specificities

CTXΦ variants	Isolated from	Chromosomal integration site	Ref.
CTXΦ^{d1}	O1 El Tor	*difA*, *dif1*	Das *et al*[29] Das *et al*[10]
CTXΦ$^{d1/2}$	O1 classical	*difA*, *dif1* and *dif2*	Das *et al*[29] Das *et al*[10]
CTXΦ^{dG}	Environmental	*difG*	Das *et al*[29] Das *et al*[10]

CTXΦ: Cholera toxin phage; *dif*: Dimer resolution sites.

es[33], which support integration of only specific type of phage, CTXΦ^{dG}. All these CTXΦ variants are different in terms of their integration specificity as well as integration efficiency. Highest level of integration efficiency was reported in between *dif1* and CTXΦ isolated from 7th pandemic El Tor cholera isolates while the lowest integration efficiency is detected in between *difG* and CTXΦ^{dG} isolated from environmental *V. cholerae*[29]. Our extensive work on the integration mechanisms revealed that integration specificity and efficiency of all CTX phages including Haitian and altered variants rely only on their *attP* sequence but not on the type of *ctxB* allele present in the core genome.

ECOLOGY OF *V. CHOLERAE DIF* SITES

Each *V. cholerae* genome carries two *dif* sites, one for each of the two circular chromosomes of the bacterium. Till now, four different *dif* alleles have been identified in *V. cholerae* strains, *difA*, *dif1*, *dif2* and *difG*[10]. *difA* and *dif1* are identical except two bases in the XerC binding site[10,13]. All *dif* sites consist of 28 bp DNA sequence, 11 bp binding sites for XerC and XerD separated by a 6 bp central region. *dif1*, *dif2* and *difG* have different bases in the overlap region at the borders of which XerC and XerD mediate strand exchanges during chromosome dimer resolution or IMGEs integration[10]. Metagenomic analysis of clinical and environmental *V. cholerae* isolates revealed *dif1* could be the integration site of several IMGEs, including phages (CTXΦ, VGJΦ, VEJΦ, VSK, fs2, f237, Vf33), satellite phages (RS1), plasmids (TLC) and small GIs[9]. Several *V. cholerae* cells have different arrangements of IMGE at *dif1* site, *e.g.*, single or multiple IMGE(s) in multiple combinations[9]. Almost all integrated IMGEs are flanked by *dif* like sequences. Several *in vivo* and *in vitro* experiments have been conducted to demonstrate the XerC and XerD mediated integration of CTXΦ, RS1, VGJΦ, and TLC[8,28,29,34]. Based on the sequence of *attP* site, IMGEs have been classified into three groups, CTXΦ-type, VGJΦ-type and TLC-type (Figure 3). Although, all of them rely on Xer recombinases for their integration, the integration mechanisms are very different (Figure 3). While the integration of CTXΦ-type IMGE rely on its folded single stranded genome, VGJΦ-type and TLC-type IMGEs use their replicative double stranded DNA for integration. Integration of CTXΦ-type IMGE are irreversible, other two could excise from the chromosomes. Both CTXΦ-type and VGJΦ-type IMGEs use only XerC catalytic activity but this is not clear for TLC. Bases in the overlap region of CTXΦ-type and VGJΦ-type *attP* have homology to *dif1* next to the XerC binding site only, but the bases of overlap region of TLC-type *attP* has homology next to both XerC and XerD binding sites. At this point it is not clear whether TLC uses catalytic activity of XerC or XerD or both. Since the XerD binding site of *attP* of TLC is very degenerative it is not clear whether any other accessory protein(s) is needed for its integration. Currently, Barre and his colleagues at CGM-CNRS, France are working on TLC to address all these unanswered issues.

CONCLUSION

Integration mechanism of CTXΦ and related elements like RS1, CUSΦ, YpfΦ are quite similar to the integration mechanism of integron cassette. In both the cases *attP* site is formed by intra-strand DNA base pairing which is recognized by tyrosine recombinases followed by catalysis of single pair of strand exchange with double stranded *attB*. The resulting pseudo-Holiday junction is resolved either by DNA replication or other DNA repairing system. Since integration of CTXΦ is irreversible, phage production from the integrated prophage genome relies on rolling circle replication. Virion production from integrated phage genome is detected only when multiple CTX prophage are present in tandem or CTX-prophages are flanked by pre-CTX or RS1 element. Recent metagenomic analysis revealed that several toxigenic *V. cholerae* isolates carry a single copy of the CTX prophage in the *dif* region. It is not clear whether these isolates are unable to produce virion or the special genotype of such toxigenic isolates could allow phage production due to excision of CTX prophage by forming cruciform structure under specific environmental conditions. Nevertheless, CTXΦ could replicate only in *V. cholerae* and few other vibrios but not in all species of *Vibrio* or other γ-*proteobacteria* like *Escherichia coli*. Further research is needed to determine what are the host factors involved in CTXΦ replication and what type of signal is recognised by the prophage to initiate rolling circle replication and virion production from toxigenic cholera pathogen. Clinical *V. cholerae* isolates carrying CTXΦ in the large chromosome also carry single or multiple copies of TLC element. Although, TLC is not essential for CTXΦ replication or integration, at least in the laboratory conditions, it is not clear why CTXΦ is always accompanied by the TLC element. More importantly, it is quite interesting to know the precise integration mechanism of TLC element, and whether the integration and excision of TLC could help CTXΦ dissemination in clinical and environmental *V. cholerae* strains. A recent study reported that TLC may promote CTXΦ integrations by contributing ideal attachment site (*dif1*) to the host chromosome[13]. Further work in this

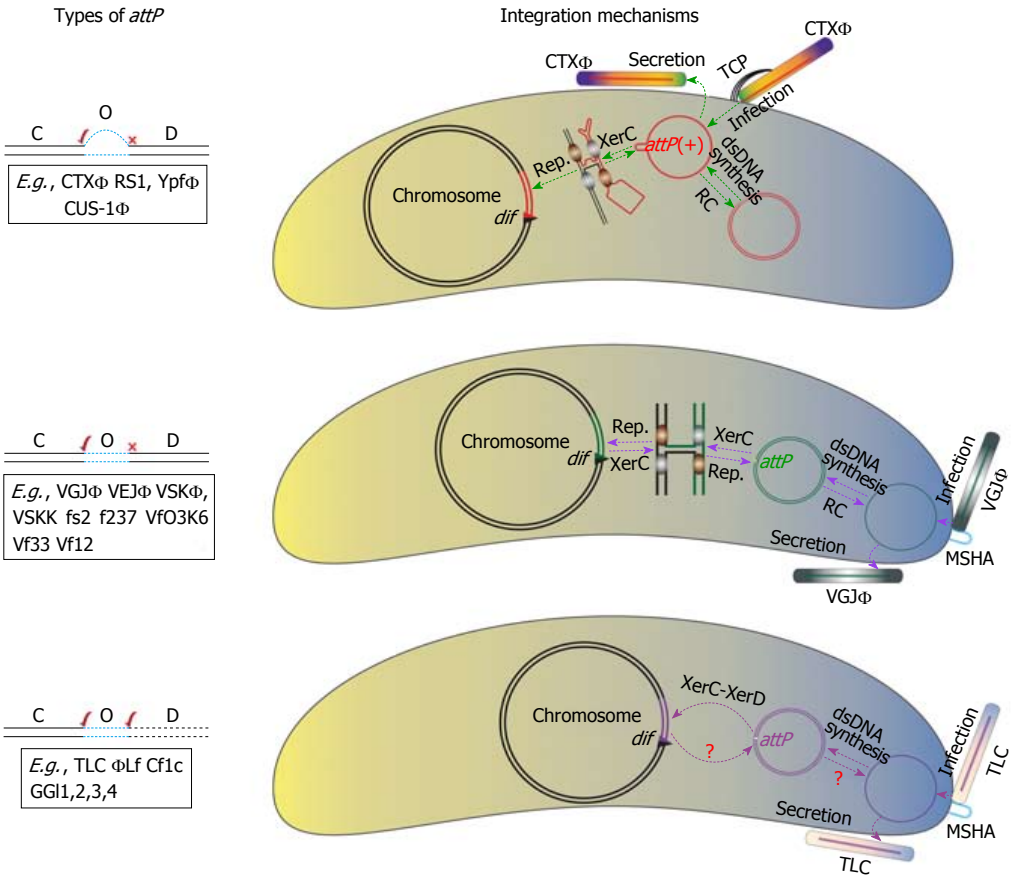

Figure 3 Schematic representation of the integration mechanisms of three classes of integrative mobile genetic elements present exclusively in the dimer resolution sites of bacterial genome. C-O-D denotes XerC-Overlap region-XerD. The XerC-XerD recombinases, attachment site of phage and bacterial genome are essential components of nucleoprotein complex. Compatibility of terminal bases *attP* with *attB* immediate after XerC or XerD cleavage is indicated by √. The nucleoprotein complex and the sequential strand exchanges are not yet characterized for TLC and related genetic elements. CTXΦ integration is irreversible. By contrast, VGJΦ and TLC integration is reversible. Key steps in the life cycle of CTXΦ, VGJΦ and TLC are also indicated. Host-encoded TCP and MSHA served as phage receptors for CTXΦ, VGJΦ and TLC, respectively. Generally, TLC exploit fs2 encoded protein for encapsulation of its ssDNA genome and produces virion. Virion recognizes specific receptor present on its host cell surfaces and delivers its single-stranded DNA genome into the host cytoplasm. Once inside the host cell, the ssDNA phage genome is either converted into a double-stranded replicative phage genome by host machineries or directly integrated into the host chromosome. TLC: Plasmids; CTXΦ: Cholera toxin phage; TCP: Toxin co-regulated pilus; MSHA: Mannose sensitive hemagglutinin A.

direction is warranted to understand the intricate biology of TLC and CTXΦ.

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