The TLCΦ satellite phage harbors a Xer recombination activation factor

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The circular chromosomes of bacteria can be concatenated into dimers by homologous recombination. Dimers are solved by the addition of a cross-over at a specific chromosomal site, dif, by 2 related tyrosine recombinases, XerC and XerD. Each enzyme catalyzes the exchange of a specific pair of strands. Some plasmids exploit the Xer machinery for concatamer resolution. Other mobile elements exploit it to integrate into the genome of their host. Chromosome dimer resolution is initiated by XerD. The reaction is under the control of a cell-division protein, FtsK, which activates XerD by a direct contact. Most mobile elements exploit FtsK-independent Xer recombination reactions initiated by XerC. The only notable exception is the toxin-linked cryptic satellite phage of Vibrio cholerae, TLCΦ, which integrates into and excises from the dif site of the primary chromosome of its host by a reaction initiated by XerD. However, the reaction remains independent of FtsK. Here, we show that TLCΦ carries a Xer recombination activation factor, XaFT. We demonstrate in vitro that XaFT activates XerD catalysis. Correspondingly, we found that XaFT specifically interacts with XerD. We further show that integrative mobile elements exploiting Xer (IMEXs) encoding a XaFT-like protein are widespread in gamma- and beta-proteobacteria, including human, animal, and plant pathogens.

Bacterial chromosomes are often circular. As a consequence, sister chromosomes can be concatenated into dimers by homologous recombination. Chromosome dimers physically impede the segregation of genetic information. They are separated at the time of cell division by a highly conserved chromosomally encoded site-specific recombination machinery, Xer (1). Xer is not essential but it allows maximal cell proliferation. In addition, Xer participates in the evolution of bacteria by horizontal gene transfer: Some plasmids rely on it for concatamer resolution; other mobile DNA elements exploit it to integrate into the genome of their host (1). Plasmids relying on Xer and integrative mobile elements exploiting Xer (IMEXs) participate in the acquisition of antibiotic resistance and pathogenicity genes (2–4). In particular, cholera toxin, the principal virulence factor of Vibrio cholerae, is encoded in the genome of an IMEX, the cholera toxin phage, CTXΦ (5–8). Nontoxic V. cholerae strains generally lack a suitable CTXΦ attachment site. In addition, CTXΦ integration is intrinsically irreversible. Nevertheless, new epidemic clones carrying potentially more potent toxins are constantly created by CTXΦ excision and reintegration cycles. Previous studies suggested that these cycles depended on another IMEX, TLCΦ, whose integration corrected the attachment site of nontoxic strains and whose excision promoted the joint elimination of CTXΦ copies. Our work brings molecular understanding to the role played by TLCΦ and suggests how similar IMEXs might participate in the evolution of other pathogenic bacteria.

Significance

Cholera toxin, the principal virulence factor of Vibrio cholerae, is encoded in the genome of an integrative mobile element exploiting Xer (IMEX), CTXΦ. Nontoxic strains generally lack a suitable CTXΦ attachment site. In addition, CTXΦ integration is intrinsically irreversible. Nevertheless, new epidemic clones carrying potentially more potent toxins are constantly created by CTXΦ excision and reintegration cycles. Previous studies suggested that these cycles depended on another IMEX, TLCΦ, whose integration corrected the attachment site of nontoxic strains and whose excision promoted the joint elimination of CTXΦ copies. Our work brings molecular understanding to the role played by TLCΦ and suggests how similar IMEXs might participate in the evolution of other pathogenic bacteria.

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See Commentary on page 18159.

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The Xer machinery generally consists of 2 related tyrosine recombinases, XerC and XerD (1). Each of them is in charge of the exchange of a specific pair of strands (1). A C and a D pathway of recombination can thus be defined depending on whether XerC or XerD catalyzes the formation of the HJ intermediate of the reaction. By default, XerD is inactive, whereas XerC can catalyze the formation and resolution of HJs (1). Chromosome dimer resolution follows the D pathway: A direct interaction with FtsK triggers the activity of XerD, leading to the formation of HJs that are resolved into product by XerC (26). On the contrary, XerD-dependent plasmids and CTXΦ exploit the default FtsK-independent pathway (8, 16, 35). Surprisingly, however, TLCΦ integration and excision reactions follow the D pathway (15).

Here, we show that TLCΦ encodes for a Xer recombination activation factor, XaT, which specifically interacts with XerD and promotes complete Xer recombination reactions by the D pathway. We further show that XaT acts independent of the sequence context of the recombination sites. Thus, XaT can promote both the integration and the excision of TLCΦ. Our results explain how TLCΦ contributes to the integration and excision cycles of CTXΦ. The discovery of XaT further permitted us to search for TLCΦ-like IMEsXs in the available databases, which revealed that they are widespread in gamma- and beta-proteobacteria, including human, animal, and plant pathogens.

**Results**

**TLCΦ Integration Depends on a 1-kbp Region Flanking Its Attachment Site.** Plasmid core recombination sites, IME attachment sites, and dif sites are composed of an 11-bp XerC-binding arm and an 11-bp XerD-binding arm, which are separated by a 6- to 8-bp overlap region at the borders of which strand exchanges occur (1). The region immediately flanking the XerD side of the attachment site of TLCΦ, attP, is devoid of ORFs, suggesting that it might contain an accessory sequence (Fig. L4). To test this possibility, we monitored the efficiency of integration of a series of truncations of a nonreplicative form of the phage genome, pTLC, in *V. cholerae*. In brief, rolling-circle replication of the phage genome was abolished by inactivating its HUH endonuclease, Cri. pTLC carries a conditional R6K origin of replication, RP4 origin of transfer, and chloramphenicol (Cm) resistance gene. Truncations were built and maintained in an *Escherichia coli* Π+ strain and delivered by conjugation to a Cm-sensitive *V. cholerae* reporter strain. Because the pTLC constructs are not replicative in *V. cholerae*, the appearance of Cm-resistant colonies of the reporter strain indicated integration. The reporter strain was equipped with a functional lacZ-dif1 in place of *dif* to verify the specificity of the integration events. Integration frequencies were normalized using the number of Cm-resistant colonies obtained with a replicative form of pTLC. Results indicated that the ~1-kbp region flanking the XerD arm of attP was not necessary for integration (Fig. 1 A, pTLC7 and 8). However, we found that an ~1-kbp region normally flanking the XerC arm of *attP* in the genome of TLCΦ was necessary and sufficient for integration (Fig. 1 A, pTLC4 and 5 and pTLC6 to 8, respectively).

**The Product of VC1465 Is Necessary and Sufficient for TLCΦ Integration.** The ~1-kbp region flanking the XerC arm of *attP* was unlikely to be an accessory sequence, because ~2 kbp of DNA separates it from *attP* in pTLC (Fig. 1A). However, it encompassed 2 complete ORFs, VC1465 and VC1466. Introduction of a stop codon by site-specific mutagenesis in VC1466 did not affect integration (Fig. 1 A, pTLC9), whereas introduction of a stop codon in VC1465 abolished it (Fig. 1 A, pTLC10). The importance of VC1465 for integration was confirmed in an N16961 derivative devoid of the colorimetric screen and of the IMEsXs (SI Appendix, Fig. S1). Ectopic production of the protein encoded by VC1465 from an arabinose promoter restored the integration of a construct harboring a stop codon in VC1465 (Fig. 1 B, pTLC10) and a construct lacking all of the other ORFs of TLCΦ (Fig. 1B, pTLC1). In addition, it permitted recombination between *attP* and *dif1* sites harbored on a plasmid in an FtsK+ *E. coli* strain producing the *V. cholerae* Xer recombinases in place of the *E. coli* recombinases (SI Appendix, Fig. S2). Taken together, these results suggested that TLCΦ encoded for its own Xer activation factor, XaT.

**XaT Promotes the Recombination of attP and dif1 In Vitro.** Most *Vibrio* species carry a *dif* site with the same sequence on their primary chromosome, *dif1* (Fig. 2A) (25). However, the ancestors of the strains at the origin of the present (sixth) and present (seventh) cholera pandemics and many of their nonoxigenic descendants are equipped with a variant, *dif1σ3*, which precludes the integration of CTXΦ (Fig. 2A) (14). In addition, most
toxigenic descendants of the present pandemic are equipped with a third variant, dif1GT (Fig. 2A) (8). We reconstituted the Xer recombination reaction leading to the integration of TLCφ using 34-bp synthetic double-stranded DNA (dsDNA) fragments containing any of these sites (Fig. 2B, S2), a 152-bp dsDNA fragment containing attP (Fig. 2B, S1), and purified Xaft and V. cholerae XerC and XerD peptides. The attP substrate was created by PCR using pTLC as a template. The dif1 substrates were assembled by annealing complementary oligonucleotides. The 5' and the 3' ends of the oligonucleotides corresponding to the strand normally processed by XerD were labeled with Cy3 and Cy5, respectively. Denaturing gel electrophoresis served to monitor the appearance of singly labeled DNA strands resulting from XerD catalysis (Fig. 2B, P1 and P2). Recombination was only observed when XerC, XerD, and Xaft were added to the reactions (Fig. 2B). Taken together, these results suggested that no other protein factor than Xaft was necessary to promote Xer recombination reactions between attP and the 3 most common V. cholerae dif1 variants.

**TLCφ Integration Follows the D Pathway.** Singly labeled dsDNA products with a size corresponding to the addition of a cross-over between attP and dif1 (Fig. 3A, S1, S2, P1, and P2 schematic) were observed on nondenaturing gels (Fig. 3A, Top). They were not detected when XerC or XerD was replaced by catalytically inactive mutants, suggesting that they resulted from the combined action of XerC and XerD (Fig. 3A, Top, KQ lanes). We also observed the apparition of a highly retarded product carrying both the Cy3 and Cy5 labels (Fig. 3A, Top). This product corresponds to an HJ between attP and dif1 (Fig. 3A, HJ schematic). The amount of HJs was much greater than the amount of cross-over products (Fig. 3A, Top). It remained very high when the catalytic activity of XerC was abolished, but no HJs could be detected when the catalytic activity of XerD was abolished (Fig. 3A, Top, KQ lanes). The very faint amount of attP/dif1 HJs created by XerC and XerD in the absence of Xaft probably corresponds to the default activity of XerC (Fig. 3A, Top). Taken together, these results suggested that Xaft promoted the formation of attP/dif1 HJs by XerD catalysis, which were resolved into product by XerC catalysis (Fig. 3A).

**Xaft Promotes Recombination between 2 dif1 Sites.** The XerD-binding arm of attP significantly differs from the canonical XerD-binding arm of dif sites (Fig. 2A). To check the possibility that it might contribute to the activation of XerD by Xaft, we analyzed the products of in vitro recombination reactions between a short, 34-bp Cy3- and Cy5-labeled dif1 substrate and a long, 152-bp dif1 substrate. The 152-bp substrate was produced by PCR using as a template pTLC/dif1, a pTLC plasmid in which attP had been replaced by dif1. XerC and XerD promoted the formation of dif1/dif1 HJs and cross-over products in the presence of Xaft (Fig. 3A, Bottom). No dif1/dif1 cross-over products were observed when the catalytic activity of XerC or XerD was suppressed by KQ mutations (Fig. 3A, Bottom). Inactivation of XerC catalysis barely affected the amount of dif1/dif1 HJs, but inactivation of XerD catalysis dramatically decreased it (Fig. 3A, Bottom). The low amount of HJs observed with the XerD-catalytic mutant or in the absence of Xaft probably results from the default basal XerC activity on dif1 sites (18, 36). Thus, the XerD-binding arm of attP does not contribute to the integration mechanism of TLCφ. On the contrary, the XerD-binding arm of attP seemed to be detrimental to recombination, since a higher amount of HJ intermediate and cross-over products was observed in the dif1/dif1 reactions than in the attP/dif1 reactions (Fig. 3A, Top and Bottom). This is probably explained by the poor binding of XerD to the XerD-binding arm of attP (15). Taken together, these results confirmed that Xaft acts as a XerD activation factor.

**No Flanking DNA Is Required for Xaft-Mediated Recombination.** Our in vivo integration assays suggested that no specific flanking DNA was required for Xaft-mediated recombination (Fig. 1A, pTLC7). Correspondingly, the dif1, dif1AT, or dif1GT sites of the short synthetic recombination substrates that we used for our in vitro recombination assays were only flanked by 3-bp GC clamps. However, the XerD and XerC sides of attP and dif1 in the 152-bp PCR substrates were flanked by 89 bp of theophage genome and 35 bp of the pTLC vector, respectively. To determine the minimal DNA requirements for the action of Xaft, we tested recombination between the short Cy3- and Cy5-labeled synthetic dif1 substrate and synthetic dif1 substrates with only 3 bp on the XerC side and 5 to 28 bp on the XerD side (Fig. 3B, S1 and S2 schematics). The expected Cy5-labeled recombination product has the same length as the Cy3- and Cy5-labeled substrate (Fig. 3B, S2 and P2 schematics). Nevertheless, we could detect its apparition using a sequencing gel because Cy3 and Cy5 labels retard the migration of DNA. The XerD cleavage strand of the Cy5-labeled 34-bp product migrated ∼5 nt below the doubly labeled XerD cleavage strand of the substrate (Fig. 3B, Right). The lengths of the XerD cleavage strand of the Cy5-labeled recombination products ranged from 36 to 59 nt (Fig. 3B, P1 schematic). The 5 longer ones migrated above the doubly labeled XerD cleavage strand of the substrate (Fig. 3B, Left). However, the shorter one migrated ∼1 nt below it (Fig. 3B, Left).
XafT Directly Interacts with XerD. As no accessory DNA was required for XafT-mediated Xer recombination, we suspected that XafT might directly interact with the recombinases. We tested this possibility using the yeast 2-hybrid assay. The XafT gene was cloned in-frame with the GAL4 activation domain (AD) in a vector carrying the LEU2 gene and introduced into a MATα his3-200 ade2-101 try1-901 leu2-3 gal4Δ gal80Δ yeast strain. The XafT, XerC, and XerD genes were cloned in-frame with the GAL4 DNA-binding domain (DBD) in a vector carrying the TRP1 gene and introduced into a MATα trpl-901 leu2-3 his3-200 Gal4Δ gal80ΔLYS2::GAL1 α-Gal1-TATA-His3 GAL2Δα-Gal2-TATAT-Ade2 yeast strain. The resulting strains were crossed and the diploids were selected on media lacking leucine and tryptophan. The diploids carrying the AD-XafT and DBD-XafT or DBD-XerD production vectors were white, indicating that the fusions permitted transcription of the ADE2 gene from the GAL2UAS promoter (Fig. 4A, Left). In addition, they could grow on a medium lacking adenine and histidine, indicating that they also restored transcription from the GAL1UAS promoter (Fig. 4A, Right). Taken together, these results suggested that XafT formed multimers that interacted with XerD. We next tested whether XafT could directly interact with XerD using an in vitro pull-down assay. We used as baits MBP fusions to XerC, XerD, and a protein from VGJ unrelated to tyrosine recombinases (Cont). The fusions were bound to magnetic beads covalently coupled with amyllose. Our purified XafT peptide slightly stuck to the amyllose beads itself (Fig. 4B). We recovered 3 times more XafT with MBP-XerD-coated beads (Fig. 4B). No significant enrichment in XafT was observed in the MBP-XerC and MBP-Cont controls (Fig. 4B). Taken together, these results suggested that XafT directly and specifically interacts with XerD.

XafT Homologs Are Found in the Genome of Many IMEs. There is no sequence homology between FtsK and XafT. XafT consists of an HTH domain from the XRE family of transcriptional regulators and a domain of unknown function, DUF3653. XerD activation was independent of DNA binding, suggesting that its action was due to DUF3653 (Fig. 3B). We recovered 179 different DUF3653-containing proteins in the NCBI, InterPro, and UniProt databases using a sensitive profile–profile search procedure (SI Appendix, Fig. S3). DUF3653-containing proteins are spread over the major orders of the γ- and β-proteobacteria, including many human, animal, and plant pathogens (SI Appendix, Fig. S4). Two hundred
XafT Promotes Recombination between Apparently Defective Sites. The XerD-binding arm of the attachment site of TLCΦ precludes the efficient binding of the recombinase (15). Nevertheless, XafT can promote its recombination with the 3 most common dif sites found on the primary chromosome of *V. cholerae*. It includes *dif*1, the apparently defective site of the primary chromosome of the ancestors of the sixth and seventh pandemics and most of their nontoxicogen derivaties (Fig. 2) (14). XafT permits the integration of TLCΦ into *dif*1, which corrects it into a site suitable for the integration of CTXΦ, *dif*1 (Fig. 5B). The subsequent integration of CTXΦ then replaces *dif*1 by *dif*1 (Fig. 5B).

**XafT Is Both an Integration and an Excision Factor.** The attachment site of CTXΦ consists of the stem of a hairpin formed by its ssDNA genome. Conversion to dsDNA masks the site in the integrated prophage, which ensures the directionality of the reaction: Xer can integrate the ssDNA genome of CTXΦ but cannot excise the integrated prophage. No such process can impose directionality on the reaction promoted by XafT, since the attachment site of TLCΦ is composed of ssDNA (Fig. 2). Xer-mediated plasmid dimer resolution requires the assembly of a nucleoprotein complex with a specific topology. It ensures directionality of the recombination reaction because the complex can only be formed if the resolution sites belong to the same DNA molecule. No such process can impose directionality on the reaction promoted by XafT, which directly interacts with XerD (Fig. 4) and activates it independent of the sequence context of the sites (Fig. 3). Thus, the Xer reaction promoted by XafT is intrinsically reversible. Correspondingly, we found that TLCΦ is able to excise from its host genome (15). However, excision was less efficient than integration (15). Future work will need to investigate the amount of XafT produced by XafT and integrated copies of TLCΦ and the possible role of the sequences surrounding *attP* and *dif*1 in the regulation of recombination.

**XafT Contributes to the Rapid Drift of the Cholera Toxin Region.** *V. cholerae* strains can be grouped into 12 distinct lineages, out of which only 1 gave rise to pandemic clones (9). The transition from the previous (sixth) to the current (seventh) pandemic was associated with a shift between 2 different phylectic subclades, the so-called classical and El Tor biotypes (9). The seventh pandemic isolates are rapidly drifting (9–13). In particular, cycles of excision and reintegration of CTXΦ promote the continuous apparition of clones producing new potentially more active forms of cholera toxin, which is a major clinical concern. The presence of an integrated copy of CTXΦ limits the possibilities for integration of new phage variants because it limits rolling-circle amplification of CTXΦ phages from the same incompatibility group (36–39). Thus, excision of previously integrated CTX copies is crucial for the spreading of new toxin variants. However, CTXΦ integration is intrinsically irreversible (7, 8). Infection by the RS1 satellite phage was found to favor CTXΦ excision (17). Homologous recombination between CTXΦ and a newly integrated copy of RS1, which contains ~3 kbp of homology with CTXΦ, could explain some of the excision events (17, 40). However, many others are accompanied by the joint elimination of TLCΦ and preexisting RS1 copies (14). Our discovery that

and fifty genome hits containing at least one homolog of a DUF3653 were obtained from 51 species in which *dif*1 had been annotated (Fig. 5A). Most of the hits were located in the immediate vicinity of *dif*1, suggesting that they belonged to IMEs (Fig. 5A). In particular, we found all of the lysogenic phages integrated at *dif*1 in Xanthomonas plant pathogens (6). Most of the hits were not associated with HTH domains (as seen from the architectures of DUF3653-containing proteins in PFAM), strengthening the proposition that DUF3653 but not the HTH domain is responsible for the activation of XerD in XafT.

**Discussion.**

**TLCΦ Harbors Its Own XerD Activation Factor, XafT.** Some mobile elements codify their own integrases, but others rely on recombinases from the host for integration. The Xer machinery is highly conserved and extremely versatile, representing an advantage for the mobile elements that can make use of it. Most mobile elements exploit FtsK-independent Xer recombination reactions initiated by XerC. The discovery of XafT, which interacts with XerD and promotes recombination by the D pathway, unveils a new paradigm for the exploitation of Xer recombination (Figs. 1, 2, 3, and 4). Chromosome dimer resolution is promoted by a direct contact between XerD and the extreme C-terminal domain of FtsK, FtsKY. However, FtsKY can only efficiently activate XerD when it is directed toward the complex by FtsK translocation or when it is fused to the recombinases, which limited the analysis of the molecular switch it operates in the reciprocal control exerted by XerC and XerD on their catalytic activities (27, 31, 32). The discovery of XafT, which can efficiently promote the recombination between *dif* sites without being fused to the recombinases, opens new possibilities for dissecting the mechanisms of activation of XerD (Fig. 3).
XaTf can promote recombination between attP and dif\textsubscript{GTS} providing an explanation for these events (Fig. 5B).

**TGCI-like IMEs Are Widespread.** We tracked the presence of XaTf-like homologs comprising the DUF3653 superfamily in available databases. The proportion of DUF3653 identified in close vicinity to a dif site is remarkable and strongly suggests that the function of DUF3653 in Xer recombination is common to most members of the superfamily (Fig. 5A). The details of the ORFs’ ID number, position of the dif site, and genome ID number can be accessed from the information provided in an interactive tree of life (SI Appendix, Fig. S5; https://itol.embl.de/tree/13216652179197671505892051) (41). The position and orientation of ORFs in DUF3653 supergroups with respect to dif consistently vary with the different DUF3653 subclasses, which could correspond to subclass-specific mechanistic constraints. Our findings will help monitor IMEs’ dynamics. For instance, multiple occurrences of DUF3653 could be detected next to dif in *Xanthomonas oryzae*, suggesting multiple integration events (SI Appendix, Fig. S5; GenBank accession no. CP011955). Generalization of the role of XaTf-like proteins may also help account for the integration of the Neissera gonococcal genetic islands (GGIs), which has long remained elusive (33). The GGIs are inserted between the dif site of their host chromosome and a dif-like site with a degenerate Xer-binding arm, which prevents FtsK-dependent Xer-mediated excision events (42, 43). Strikingly, 4 out of 8 bp of the XerD-binding arm of the dif-like site of one of the GGIs, dif\textsubscript{GGIs}, are identical to the bases found in the attP XerD arm (Fig. 5C). The GGIs do not carry a XaTf-like protein, but a XaTf-like protein encoded by another IMEX could have promoted their integration in *trans* in the same manner as production of XaTf from an ectopic vector can complement for the integration of pTLC1 (Fig. 1). Alternatively, the GGIs could harbor a Xer recombination activation factor from a superfamily different from DUF3653.

**Materials and Methods.** Relevant strains, plasmids, and oligonucleotides are described in SI Appendix, Tables S1–S3, respectively. In vivo integration assay in *V. cholerae*, plasmid recombination assays in *E. coli*, protein purification, in vitro recombination, and pull-down assays, and the sensitive profile–profile search procedure for DUF3653 sequences in available databases are detailed in SI Appendix.

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8. B. Das, J. Bisherour, M.-E. Val, F.-X. Barre, Molecular keys of the tropism of integration of the Neisseriales gonococcal genetic islands (GGIs), which has long remained elusive (33). The GGIs are inserted between the dif site of their host chromosome and a dif-like site with a degenerate Xer-binding arm, which prevents FtsK-dependent Xer-mediated excision events (42, 43). Strikingly, 4 out of 8 bp of the XerD-binding arm of the dif-like site of one of the GGIs, dif\textsubscript{GGIs}, are identical to the bases found in the attP XerD arm (Fig. 5C). The GGIs do not carry a XaTf-like protein, but a XaTf-like protein encoded by another IMEX could have promoted their integration in *trans* in the same manner as production of XaTf from an ectopic vector can complement for the integration of pTLC1 (Fig. 1). Alternatively, the GGIs could harbor a Xer recombination activation factor from a superfamily different from DUF3653.

**Materials and Methods.** Relevant strains, plasmids, and oligonucleotides are described in SI Appendix, Tables S1–S3, respectively. In vivo integration assay in *V. cholerae*, plasmid recombination assays in *E. coli*, protein purification, in vitro recombination, and pull-down assays, and the sensitive profile–profile search procedure for DUF3653 sequences in available databases are detailed in SI Appendix.

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