The Unconventional Xer Recombination Machinery of Streptococci/Lactococci

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Homologous recombination between circular sister chromosomes during DNA replication in bacteria can generate chromosome dimers that must be resolved into monomers prior to cell division. In *Escherichia coli*, dimer resolution is achieved by site-specific recombination, Xer recombination, involving two paralogous tyrosine recombinases, XerC and XerD, and a 28-bp recombination site (dif) located at the junction of the two replication arms. Xer recombination is tightly controlled by the septal protein FtsK. XerCD recombinases and FtsK are found on most sequenced eubacterial genomes, suggesting that the Xer recombination system as described in *E. coli* is highly conserved among prokaryotes. We show here that *Streptococci* and *Lactococci* carry an alternative Xer recombination machinery, organized in a single recombination module. This corresponds to an atypical 31-bp recombination site (difSL) associated with a dedicated tyrosine recombinase (XerS). In contrast to the *E. coli* Xer system, only a single recombinase is required to recombine difSL, suggesting a different mechanism in the recombination process. Despite this important difference, XerS can only perform efficient recombination when difSL sites are located on chromosome dimers. Moreover, the XerS/difSL recombination module can be considered as a landmark of the separation of *Streptococci/Lactococci* from other firmicutes and support the view that Xer recombination is a conserved cellular function in bacteria, but that can be achieved by functional analogs.

Introduction

Chromosome replication is a key function in living cells, and any factor that impedes progression of replication forks can result in mutagenesis and genome instability. Several strategies have evolved to rescue replication forks stalled by DNA damage. Most of these depend on homologous recombination pathways but are not necessarily accompanied by strand exchange [1]. However, in cases where replication fork repair does lead to sister chromatid exchange, bacteria with circular chromosome(s) are faced with a potential topological problem because an odd number of crossovers between sister chromatids generates chromosome dimer, which must be converted back to monomers for a correct segregation to daughter cells. In *E. coli*, chromosome dimer formation occurs in 15% of the cell population [2,3], and conversion to monomers is carried out by the Xer site-specific recombination system (for recent reviews see [4,5]). This is composed of two paralogous tyrosine recombinases (integrases), XerC and XerD, which cooperatively catalyze strand exchanges at a 28-bp DNA sequence, the dif site, which must be located at the junction of the two replichores to be functional [3,6–8]. Xer recombination is intimately coupled to cell division [9] through the septal protein FtsK [10–12], a DNA translocase [8] with an essential N-terminal transmembrane domain involved in its localization at the septum [13], and a C-terminal DNA motor domain involved in positioning and synapsing the two dif sites of the chromosome dimer at the division septum [12,14–19] as well as in activating the strand exchange [8] by direct interaction with XerD [20,21].

Homologs of XerCD recombinases and FtsK are found in most eubacterial phyla and some archael lineages [22] as well as the canonical dif site [23]. Moreover, interactions between the *E. coli* dif site and the XerCD recombinases of *Haemophilus influenzae* [24], *Pseudomonas aeruginosa* [25], *Bacillus subtilis* [26], *Proteus mirabilis* [27], and *Caulobacter crescentus* [28] have been experimentally demonstrated in vitro. These observations led to the general view that Xer recombination is a function conserved among bacteria harboring circular chromosome(s). However, regulation of strand exchange may differ, depending on the bacterial species: FtsK-mediated activation of Xer recombination in *H. influenzae* obeys the *E. coli* paradigm [21], whereas in *B. subtilis*, the model bacteria for firmicutes (formerly known as low GC-content Gram-positives bacteria), neither of the two FtsK analogs (SpoIIIIE and YtpT) appears able to drive Xer recombination [26]. Several attempts have...
been made to identify the Xer recombination machinery in Streptococci, a taxonomic group belonging to firmicutes and comprising major pathogens [29] as well as innocuous food-grade species of major industrial importance [30,31]. These studies revealed putative XerCD recombinases but failed to identify a dif site [32,33]. We show here, by comparative genomics and functional analyses in L. lactis, S. pneumoniae, and E. coli, that Streptococci possess alternative Xer recombination machinery phylogenetically unrelated to the classical XerCD/dif system. This machinery involves a single tyrosine recombinase (XerS) and an atypical dif site (dif_SL), both located on a single genetic module. We also show that, in contrast to B. subtilis, the streptococcal FtsK protein localizes at the division septum and controls the XerS/dif_SL recombination.

### Results

#### Identification of the Streptococcal dif Site by Comparative Genomics

Assuming that Xer recombination is highly conserved in eubacteria with a significant homology of the dif sites even between distantly related species [26], we performed an in silico search for putative dif sites in several completely sequenced firmicutes genomes. Candidate dif sites should: (i) have a significant similarity with that of B. subtilis (difBS), (ii) occur only once per genome, and (iii) be localized in the replication terminus (terC), defined as the chromosomal region located opposite the replication origin (oriC) where compositional strand biases switch sign [34,35]. Using these rules, a canonical dif site could be identified in all species analyzed except for Streptococci and Lactococci (Table 1). We therefore used an alternative three-step approach based on comparative genomics to identify the streptococcal/dif site (Figure 1). The terC region for three streptococcal genomes was localized (Figure 1A) using a cumulative GC skew diagram [34], and a comparison of the 10-kb region encompassing the GC skew shift was performed (Figure 1B).

This analysis revealed a 2-kb segment that showed significant similarity within the three species (>70% identity at the DNA level) and included a 356-amino-acid tyrosine-recombinase–encoding gene (annotated ymfD) on the L. lactis IL1403 genome [36] but hereafter renamed xerS) preceded by a ~50-bp highly (>90%) conserved sequence. When used to scan 49 genomes of other streptococcal species (Figure 1C), this ~50-bp fragment revealed a 31-bp consensus sequence (hereafter named difSL) with weak homology to the B. subtilis or E. coli dif sites, but with an overall structure resembling the DNA targets for tyrosine recombinases (i.e., two imperfect inverted repeats separated by a 6–8-bp central sequence). Comparative analysis of the genetic context in the 10-kb terC region of different streptococcal species revealed notable features strongly suggesting that streptococcal Xer recombination machinery is defined by one genetic module, corresponding to the difSL site followed by one of its dedicated recombinases (Figure 2). The physical link between difSL and xerS open reading frame (ORF) was found to be preserved among all Streptococci for which there is sequence data, and no genetic element other than difSL-xerS was conserved in the 10-kb terC region. In addition, the genes surrounding difSL-xerS did not show a preferred orientation that might indicate possible cotranscription with the xerS gene. Moreover, the xerS ORF often exhibits a putative p-independent transcription terminator at its end. These observations indicate that xerS is unlikely to be part of an operon and suggest that the difSL-xerS pair behaves as an individual genetic module.

#### In Vivo Characterization of the difSL Site

The candidate difSL site was tested for its ability to support site-specific recombination in L. lactis and S. pneumoniae by intermolecular recombination assays. A 37-bp synthetic sequence encompassing the putative 31-bp lacticoccal difSL site was cloned in a plasmid that does not replicate in firmicutes (pCL52, Table S1). The resulting construction (pCL235, Table S1) was used to transform a wild-type (WT) strain of L. lactis (MG1363, Table S1). In contrast to pCL52, which did not yield transformant, pCL235 produced transformants at an efficiency representing 1% of the efficiency attained with a replicative plasmid (unpublished data). This demonstrates that the putative difSL site was capable of rescuing pCL235, presumably by promoting integration of exogenous DNA into the lacticoccal chromosome. When transformed into a recA strain (VEL1122, Table S1), plasmid pCL235 was also rescued with the same efficiency as in the WT strain (unpublished data), indicating that plasmid integration occurred in a RecA-independent manner. Moreover, as judged by Pulsed-Field gel Electrophoresis analysis (Figure S1, lanes 2 and 4), pCL235 integrated into the chromosome of both strains at the location predicted for the native difSL site. Thus, the 37-bp sequence appeared to contain the DNA target of a site-specific recombination system. The difSL-mediated site-specific integration was also demonstrated to be a general process in Streptococci, since plasmid pCH9, a temperature sensitive replication (repKts) mutant containing either the lacticoccal 37-bp sequence described above (pCL231, Table S1) or its pneumococcal counterpart (pCL403, Table S1), integrated into the chromosomes of L. lactis and S. pneumoniae under nonpermissive conditions with comparable efficiencies (respectively 4.88 ×
Table 1. Localization of dif sites in some Firmicutes

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Sequence</th>
<th>Site Position*</th>
<th>GC Skew Shift Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CodV (XerC) Arm</td>
<td>Core</td>
<td>RipX (XerD) Arm</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>acttcctataaa</td>
<td>tatata</td>
<td>ttatgtaaact</td>
</tr>
<tr>
<td>B. anthracis Ames</td>
<td>actgcctataaa</td>
<td>tatata</td>
<td>ttatgtaaact</td>
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<td>actgcctataaa</td>
<td>tatata</td>
<td>ttatgtaaact</td>
</tr>
<tr>
<td>B. anthracis Sterne</td>
<td>actgcctataaa</td>
<td>tatata</td>
<td>ttatgtaaact</td>
</tr>
<tr>
<td>C. c. ATCC 10987</td>
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<td>tatata</td>
<td>ttatgtaaact</td>
</tr>
<tr>
<td>C. c. ATCC 14579</td>
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<td>ttatgtaaact</td>
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<td>ttatgtaaact</td>
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<td>ttatgtaaact</td>
</tr>
<tr>
<td>L. lactis IL1403</td>
<td>No homology</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Streptococcus (nine species)</td>
<td>No homology</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Variable nucleotides are underlined. Homology search was performed using the B. subtilis (difB) site as query against complete genomes available at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), parameters with "Megablast" designated as "off" and "Filter" as "none").

* indicates first bp of the dif site.

NA, not applicable.

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$10^{-2}$ [± 2.33 × 10$^{-2}$] cell$^{-1}$ and 2.67 × 10$^{-2}$ [± 1.55 × 10$^{-2}$] cell$^{-1}$). However, it should be mentioned that location of the insertion site of pCL403 has not been verified in S. pneumoniae.

The minimal size of the difSpl site was determined in L. lactis by scoring the integration efficiency of pGh9 containing variants of the difSpl site (Table 2). Reducing the length of the difSpl region from 48 to 31 bp did not alter the plasmid integration efficiency, indicating that the strongly conserved T located 13 bp away from the 31-bp consensus sequence (Figure 1C) was not part of the difSpl site. However, removing the two external bp from both sides of the 31-bp consensus sequence (Table 2, dif-8) led to a 100-fold decrease in integration efficiency, though this sequence was still proficient in site-specific recombination at the native difSpl site (Figure S1, lanes 3 and 5). Finally, deleting two nucleotides at either side of the 31-bp consensus sequence led to a 2-fold (Table 2, compare dif-7 to dif-5; Wilcoxon test, p < 0.003) or 4-fold (Table 2, compare dif-6 to dif-5; Wilcoxon test, p < 0.01) reduction in integration frequency. Together, these results led us to propose that the 31-bp consensus sequence defines the authentic difSpl site.

Recombination at difSpl Requires One Recombinase, XerS

Given that predictive analyses revealed XerS as the prime candidate for the actual Xer recombinase, a recombination assay was performed in S. pneumoniae to test if XerS was needed for recombination at difSpl. This streptococcal species was selected mainly for its facility to construct mutants compared to L. lactis. The recombination at the difSpl site was totally abolished in a xerS mutant (strain S501, Table S1; Materials and Methods), with undetectable integration of pCL403, demonstrating that XerS was one catalytic recombination of the XerS/difSpl system. To test whether XerS was the only recombinase involved in the Xer catalytic machinery, XerS/difSpl recombination was assayed in E. coli using an excision assay previously developed for the genetic analysis of the E. coli dif site activity [37]. Briefly, the native E. coli dif site was replaced by a cassette containing two directly repeated lactococcal difSpl sites flanking a kanamycin resistance (Km$^R$) gene (strain E359, Table S1), and the excision frequency (cell$^{-1}$ generation$^{-1}$; Materials and Methods) was determined by counting the number of Km$^R$ recombinants at different generations during serial cultures (Figure 3A). In absence of the lactococcal XerS recombinase, almost no recombination was observed (<0.006% cell$^{-1}$ generation$^{-1}$) indicating that XerCD of E. coli do not recombine difSpl. In contrast, introduction of a plasmid expressing the lactococcal xerS gene (pCL297, Table S1) increased the excision frequency to 10% cell$^{-1}$ generation$^{-1}$ (Figure 3A), a value close to the excision frequency observed in E. coli when using the native XerCD/dif system [3]. In addition, the excision frequency was not significantly altered in E. coli xerC or xerD mutant (Figure 3A). This indicates that fortuitous interaction between XerS and E. coli XerC or XerD recombinases is unlikely to account for recombination at difSpl sites. However, as recombination assay has not been performed on a xerC xerD double mutant, this cannot be totally ruled out. XerS also promoted

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intermolecular recombination between one lactococcal difSL site replacing the native E. coli dif site on the chromosome (strain E368, Table S1) and a second difSL site located on a nonreplicative plasmid (unpublished data). Together, these data demonstrated that XerS is the only streptococcal tyrosine recombinase required to catalyze intra- and intermolecular recombination between difSL sites.

A phylogenetic analysis of all tyrosine recombinases present in the sequenced genome of five streptococcal species revealed another integrase conserved among Streptococci. This atypical recombinase, more related to phages’ integrases (Figure S2) and previously identified as XerD in S. pneumoniae [33], lacks the extreme C-terminal region and two amino acids of the catalytic tetrad R-H-R-Y [38]. When tested alone...
Xer Recombination in Streptococci

Although the XerS/dif\textsubscript{SL} system involves only one recombination process, as do the CrecloX\textsubscript{P} and Fip/FRT systems, its location at the ter\textsubscript{C} region of streptococcal chromosomes strongly suggests that it functions to resolve chromosome dimers. To examine whether XerS/dif\textsubscript{SL} can substitute the XerCD/dif\textsubscript{SL} system in E. coli, we constructed FtsKSL (Figure 3B) previously developed to show that XerCD/dif\textsubscript{SL} can perform efficient recombination only when dif\textsubscript{SL} sites are located on chromosome dimers.

XerS/dif\textsubscript{SL} Recombination Depends on the Septal Protein FtsK

All streptococcal genomes sequenced so far contain one ORF encoding a protein homologous to the 787-amino-acid B. subtilis protein SpoIIIIE. These SpoIIIIE-like proteins (hereafter named FtsK\textsubscript{SL}) range from 758 (S. mutans) to 816 (S. agalactiae) amino acids in length and retain the structural signatures of proteins from the FtsK-HerA superfamily [40]: they contain a \(~180\)-amino-acid N-terminal region of weak similarity that includes four predicted transmembrane domains and a strongly conserved \(~500\)-amino-acid C-terminal region corresponding to the DNA translocase domain (unpublished data). The cellular localization of FtsK\textsubscript{SL} was determined in L. lactis using GFP fusions,

\begin{table}[ht]
\caption{Chromosomal Integration Frequencies of dif\textsubscript{SL} Variants in L. lactis}
\begin{tabular}{lll}
 Name (Plasmid\textsuperscript{a}) & Size (bp) & Sequence\textsuperscript{b} & Integration Frequency (Integration Frequency per Cell)\textsuperscript{c} \\
\hline
dif-1 (pCL247) & 48 & TAACATTTTC\textsuperscript{c}gAAAAAACGTTAAATTTTTTGGTCATTgATATCT & 2.86 \times 10^{-4} (\pm 7.61 \times 10^{-4}) \textsuperscript{d} \\
dif-5 (pCL251) & 31 & ATCTTTAC\textsuperscript{c}gAAAAAACGTTAAATTTTTTGGTCAT & 2.67 \times 10^{-4} (\pm 1.03 \times 10^{-4}) \\
dif-8 (pCL279) & 29 & CTTTTTAC\textsuperscript{c}gAAAAAACGTTAAATTTTTTGGTCAT & 5.12 \times 10^{-5} (\pm 2.58 \times 10^{-5}) \\
dif-7 (pCL281) & 28 & ATCTTTAC\textsuperscript{c}gAAAAAACGTTAAATTTTTTGGTCAT & 9.28 \times 10^{-6} (\pm 2.98 \times 10^{-6}) \\
dif-8 (pCL233) & 27 & CTTTTTAC\textsuperscript{c}gAAAAAACGTTAAATTTTTTGGTCAT & 2.34 \times 10^{-6} (\pm 1.45 \times 10^{-6}) \\
\end{tabular}
\textsuperscript{a}See Materials and Methods for detailed construction of the plasmids.
\textsuperscript{b}Conserved nucleotides among Streptococci are indicated as in Figure 1C.
\textsuperscript{c}See Materials and Methods. Plasmid pDH9 integration frequency was 2.61 \times 10^{-3} (9.53 \times 10^{-4}).
\textsuperscript{d}Standard deviation ($\sigma$).
\textsuperscript{e}doi:10.1371/journal.pgen.0030117.t002
\end{table}

in E. coli, YnbA (the lactococcal ortholog of S. pneumoniae XerD) showed no intra- or intermolecular recombination activity on dif\textsubscript{SL} and did not influence the recombination process when coexpressed with XerS. Moreover, it did not bind specifically to the lactococcal dif\textsubscript{SL} site in vitro (unpublished data). Therefore, YnbA is unlikely to belong to the streptococcal Xer system.

XerS/dif\textsubscript{SL} and Chromosome Dimer Resolution in E. coli

Although the XerS/dif\textsubscript{SL} system involves only one recombination process, as do the CrecloX\textsubscript{P} and Fip/FRT systems, its location at the ter\textsubscript{C} region of streptococcal chromosomes strongly suggests that it functions to resolve chromosome dimers. To examine whether XerS/dif\textsubscript{SL} can substitute the XerCD/dif\textsubscript{SL} system in E. coli, we used the growth competition assays (Figure 3B) previously developed to show that XerCD/dif\textsubscript{SL} resolved chromosome dimers in E. coli [3,39]. For that purpose, we constructed E. coli strains containing or not one lactococcal dif\textsubscript{SL} site replacing the native dif site. The strain harboring a complete streptococcal Xer system (E368, Table S1) showed a growth advantage of 10% generation\textsuperscript{−1} when competed with its isogenic strain missing the dif\textsubscript{SL} site (E367, Table S1). As found for XerCD in E. coli [37], this selective benefit matches the excision frequency of the Km\textsuperscript{R} cassette in the excision assay described above. In addition, strain E368 showed no growth defect compared to a strain harboring a functional XerCD/dif\textsubscript{SL} system (E375, Table S1). These results were correlated with cell morphology changes: strain E367 retained the filamentation phenotype of an E. coli strain defective in Xer recombination, while the strain harboring the complete XerS/dif\textsubscript{SL} system displayed a WT cell morphology (unpublished data). Thus, the XerS/dif\textsubscript{SL} system can substitute XerCD/dif in E. coli to resolve chromosome dimers.

Chromosome dimers in E. coli are mostly formed by homologous recombination [2]. As a recA mutation also drastically reduces the XerCD-mediated recombination at dif [2,37], this argued toward the fact that chromosome dimer is mandatory for creating the conditions necessary for a recombination between two directly repeated dif sites. Such dependence was investigated for the XerS system using the same E. coli excision assay [37]. The frequency of the Km\textsuperscript{R} cassette excision by the XerS system fell from 10% to less than 0.6% cell\textsuperscript{−1} generation\textsuperscript{−1} (Figure 3A) in a recA derivative of the E359 strain (strain E379, Table S1). As this 20-fold reduction of recombination efficiency was similar to that observed in E. coli [37], this strongly suggests that, as for the XerCD/dif\textsubscript{SL} system, XerS can perform efficient recombination only when dif\textsubscript{SL} sites are located on chromosome dimers.

Figure 2. Gene Context Analysis of the 10-kb terC Region of Different Streptococcal Species

Lla, L. lactis IL1403; Spn, S. pneumoniae R6; Spyo, S. pyogenes M1 GAS; Sagal, S. agalactiae NEM316; Sth, S. salivarius subsp. thermophilus CNRZ1066; Smut, S. mutans UA159. The ORF coding for the putative tyrosine recombinase is shown in black. Only putative \textsuperscript{R} genes are indicated.

Table 2. Chromosomal Integration Frequencies of dif\textsubscript{SL} Variants in L. lactis

See Materials and Methods. Plasmid pDH9 integration frequency was 2.61 \times 10^{-3} (9.53 \times 10^{-4}).

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corresponding to the GFP protein fused to the C-terminal of the full-length (FtsK\textsubscript{C1-C0GFP}) or N-terminal region (FtsK\textsubscript{C181-C0GFP}) of lactococcal FtsKSL. Both GFP fusions clearly localized at the septum of \textit{L. lactis} (Figure 4), indicating that as expected, FtsKSL localizes at the lactococcal division septum and its 181 amino acids containing the four transmembrane domains were sufficient to drive this localization.

The control of XerS-mediated recombination by FtsK was examined in \textit{S. pneumoniae} and \textit{E. coli}. For that purpose, strains expressing FtsK proteins deleted of their C-terminal part were constructed (\textit{ftsK\textsubscript{C}} mutants, only the first 405 amino acids and the first 316 amino acids of FtsK are synthesized in \textit{S. pneumoniae} and \textit{E. coli} respectively; Materials and Methods). Surprisingly, during the construction of the \textit{ftsKC} mutants in \textit{S. pneumoniae}, insertions of the Mariner minitransposon were obtained into the C-terminal or the N-terminal domain of FtsKSL. This suggests that neither the C- nor the N terminus is essential for the growth of this bacterium, though all pneumococcal \textit{ftsK} mutants were severely impaired in growth rate and cell viability as \textit{xerS} mutants (unpublished data). In \textit{S. pneumoniae}, XerS/dif\textsubscript{SL} recombination depended on the C-terminal part of FtsKSL, because integration of the repA\textsuperscript{ts} plasmid containing the pneumococcal dif\textsubscript{SL} site (pCL403), though not totally abolished as for the \textit{xerS} mutant, became severely impaired in the \textit{ftsKC} mutant (S502, Table S1), with an efficiency of $1.52 \times 10^{-3} (\pm 3.5 \times 10^{-4})$ cell\textsuperscript{-1} corresponding to less than 1% of the integration efficiency of the WT strain. Similar observations were made in the \textit{ftsKC} mutant of

**Figure 4.** Subcellular Localization of FtsKSL-GFP Proteins in \textit{L. lactis}
Phase-contrast (A) and fluorescence (B) microscopy of \textit{L. lactis} NZ900 overexpressing full-length lactococcal FtsK-GFP (upper) or N-ter Ftsk-GFP (lower) are presented. Cells were analyzed by microscopy on mid-exponential growth phase.
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E. coli (E372, Table S1), using the excision assay. The excision frequency of the difSL-Km cassette dropped from 10% in WT strain to 0.1% cell\(^{-1}\) generation\(^{-1}\) in the \(f_{\text{tsK}}\) strain (Figure 3A). This decrease was unambiguously associated to the lack of the C-terminal part of FtsK, since expression of the full-length E. coli \(f_{\text{tsK}}\) gene carried on a pBAD-derivative plasmid (pCL263, Table S1) restored Km\(^{\text{R}}\) cassette excision to nearly the WT frequency (Figure 3A). In addition, results from growth competition assays (Figure 3B) or cell morphology observations (unpublished data) also showed that the XerS system was unable to resolve chromosome dimers in E. coli in absence of the C-terminal domain of FtsK. Together our data demonstrated that XerS/difSL recombination in S. pneumoniae and E. coli, as well as dimer resolution in E. coli, depends on FtsK.

**Discussion**

In this work, we provide experimental evidence that Streptococci possess an unconventional Xer recombination machinery that requires only one tyrosine recombinase, XerS, to catalyze the site-specific recombination at a 31-bp sequence difSL. This raises an important question as to whether this system is orthologous to the “classical” E. coli XerCD system found in most bacterial species, including many other firmicutes. Not only does XerS differ significantly in length and primary sequence from members of the XerCD recombinases family (unpublished data), but difSL also differs in length and shows a weak similarity with the E. coli or B. subtilis dif sites (Figure 1C). Moreover, difSL is located immediately upstream the xerS coding sequence in all streptococcal and lactococcal species analyzed. Such genetic organization contrasts with that of classical XerCD systems, with the two recombinases encoded by genes located far from each other and distant from dif, and is more comparable to the integration modules of mobile elements such as integrons [41], bacteriophages such as P1 [42] or mycobacteriophage L5 [43], and some ICEs such as the cle element from *Pseudomonas* [44] or CTnDOT from *Bacteroides* [45]. As Streptococci and Lactococci (together defining the taxonomic family of *Streptococcus*-cosecaceae) represent a rather homogeneous phylogenetic group among firmicutes when compared to other genera such as *Clostridium* or *Lactobacillus* [46,47], we speculate that acquisition of the XerS system might have replaced the “classical” XerCD system at the time of or soon after their split from other firmicutes, with this event representing one landmark of this separation.

As demonstrated in this study, the cis-organization of the difSL-xerS module is not mandatory for efficient recombination, but this probably reveals a selective pressure to maintain that arrangement. Although at present the xerS transcription start point location is unknown, we speculate that difSL either lies between the xerS ORF and its promoter or is part of the xerS promoter. If this is true, this unusual arrangement might reflect a regulatory mechanism in which binding of XerS to difSL might autoregulate xerS expression. Alternatively, as it has been recently observed that some filamentous phages [48] or genetic islands [49] can divert the XerCD recombination system to integrate themselves at the chromosomal dif site of several bacteria, another hypothesis could be that the difSL-xerS arrangement might serve to prevent insertion of additional genetic material at difSL, because such event would separate the xerS ORF from its promoter and lead to inactivation of the chromosome dimer resolution system.

With only one catalytic recombinase involved in the recombination reaction, the XerS system is more similar to Cre/loxP and Flp/FRT than to XerCD/dif. However, XerS retains particular features that could indicate alternative mechanism in the recombination process. For instance, in vivo characterization of the difSL site in *L. lactis* revealed an asymmetry in its organization, with left and right arms differing in length (the left arm being 2-bp longer than the right one) as well as in nucleotide sequence (Table 2). This differs from loxP and FRT sites, which contain two perfectly symmetrical 13-bp arms surrounding the core region [50,51]. How a single recombinase can accommodate dissimilar binding sites to perform the DNA strand exchange reaction without accessory factor has to be analyzed, but we speculate that asymmetry of the difSL site might have a role in the control of this strand exchange order.

Though we did not provide direct experimental data demonstrating that XerS/difSL is involved in chromosome dimer resolution in Streptococci, several lines of evidence strongly suggest that dimer resolution is the primary task of this site-specific recombination system. First, classical XerCD recombinases and canonical dif site are not present in streptococcal/lactococcal genomes but substituted by the XerS/difSL recombination module at the chromosomal location predicted for a site-specific recombination system acting on chromosome dimer resolution. Second, to catalyze the strand exchange reaction XerS seems to require at least one of the two difSL sites located on the chromosome, because recombination between two difSL sites contained within a multicopy plasmid with theta replication (pSC101 derivative in *E. coli* and pAMB1 in *L. lactis*, unpublished data) could be detected neither in E. coli nor in *L. lactis*. At last, not only is XerS/difSL able to resolve chromosome dimers in E. coli as efficiently as the native XerCD/dif system (Figure 3B), but XerS/difSL recombination also hinged on formation of chromosome dimers, as revealed by the RecA-dependency of the Km\(^{\text{R}}\) cassette excision (Figure 3A), with the excision efficiency exactly matching the frequency of chromosome dimers resolution.

We also demonstrated that, in contrast to SpoIIIIE from *B. subtilis* that only infrequently (∼6%) concentrates at the vegetative septum [52] and is not involved in the Xer recombination [26], the streptococcal FtsK\(^{\text{L}}\) protein localizes at the division septum and still directs the XerS/difSL recombination, as dimer resolution and intra- or intermolecular recombination were drastically inhibited in *E. coli* and *S. pneumoniae* cells lacking the C-terminal part of FtsK. Although our preliminary analyses of the pneumococcal ftsK mutants need to be confirmed, the ability to obtain viable cells depleted of FtsK suggests that neither the N- nor the C-terminus of the protein is essential in Streptococci. As essentiality of FtsK seems to be species dependent, with only the N-terminal part in *E. coli* and the C terminus in *C. crescentus* being essential [53,54], we hypothesize that activity of FtsK, though still involved in cell division and DNA translocation, could slightly differ among the different bacteria. However, it appears that no correlation can be done between essentiality and localization of FtsK to the division septum. In *E. coli* and some other γ-proteobacteria, the C-terminal part of FtsK drives the XerCD recombination...
reaction in two ways: by participating to the formation of the recombination synapse through its DNA translocase activity [14–16] and activating the recombination reaction by direct interaction with XerD [8]. Some of our data strongly indicated that such interaction between FtsKSL and XerS is unnecessary to activate the XerS/difSL recombination in Streptococci, though this cannot be totally ruled out. First, the XerS-mediated intramolecular recombination frequency at difSL in E. coli (Figure 3A) was close to that measured with XerCD/dif [8], suggesting no specific specificity for FtsK requirement. This observation contrasts to that made in E. coli where the H. influenzae FtsK was inefficient in activation of the E. coli XerD and vice versa, implying that the FtsK-XerD interaction is highly species specific in these bacteria [21]. In addition, both pneumococcal and lactococcal XerS protein sequences do not contain the amino acids motif (RQ–QQ) found in E. coli XerD and involved in its specific interaction between with FtsK [29]. At last, the cassette excision by recombination at difSL in E. coli, as well as plasmid integration in S. pneumoniae, was not totally abolished in ftsKSL mutants but dropped to 1% of the recombination activity of WT strains, suggesting that some productive recombination synapses might form independently of FtsK, most probably by the random collision of two dif sites. This observation also contrasts the results obtained with the cassette excision assay performed with the E. coli XerCD/dif system, wherein no recombination was detected in an ftsK mutant [55], suggestive of the FtsK-mediated activation of the recombination. Our data are more easily accommodated to a model where XerS is unable to form a productive synapse and requires the DNA translocase activity of FtsKSL to bring the two difSL sites of a chromosome dimer close to each other and in an active geometry before performing the strand exchange. However, the recombination would not need direct activation by protein interaction between FtsKSL and XerS. However, as for the XerCD model [11], our model cannot provide satisfactory explanation to how FtsK is involved in the intermolecular recombination between one difSL site located on a suicide (or repA+) plasmid and the chromosomal difSL site, and the mechanism of the FtsKSL-mediated control has to be analyzed further.

In conclusion, the discovery of a Xer recombination system phylogenetically unrelated to the classical XerCD system reinforces the idea that chromosome dimer resolution can be viewed as a housekeeping function conserved among bacteria with circular chromosomes(s), but that some species can use functional analogs to perform this task. We expect that other bacterial species among those whose genome(s) are missing a canonical dif site also contain alternative chromosome dimer resolution systems. Finally, we note that the particularity of the XerS system makes it a valuable candidate for the development of new antibacterial drugs specifically directed against the pathogenic Streptococci.

Materials and Methods

Plasmids, bacterial strains, and growth conditions. The plasmids and bacterial strains used in this study are listed in Table S1. E. coli strains and plasmids containing synthetic lactococcal or pneumococcal variants of difSL sites were constructed using the procedure provided in Text S1. E. coli strains were grown at 30 °C in LB medium. Antibiotics were used at the following concentrations: erythromycin (Em) 150 μg ml⁻¹, chloramphenicol (Cm) 20 μg ml⁻¹, spectinomycin (Spc) 100 μg ml⁻¹, kanamycin (Km) 50 μg ml⁻¹, and ampicillin (Ap) 25 μg ml⁻¹. L. lactis strains were grown semi-aerobically at 30 °C in M17 broth (Merck KGaA, http://www.merck.de) supplemented with 0.5% (w/v) glucose (GM17) and transformed as previously described [56]. Antibiotics used for selection of lactococcal transformants were: Em 1 μg ml⁻¹, Cm 3 μg ml⁻¹, and Spc 200 μg ml⁻¹. S. pneumoniae strains were grown in Todd-Hewitt broth (DifcoBD Biosciences, http://www.biosciences.com) supplemented with 0.1% yeast extract (THY) and transformed using synthetic competence-stimulating peptide (CSP) as described [57]. Antibiotic concentrations used for selection of pneumococcal transformants were: Em 0.2 μg ml⁻¹ and Km 500 μg ml⁻¹.

DNA manipulation. Restriction and modification enzymes were purchased from New England Biolabs (http://www.neb.com) and used as recommended by the supplier. Plasmid DNA from E. coli was isolated with the Qiaprep spin kit (QIAGEN, http://www.qiagen.com). Chromosomal DNA from L. lactis, L. casei, and S. pneumoniae was isolated with the DNeasy tissue kit according to the manufacturer’s instructions (QIAGEN). Preparation of lactococcal genomic DNA embedded in agarose matrix, Pulsed-Field gel Electrophoresis, and Southern blot with dried agarose gels were performed as previously described [58]. Hybridization signals were detected with a Bioimaging BAS1000 analyzer system (FUJI Photo Film, http://www.fujifilm.com) and analyzed with TINA version 2.07c software (Raytest Isotopen Berger GMBH, http://www.raytest.de).

Genome sequences analyses. Nucleotide sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/Genome/static/euch_gene.html), Pseudoallescheria genomix-gpi-pfam.org), and Laboratoire de Biologie Moléculaire des Eucaryotes, (http://www.sanger.ac.uk/Projects/Microbes). Cumulative GC skew were obtained from an in-house build program (Laurent Lestrade, Laboratoire de Biologie Moléculaire des Eucaryotes, Toulouse, France). Multiple DNA comparison was performed using Multitau of the MultipipMaker program [59].

Intermolecular recombination assays in L. lactis and S. pneumoniae. Chromosomal integration assays of repA⁺ plasmid pGh9 or difSL- containing derivatives in L. lactis were performed according to [60]. For S. pneumoniae, Frozen strains containing the pg9 or derivatives were grown at 39 °C (water bath) to an OD₆₀₀ = 0.5 in THY without antibiotics. Appropriate dilutions were plated on medium [57] containing 2% of defibrinated horse blood (bioMérieux, http://www.biomerieux.com) and supplemented when appropriate with Em, and plates were incubated at 39 °C. Integration of the pg9 plasmid was undetectable in S. pneumoniae (no colonies were observed when plating 0.1 ml of the undiluted bacterial culture). The integration frequency per cell (ipc) was calculated as the geometric mean of the ratio of colonies on selective versus nonselective plates obtained from five to 19 independent experiments.

In vitro mariner mutagenesis in S. pneumoniae. Mutagenesis was carried out as described [61]. The target DNA for in vitro transposition of the Km⁺ mariner minitransposon pTr10 [61] were obtained by PCR reactions using R800 chromosomal DNA as template. The sizes of PCR fragments were: 2012 bp for xerS gene (primers: forward, 5'-TAAAGAACGCTTATTCCAGAATGATC-3'; reverse, 5'-GAGAAAGGAAGCAATTCTGACAAATC-3'), and 952 bp for ftsKSL gene (primers: forward, 5'-AAAAACAAAAGCTTCCTGGTGCCT-3'; reverse, 5'-CTTTGCGAACAAAGGGGA-3'). The mutagenized PCR fragments were then transformed into strain R800. For each mutagenesis, ten Km⁺ transformants were checked by PCR and shown to carry a mariner insertion. The accurate insertion position of the transposon, as well as its orientation, was determined by PCR and DNA sequencing as described [61]. The resulting chromosome structures of the selected mutants were: R800 xerS, insertion of mariner 167 bp downstream the ATG (insertion allows the synthesis of only the first 55 amino acids of XerS); R800 ftsKSL, insertion of mariner 1217 bp downstream the ATG (insertion allows the synthesis of only the first 405 amino acids of FtsK).

difSL-Km⁺-difSL cassette excision in E. coli. XerS-mediated excision of the difSL-Km⁺-difSL was performed according to [37]. Briefly, E. coli strains transformed with the XerS expression plasmid pCL297 (or cotransformed with pCL297 and pCL263) were grown in serial cultures at 30 °C (because of the thermosensitive replication of plasmid pCl297) for 60 generations in nonselective LB medium. The ratio of Km⁺/total colonies was measured each 20 generations, and excision frequencies (cell⁻¹ generation⁻¹) deduced according to Perales et al. [37]. For low excision frequencies (cell⁻¹ generation⁻¹), such as those obtained in the ATG region in repA or ftsKSL, single mutants and repA ftsKSL double mutants, 200 colonies picked from nonselective plates after 85 generations of serial growth were replica plated on Km-containing LB plates, the ratio of Km⁻
total colonies was measured, and used to estimate the excision frequencies per generation.

**Growth competition assays in E. coli.** Coculture experiments were performed as described by Perals et al. [3], except that the growth temperature was 30 °C (because of the thermosensitive replication of plasmid pCL235). The growth advantage per generation, corresponding to the chromosome loss resolution, was calculated from the slope of each competition curve as according to [3]. The strains containing one dif site located at the native position of the E. coli dif site correspond to Km* strains obtained from replica plating from nonselective plates in the dif*/Km* dif* excision experiments described above.

**Subcellular localization of FtsK-GFP fusions in L. lactis.** The laccotaxis ftsK gene was amplified by PCR from chromosomal DNA of L. lactis MG1363. The 575-bp PCR fragment (encoding the first 199 amino acids of FtsK; primers: forward, 5′-CATGCTATGCAAGTTGCTCGAAATG-3′; reverse 5′-CTGATTGATTTTGCTTGTGTAACAAAC-3′) and the 2303-bp PCR fragment (full-length ftsK gene; primers: forward, 5′-CATGGCATATGCAAGTTGCTCGAAATG-3′; reverse 5′-CTGATTGATTTTGCTTGTGTAACAAAC-3′) were cloned into the cloning sites of pFtsKNter and pFtsKFL yielding plasmids containing the gfpmut1 gene; primers: forward, 5′-gAAAAgTCC TggAA-3′.

**Physical Analysis of L. lactis Chromosome** and genome sequence, pulse time in Tris/borate/EDTA 0.05M). As predicted from the figures used Adobe Photoshop version 6.0. Technologies, http://www.axiontech.com), and assembly of the final Images were captured with an Axion Vision camera (Axion zeiss.com), and the fluorescent signal of GFP was detected using filter set 09 (excitation, 450–490 nm and emission, 520 nm) and long-pass interference filters, cells from an overnight culture were diluted (1:100) into fresh corresponding sites of pFtsKNter and pFtsKFL yielding plasmids containing the gfpmut1 gene; primers: forward, 5′-CATGCTATGCAAGTTGCTCGAAATG-3′; reverse 5′-CTGATTGATTTTGCTTGTGTAACAAAC-3′) and the 2303-bp PCR fragment (full-length ftsK gene; primers: forward, 5′-CATGGCATATGCAAGTTGCTCGAAATG-3′; reverse 5′-CTGATTGATTTTGCTTGTGTAACAAAC-3′) were cleaved with NotI (bold) and EcoRV (underlined) and cloned into corresponding sites of pNG08148 [62], generating respectively pFtsKNter and pFtsKFL. The Clal-XhoI fragment containing the gfpmut1 gene of pGS1154 [63] was cloned into the corresponding sites of pFtsKNter and pFtsKFL yielding plasmids pKNOpt and pKNOptFL, respectively. For fluorescence microscopy, cells from an overnight culture were diluted (1:100) into fresh medium and grown at 30 °C with agitation. At OD600 = 0.5, supernatant of the nisin-producing strain NZ9700 [64] was added at dilution 1:1,000. At OD600 = 1, 2 μl of culture was directly applied to a polystyrene microscope slide (Omnilabo, http://www.omnilabo.nl) and covered with a cover glass. Cells were examined using a 100× oil immersion objective on a Zeiss microscope (Carl Zeiss, http://www.zeiss.com), and the fluorescent signal of GFP was detected using filter set 09 (excitation, 450–490 nm and emission, >520 nm) from Zeiss. Images were captured with an Axion Vision camera (Axion Technologies, http://www.axiontech.com), and assembly of the final figures used Adobe Photoshop version 6.0.

**Supporting Information**

**Figure S1.** Physical Analysis of difS5-Mediated Integration Site in L. lactis Chromosome

Shown is Pulsed-Field gel Electrophoresis of SmaI-digested chromosomal DNA (A) and corresponding Southern hybridization (B) of WT strain (MG1363) and its recA-derivative (VEL1122) after dif-mediated integration of plasmids pCL235 or pCL237. Lanes: 1, MG1363; 2, MG1363:pCL235; 3, MG1363:pCL237; 4, VEL1122:pCL235; and 5, VEL1122:pCL237. (Electrophoresis conditions: 10 V/cm for 21 h at 14°C).

**References**

DNA motifs that control E. coli chromosome segregation by orienting the FtsK translocase. EMBO J 24: 3770–3780.


