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SSB recruitment of Exonuclease I aborts template-switching in *Escherichia coli*

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Abstract

Misalignment of a nascent strand and the use of an alternative template during DNA replication, a process termed “template-switching”, can give rise to frequent mutations and genetic rearrangements. Mutational hotspots are frequently found associated with imperfect inverted repeats (“quasipalindromes” or “QPs”) in many organisms, including bacteriophage, bacteria, yeast and mammals. Evidence suggests that QPs mutate by a replication template-switch whereby one copy of the inverted repeat templates synthesis of the other. To study quasipalindrome-associated mutagenesis (“QPM”) more systematically, we have engineered mutational reporters in the *lacZ* gene of *Escherichia coli*, that revert to Lac⁺ specifically by QPM. We and others have shown that QPM is more efficient during replication of the leading strand than it is on the lagging strand. We have previously shown that QPM is elevated and that the leading-strand bias is lost in mutants lacking the major 3′ ssDNA exonucleases, ExoI and ExoVII. This suggests that one or both of these exonucleases more efficiently abort template-switches on the lagging strand. Here, we show that ExoI is primarily responsible for this bias and that its ability to be recruited by single-strand DNA binding protein plays a critical role in QPM avoidance and strand bias. In addition to these stand-alone exonucleases, loss of the 3′ proofreading exonuclease activity of the replicative DNA polymerase III also greatly elevates QPM. This may be because template-switching is initiated by base misincorporation, leading to polymerase dissociation and subsequent nascent strand misalignment; alternatively or additionally, the proofreading exonuclease may scavenge displaced 3′ DNA that would otherwise be free to misalign.

Keywords

quasipalindrome; mutagenesis; DNA replication; nuclease

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INTRODUCTION

Ensuring genetic stability is vital to the survival of all organisms and cells employ conserved mechanisms to avoid mutations that might arise during DNA replication. This includes proofreading activities associated with DNA polymerases and a post-replication mismatch repair system. Even so, mutational hotspots, sites of frequent spontaneous mutation, are observed in within certain genes. One type of mutation hotspot arises in imperfect inverted repeat sequences or “quasipalindromes” (“QPs”) where the observed mutation produces a more perfect palindrome. Examples of QP-associated mutagenesis (“QPM”) are seen in the mutational spectra of the *tpsL* [1–3] and *thyA* [4] genes of *E. coli* and have been documented in bacteriophage [5], yeast [6] and humans [7, 8]. Genetic evidence suggests a template-switch mechanism for QPM [2, 9, 10], where one arm of the inverted repeat acts as a template for the other, either by formation of an intramolecular hairpin structure on the nascent strand or by misalignment of the nascent strand across the fork to the alternative parental strand (Fig. 1). QPM is elevated by problems during DNA replication [1, 11], caused either by replication inhibitors or by mutations that impair the replication machinery. In the yeast *Saccharomyces cerevisiae*, QPM is associated with double-strand break repair and in loci undergoing high levels of transcription [12, 13]. Despite the prevalence of QPM, mechanisms for avoidance of this class of mutation are not fully understood. In *E. coli*, the ssDNA-exonucleases exonuclease I (ExoI) and exonuclease VII (ExoVII) play a partially redundant role in avoidance of QPM [10, 14, 15] and we have proposed that both enzymes scavenge 3′ single-strand DNA (ssDNA) in the cell before it has an opportunity to misalign with an alternative template and promote templated mutagenesis.

In previous work, we developed a pair of *lacZ* alleles in *E. coli* that specifically revert to Lac⁺ by a template-switch event (Fig. 2), one during leading strand synthesis and the other during lagging strand synthesis [15]. Such mutational reporters facilitate the systematic examination of factors that influence mutagenesis. Using these we examine here the roles of ExoI and ExoVII, as well as the proofreading exonuclease activity of DNA polymerase III (DnaQ), in the avoidance of QPM.

MATERIALS AND METHODS

Growth media

Strains were grown routinely in LB broth [16]. Minimal medium and dilution buffer consisted of 56/2 salts [17], with lactose added to 0.2% for Lac reversion assays. To facilitate counting of colonies, X-gal (40 mg/ml) and IPTG (0.1 mM) were added to plate minimum medium.

Strain construction

All strains (Table 1) were constructed in the MG1655 background by P1 *virA* transduction [18] and carried one of two QPM *lacZ* reporter alleles previously described [15]. Selections for tetracycline resistance employed 30 µg/ml tetracycline in LB; those for kanamycin resistance used kanamycin at 30 µg/ml. Strains deficient in exonuclease I and/or exonuclease VII were constructed using knockout mutations from the Mori collection [19]. The *xonA*

gene and its natural promoter were amplified by PCR using primers 5' GGGGac aagtt tgtac aaaaa agcag gcfTC CAGCA AACCC TCAGG AGTTT C and 5' GGGGa ccact ttgta caaga aagct gggtC TTAGA CAATC TCTTC CGCGT ACT and inserted first into plasmid pDONR221 (ThermoFisher Scientific) and then into Tn7 vector pRG37 [20] by GATEWAY [21] cloning. Alleles *xonA* R148A and A183V were introduced into the pDONR221 clone by site-directed mutagenesis using procedures provided by the vendor (QuikChange II, Agilent). Plasmid DNA was introduced into *xonA xseA* strains by electroporation [22] and integrants were identified by PCR of chromosomal DNA using primers 5' GATGC TGGTG GCGAA GCTGT and 5' GATGC TGGTG GCGAA GCTGT flanking the *attTn7* site. This integration of the *xonA* gene and its promoter into the chromosome avoids the toxicity associated with expression of *xonA* from plasmids.

Mutation rate determination

Mutation rates were determined by a fluctuation analysis assay as previously described [15]. Mutation rates and 95% confidence interval were calculated using the Ma-Sandri-Sarkar Maximum Likelihood method [23] as described in Rosche and Foster [24], using the FALCOR web resource [25]. To confirm the QP mutation specificity of each reporter, the *lacZ* gene from ten revertants from each of the strains was recovered by PCR (GoTaq Green Master Mix; Promega) and subjected to DNA sequence analysis (Genewiz).

RESULTS AND DISCUSSION

We have previously constructed *E. coli lacZ* strains that revert to Lac⁺ by a template-switch reaction in a 18 bp quasipalindrome sequence (Fig. 2); “QP5” reports a template-switch on the leading strand whereas “QP6” reports QPM on the lagging strand [15]. Using these, we confirmed that QP mutations are recovered more efficiently on the leading strand, as documented previously [2, 3, 15, 26, 27], with a higher reversion rate of QP5 than QP6 (Fig. 3A). Inactivation of both ExoI and ExoVII greatly elevates mutation rates, consistent with our earlier observations with a QPM hotspot in *thyA* and with the *lacZ* reporters [10, 15]. Of the two exonucleases, ExoI appears to be the major contributor to mutation avoidance, as a single *xonA* mutation elevated mutation rates for QP5 and QP6 8- and 25-fold, respectively, whereas knockout of ExoVII (*xseA*) had a lesser effect, elevating QPM assayed with QP5 and QP6, 2- and 6 -fold, respectively. In the *xonA* mutant, the strand bias of QPM was lost relative to that of wild-type strains, with lagging strand QPM recovered at slightly higher rates (QP5 reversion 3.2×10^{-7} ; QP6 reversion 4.1×10^{-7}). Inactivation of both ExoI and ExoVII is additive or synergistic in elevating mutation rates, 10-fold and 46-fold for QP5 and QP6, respectively, yielding QP5 reversion at 3.8×10^{-7} and QP6 reversion at 7.4×10^{-7} . This indicates that both enzymes independently contribute to QPM avoidance, especially on the lagging strand, with ExoI playing the larger role.

A possible explanation for the strand bias of QPM is that the lagging strand recruits and stimulates ExoI through its interaction with SSB, expected to be more prevalent on the lagging strand template of the replication fork. To investigate this, we performed complementation tests by introducing various *xonA* alleles, expressed from the natural *xonA* promoter, into the Tn7 chromosomal attachment site [20] in a *xonA xseA* genetic

background. Keck and colleagues have previously shown that a R128A mutation in ExoI abolishes its interaction with SSB, while not affecting its exonuclease activity [28]; an A183V mutation (also known as “*sbcB15*”) eliminates exonuclease activity while retaining DNA binding [29]. Introduction of the wild-type *xonA* allele suppressed QPM 6–7 fold, as detected with both QP5 and QP6 reporters, relative to strains lacking *xonA* completely (Fig. 3B). In contrast, *xonA*-A183V failed to complement; introduction of *xonA*-A183V had no significant effect on mutation rates relative to *xonA xseA* strains and yielded QPM rates 5–6 fold higher than those carrying the *xonA*⁺ allele. The R128A allele of ExoI had partial effects; it failed to lower rates of mutation fully to wild-type levels, which was especially evident with the lagging strand QPM reporter QP6. Relative to strains expressing *xonA*⁺, the *xonA* R128A-carrying strain exhibited QPM rates 2-fold higher for the leading strand reporter and 4-fold higher for the lagging strand reporter. This indicates that SSB interaction is required for full avoidance of QPM, especially on the lagging strand. Although it is known that SSB stimulates ExoI activity in vitro [28], this provides the first evidence that ExoI’s interaction with SSB is important for its function in vivo. That SSB interaction was required, in part, for QPM avoidance on the leading strand was somewhat surprising. However, if QPM is sometimes associated with replication stall events that generate tracts of ssDNA gaps, SSB-ExoI recruitment may partially contribute to avoidance of QPM, even on the leading strand.

Both ExoI and ExoVII are strongly specific for ssDNA (reviewed in [30]) and may therefore function in the cell to scavenge unwound 3’ strands from the replication fork, a likely intermediate during the template-switch events. ExoI is in the DnaQ family of exonucleases, some of which are associated with DNA polymerases as “proofreading” functions. We asked whether DnaQ itself, the epsilon subunit of DNA polymerase III, plays a role in the avoidance of template-switching by introducing the *dnaQ*T151 allele (“*mutD5*”, [31]) into our QPM reporter strains. With both QP5 and QP6 reporter strains we observed a large stimulation, 27- and 100-fold, respectively, of template-switch mutagenesis by this *mutD5* mutation (Fig. 4). Interestingly, the leading vs. lagging strand bias is lost or reversed by *mutD5* as well. The loss of strand bias may be because the rate of template-switching is so high in *mutD5* strains as to saturate the capacity of ExoI/ExoVII to abort the events. The *mutD5* allele causes a large increase in rates of mutations of all types, both directly by the failure to correct misincorporation errors by Pol III and indirectly by the saturation of the mismatch repair system [32]. Because the QPM detected with our reporters is not subject to mismatch repair avoidance [15], the effect of *mutD5* must be direct via effects on DNA Pol III. We note that in the switched configuration, the templated mutation is not expected to be a misincorporated base. Lack of proofreading reduces the processivity of polymerization by DNA Pol III [33], which might provide increased opportunity for the nascent strand to isomerize and pair with itself, a critical intermediate in the template-switch reaction. Alternatively, the DnaQ proofreading exonuclease may serve to scavenge ssDNA, aborting QPM much in the same way that we have proposed for the stand-alone exonucleases ExoI and ExoVII. The strong effects of *mutD5* also suggests that DNA polymerase III is the enzyme that is engaged prior to the template-switch polymerization reactions.

Conclusions

These results suggest that, in the absence of exonuclease surveillance, template-switch reactions at imperfect palindromic repeats occur on the lagging strand at a equal or somewhat higher frequency than on the leading strand. Because of the discontinuous nature of lagging strand synthesis, polymerase dissociation, a necessary prerequisite for template-switching, may be more frequent and, because of its greater single-stranded character, secondary structures are more likely to form. However, SSB recruitment and stimulation of Exonuclease I to the lagging strand limits this mutagenic potential, such that template-switch generated mutations are more frequently recovered from the leading strand in wild-type strains. Exonuclease VII, an exonuclease widespread among bacterial phyla, plays a more minor role in avoidance of template-switch mutations in *E. coli* than does Exonuclease I, which is found primarily in the Enterobacteriaceae. In those bacteria that lack Exonuclease I, Exonuclease VII or another unknown exonucleases may play a larger role. What enzymes may function in eukaryote cells to abort QPM is not known.

We propose that 3' exonucleases play an important role in mutation avoidance by their ability to scavenge displaced 3' ssDNA ends that are unwound from the replication fork. Exonuclease digestion aborts mispairing events that could lead to quasipalindrome-associated mutational hotspots as reported here, as it limits genetic rearrangements at tandem direct repeats, as shown previously [34]. In addition, the DnaQ-encoded, proofreading subunit of DNA polymerase III plays an important role in the avoidance of template-switch reactions, even though the templated mutations are not mispaired during their synthesis from the alternative template. DnaQ may, like ExoI and ExoVII, scavenge displaced ssDNA from the fork or it may promote processive DNA synthesis, thereby limiting Polymerase III dissociation and potential for template-switching.

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Abbreviations

DNA Pol III	DNA polymerase III
ExoI	exonuclease I
ExoVII	exonuclease VII
QP	quasipalindrome
QPM	quasipalindrome-associated mutagenesis
ssDNA	single-strand DNA
SSB	single-strand DNA binding protein

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HIGHLIGHTS

- Exonuclease I plays a major role in avoidance of template-switch mutations in *E. coli*
- In the absence of ExoI, bias of template-switching to the leading strand is lost.
- ExoI that cannot interact with SSB increases mutations and negates strand bias.
- Recruitment of ExoI by SSB aborts mutagenesis preferentially on the lagging strand.
- Loss of proofreading by DNA polymerase III increases template-switching.

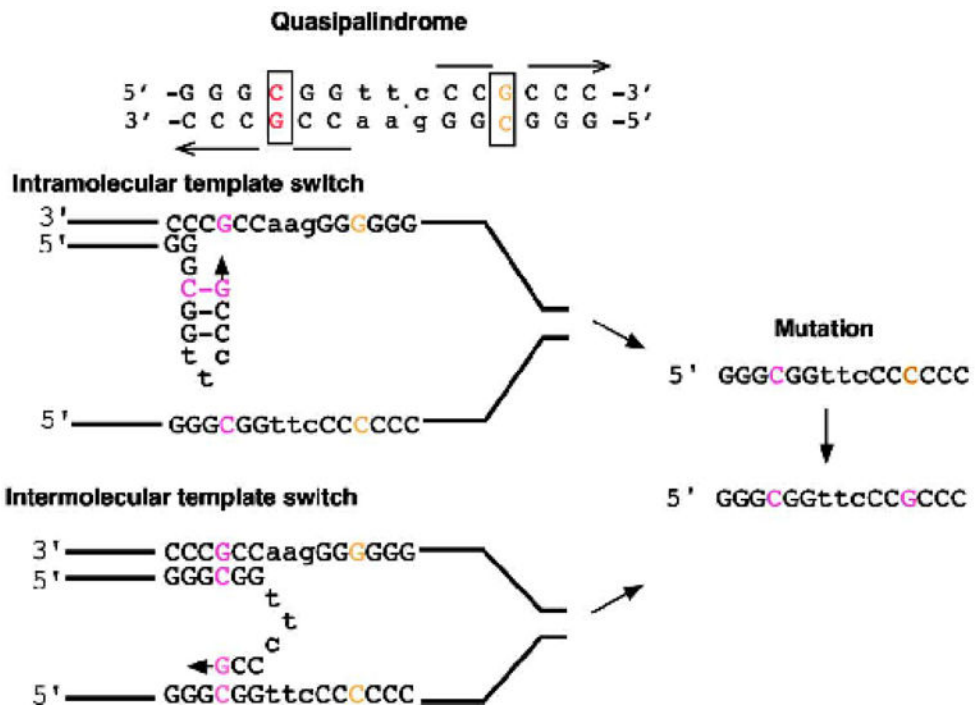


Figure 1.
QP template-switch mechanisms

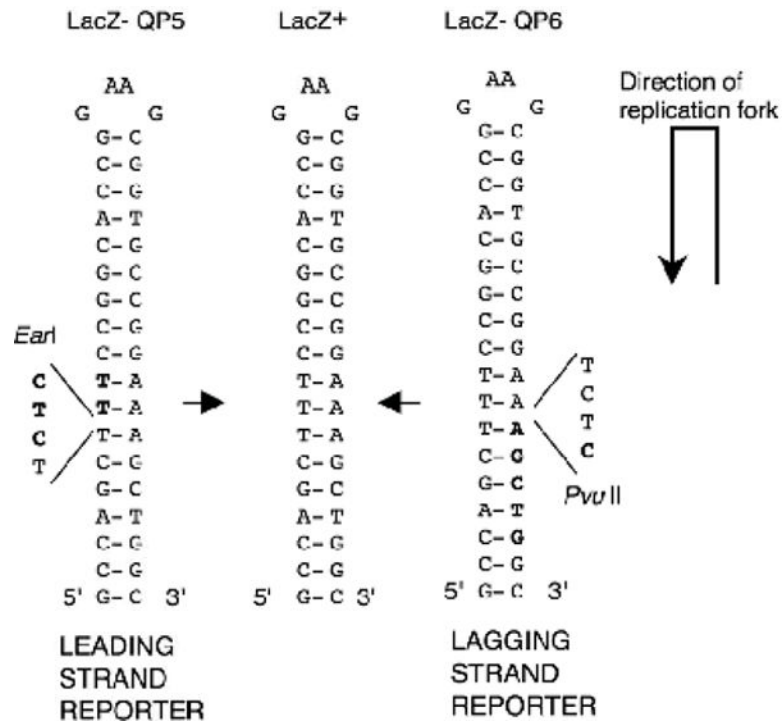


Figure 2.
QPM reporters in *lacZ*

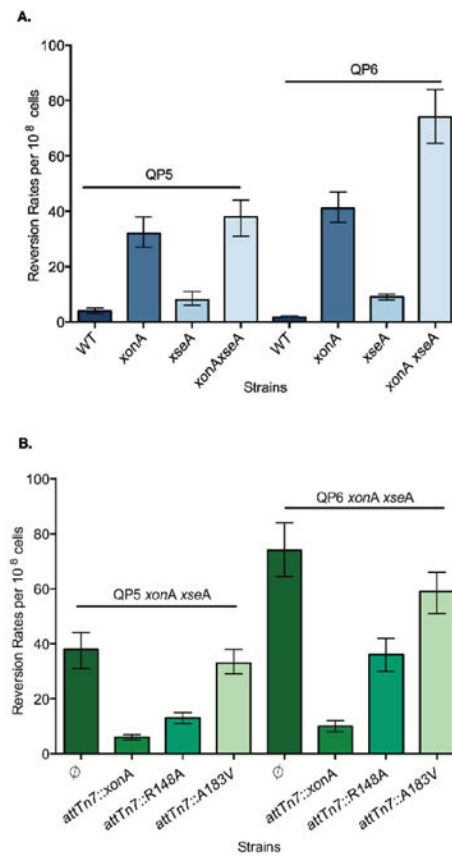


Figure 3.

Reversion rate of *lacZQP* alleles. Reversion rates of QP5 (leading strand reporter) and QP6 (lagging strand reporter) were measured in various strains. Data represents rate per 10^8 cells from 10 trials performed in three different days. Error bars represent 95% confidence interval calculated using FALCOR (A.) In exonuclease mutant strains. In wild-type QP5 reversion rate was 4.0×10^{-8} ; reversion of QP6 was 1.6×10^{-8} (B.) In exonuclease I and VII-deficient strains after integration of exonuclease I alleles.

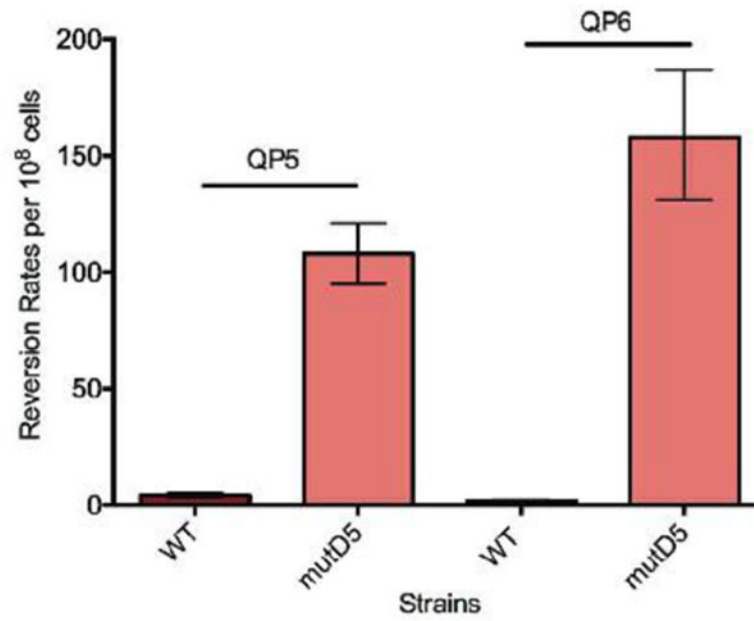


Figure 4. Reversion rates per 10⁸ cells measured in *mutD5* strains containing QP5 (leading strand) and QP6 (lagging strand) reporters. Data represents rate per 10⁸ cells from 10 trials performed in three different days. Error bars represent 95% confidence interval calculated using FALCOR.

TABLE 1

Escherichia coli K-12 strains used in this study

Strain number	Genotype	Origin of Reference
STL14776	<i>lacZ</i> (QP5) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10	Seier (2011)
STL15654	<i>lacZ</i> (QP6) <i>mhpC</i> -281::Tn10	Seier (2011)
STL15823	<i>lacZ</i> (QP6) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10	Seier (2011)
STL17685	<i>lacZ</i> (QP5) <i>mhpC</i> -281::Tn10	This work
STL18460	<i>lacZ</i> (QP5) <i>xonA</i> ::FRT <i>mhpC</i> -281::Tn10	This work
STL18462	<i>lacZ</i> (QP6) <i>xonA</i> ::FRT <i>mhpC</i> -281::Tn10	This work
STL20310	<i>lacZ</i> (QP5) <i>mutD5 mhpC</i> -281::Tn10	This work
STL20311	<i>lacZ</i> (QP6) <i>mutD5 mhpC</i> -281::Tn10	This work
STL20316	<i>lacZ</i> (QP5) <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10	This work
STL20317	<i>lacZ</i> (QP6) <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10	This work
STL20355	<i>lacZ</i> (QP5) <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> ⁺	This work
STL20357	<i>lacZ</i> (QP5) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> ⁺	This work
STL20361	<i>lacZ</i> (QP6) <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> ⁺	This work
STL20363	<i>lacZ</i> (QP6) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> ⁺	This work
STL20366	<i>lacZ</i> (QP5) <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> ⁺	This work
STL20367	<i>lacZ</i> (QP6) <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> ⁺	This work
STL20369	<i>lacZ</i> (QP5) <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> R148A	This work
STL20371	<i>lacZ</i> (QP6) <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> R148A	This work
STL20381	<i>lacZ</i> (QP5) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> R148A	This work
STL20383	<i>lacZ</i> (QP6) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> R148A	This work
STL20385	<i>lacZ</i> (QP5) <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> A183V	This work
STL20387	<i>lacZ</i> (QP6) <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> A183V	This work
STL20397	<i>lacZ</i> (QP5) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> A183V	This work
STL20399	<i>lacZ</i> (QP6) <i>xonA</i> ::FRT <i>xseA</i> ::FRT <i>mhpC</i> -281::Tn10 <i>attTn7</i> :: <i>xonA</i> A183V	This work