Role of error-prone DNA polymerases in spontaneous mutagenesis in *Caulobacter crescentus*

Alexy O. Valencia¹, Vânia S. Braz¹, Magna Magalhães¹ and Rodrigo S. Galhardo¹

¹Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, Sao Paulo, SP, Brazil.

Send correspondence to Rodrigo S. Galhardo. E-mail: rgalhard@usp.br

Abstract

Spontaneous mutations are important players in evolution. Nevertheless, there is a paucity of information about the mutagenic processes operating in most bacterial species. In this work, we implemented two forward mutational markers for studies in *Caulobacter crescentus*. We confirmed previous results in which A:T→ G:C transitions are the most prevalent type of spontaneous base substitutions in this organism, although there is considerable deviation from this trend in one of the loci analyzed. We also investigated the role of *dinB* and *imuC*, encoding error-prone DNA polymerases, in spontaneous mutagenesis in this GC-rich organism. Both *dinB* and *imuC* mutant strains show comparable mutation rates to the parental strain. Nevertheless, both strains show differences in the base substitution patterns, and the *dinB* mutant strain shows a striking reduction in the number of spontaneous -1 deletions and an increase in C:G → T:A transitions in both assays.

Keywords: DinB, ImuC, DnaE2, spontaneous mutagenesis, *Caulobacter crescentus*.

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Introduction

Spontaneous mutations, arising without exposure of cells to external genotoxic agents, arise at a constant rate in all organisms (Drake 1991; Lynch 2010). Although most organisms show a bias towards C:G → T:A transitions among spontaneous base substitutions, we have recently found that the bacterium *C. crescentus* shows a different trend, with more A:T → G:C substitutions among spontaneous mutations in the *rpoB* gene (Martins-Pinheiro et al. 2017). Nevertheless, use of *rpoB* as a mutational marker has limitations, such as the relatively small number of amino acid changes leading to the detectable phenotype (RifR) and the lack of detection of insertions and deletions.

Error-prone polymerases are widespread in nature (Ohmori et al. 2001), playing an important role in DNA damage tolerance in bacteria by promoting translesion DNA synthesis (TLS) (Fuchs and Fujii 2013). As a direct consequence of their TLS activity and their regulation by many cellular stress responses, error-prone polymerases are likely important players in the mutational processes both in growing and non-growing bacterial cells (Galhardo et al. 2007). These enzymes have been extensively studied in *Escherichia coli*, where the SOS-regulated genes *umuDC* and *dinB* encode the error prone polymerases Pol V and Pol IV respectively.

The role of these enzymes in DNA damage tolerance in *E. coli* is clear. Pol V is required for damage-induced mutagenesis after cellular exposure to a number of different DNA damaging agents, such as UV light, methyl nitrosoguanidine, and 4-NQO (Kato and Shinoura 1977; Bagg et al. 1981; Woodgate 1992). Pol IV is involved in error-free bypass of both alkylation damage (Bjedov et al. 2007) and N2-guanine adducts (Jarosz et al. 2006). Deletion of the *dinB* gene does not affect the rate of spontaneous mutations (Mckenzie et al. 2003; Kuban et al. 2004), and neither the sequences of the mutations observed in the *rpoB* gene (Wolff et al. 2004). Due to the very tight transcriptional and post-transcriptional control of Pol V activity (Goodman et al. 2016), *umuDC* genes are assumed to have little effect on spontaneous mutagenesis. On the other hand, both DinB and UmuDC have been implicated in untargeted mutagenesis in SOS-constitutive cells (Caillet-Fauquet and Maenhaut-Michel 1988; Kim et al. 1997; Kim et al. 2001), and also in stress-induced mutagenesis (Cirz et al. 2005; Petrosino et al. 2009). Additionally, *dinB* is subject to many layers of regulation in *E. coli*, being induced by the SOS response (Kenyon and Walker 1980), upon entry into stationary phase (Layton and Foster 2003) and by beta-lactam antibiotics (Perez-Capilla et al. 2005).

*C. crescentus* bears two genes encoding error-prone polymerases in its genome, *dinB*, and *imuC* (*dnaE2*), the later one being part of a conserved operon also containing *imuA* and *imuB*. Previous studies have shown that this operon is part of the SOS response both in *C. crescentus* and in other bacteria where these three genes are induced as part of the SOS response and cooperate in a
mutagenesis pathway responsible for Mitomycin C- and UV-induced mutagenesis (Boshoff et al. 2003; Galhardo et al. 2005; Warner et al. 2010). Nevertheless, constitutive transcription of imuABC in SOS-induced levels does not promote significant increases mutation rates in C. crescentus, suggesting a tight control of this mutagenesis pathway in cells experiencing DNA damage (Alves et al. 2017). Furthermore, the same study showed that the activity of ImuABC is RecA-independent, setting it apart from the paradigm of mutagenic DNA polymerase regulation in E. coli. Therefore, imuABC are thought of as functional substitutes of umuDC in bacteria lacking these genes, although its properties and regulation show considerable differences.

On the other hand, the physiological role of dinB in C. crescentus is still not understood. Differently from E. coli, this gene is not part of the SOS regulon (Galhardo et al. 2005; da Rocha et al. 2008), and is not upregulated in response to UV light, hydroxyurea and mitomycin C (Modell et al. 2011).

In an attempt to better understand the physiological role of error-prone polymerases and to obtain a better appraisal of the characteristics of spontaneous mutagenesis in C. crescentus, we implemented two forward mutational assays. With these tools, we investigated the characteristics of spontaneous mutagenesis in C. crescentus and analyzed the role of DinB and ImuC in this process. We found that DinB has a major role in the generation of spontaneous deletions in the C. crescentus genome.

Material and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. C. crescentus strains were grown in PYE or M2 glucose (Ely 1991), at 30°C with constant shaking at 250 rpm for liquid cultures. When needed, the following concentrations of antibiotics were used: ampicillin 200 μg/mL (for selection of AmpR mutants in the xylbla assay); tetracycline 4 μg/mL (for selection of TetR mutants in the cItet assay); kanamycin 5 μg/mL; nalidixic acid 20 μg/mL.

Introduction of the cI (Ind−) λpR tetA marker in the C. crescentus genome

The cI (Ind−) λpR tetA cassette, hereafter referred to as cItet marker for simplicity, was originally constructed for use as a forward mutational marker in E. coli (Bjedov et al. 2007). This marker scores mutations in the cI repressor gene, leading to constitutive expression of tetA, and therefore, tetracycline resistance. For integration of this marker in the C. crescentus chromosome, a 538 bp fragment corresponding to the region between bases 2404103 and 2404622 of the NA1000
genome was amplified using primers inter3fwd and inter3rev (Table S1). This fragment served as the homology region for recombination of the final construct on the chromosome. This amplicon was cloned in the pMCS7 integrative vector in the NdeI site, using restriction sites introduced in the primers. The resulting plasmid was digested with KpnI and SmaI to receive the cl cassette. The cl cassette was amplified from the genomic DNA of the MG1655 cl (Ind−) λpR tetA E. coli strain using the cItetfowkpn and cItetrevsac primers (Table S1) and cloned in the above construct. The resulting plasmid, pMCSCI (GentR), was introduced in E. coli S17.1 by electroporation, and passed to C. crescentus NA1000 via conjugation, resulting in strain RSG317, which yielded spontaneous TetR mutants, unlike the parental strain NA1000. dinB and imuC derivatives of RSG317 were constructed using ΦCr30 transduction using GM40 and GM50 strains as donors.

Construction of the xylbla marker

We envisaged a strategy to replace the xylX gene, necessary for the metabolism of xylose but dispensable for growth in rich media (Stephens et al. 2007a) by blaA, conferring resistance to ampicillin (West et al. 2002), to construct a novel marker for mutagenesis studies using a native C. crescentus gene. The resulting strain is phenotypically AmpR in the absence of xylose, but AmpR after mutations (i) inactivating the XylR repressor; (ii) altering the XylR operator sequences in the PxylX promoter (Figure 1). This marker is referred to throughout the text as xylbla.

To replace xylX by blaA, we constructed plasmid pxylblaE2, containing blaA flanked by 5´ and 3´ homology regions surrounding xylX, as follows. First, blaA and blaB primers were used for the amplification of blaA flanked by EcoRI and BamHI restriction sites. A region of homology immediately 3´ of xylX was amplified using primers xylC and xylD, which introduced BamHI and SpeI restriction sites 5´ and 3´ respectively. A large fragment containing the whole xylX gene and flanking regions was obtained with primers xylA (introducing a HindIII site in the 5´portion) and xylD. This amplicon possesses a natural EcoRI site. Digestion of this fragment with HindIII and EcoRI produced a shorter fragment of 636 pb, corresponding to the region immediately 5´ to xylX plus the first 18 bp of the open reading frame. Ligation of the three fragments in pBC KS + yielded a construct containing blaA flanked by regions of homology to the 5´ and 3´ of xylX. This fragment was subcloned in pNPTS138 to produce the pNPTxylblaE2 construct.

The first recombination event, integrating pNPTxylblaE2 into the C. crescentus chromosome, was obtained by conjugation of C. crescentus CS606 (ΔblaA) with E. coli S17.1 carrying pNPTxylblaE2, selecting for KanR NalR conjugants. Afterwards, a second recombination event, leading to plasmid loss, was selected by plating cells in PYE medium supplemented with 3% sucrose. The resulting clones were screened for xylose auxotrophy, to look for strains with the substitution of xylX by blaA. As expected, these recombinants yielded spontaneous AmpR mutants, unlike the
parental strain CS606. One of these recombinants, designated RSG113, was chosen for the experiments. *dinB* and *imuC* derivatives of RSG113 were constructed using ΦCr30 transduction, using GM40 and GM50 strains as donors.

**Fluctuation tests and determination of mutation sequences**

Fluctuation tests for measurement of mutation rates were initiated by diluting a saturated culture to $\sim 10^2$ cells/mL in PYE medium. This diluted culture was split in 11 tubes containing 1 mL of cell suspension each, which were grown for 48h at 30°C. Cell viability was determined by serial dilution and plating on PYE. The number of mutants was determined as follows for each of the markers used for mutagenesis studies. Due to the high frequency of mutants, in the assays using the *xylbla* and *cItet* markers, 100 μL of each culture was plated in duplicate in PYE Amp and PYE Tet respectively. Mutation rates were calculated by the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) using the FluCalc web tool (Radchenko et al. 2018).

All the Tet$^R$ and Amp$^R$ mutants sequenced for the determination of the mutational spectrum come from independent cultures in the fluctuation assays, to ensure independent mutations were assessed. Mutations were detected and analyzed for sequence quality using the Geniou's R8 software (Biomatters).

To determine the sequences of the Tet$^R$ mutations, the *cI* gene was amplified using primers cItetfwd and cItetrev. Purified PCR products were sequenced using primers cIfwd, cIrev, cItet-Seq and cItet-int.

Since two classes of mutants are detectable in the *xylbla* assay, we first identified the two classes of mutants using a previously reported inability of *xylR* mutants to grow on minimal medium containing glucose as a carbon source. Therefore, Amp$^R$ colonies were first spotted on PYE and M2 glucose media. Mutants unable to grow on minimal media were sequenced for mutations in *xylR*, and the remainder were sequenced for mutations on PxylX. To sequence *xylR*, a PCR product was obtained with primers xylR-fwd and xylR-rev, and subsequently sequenced with the same primers and xylR-seq-final, xylR-seq-1. Mutations in PxylX were determined by PCR with primers Pxylx-fwd and blaB, and sequenced with Pxylx-fwd and PxylX-seq.

**Results**

**Forward mutational assays in C. crescentus**

In order to have better experimental tools for the study of mutagenesis in *C. crescentus*, we constructed two sets of strains (parental, and its *dinB* and *imuC* derivatives) containing different
forward mutational assays. The first set contains the cItet marker, previously developed for mutagenesis studies in E. coli (Bjedov et al. 2007). The other contains a newly developed marker, xylbla (Figure 1). It uses the well-known xylose inducible xylX promoter, which is controlled by the LacI family repressor XylR (Meisenzahl et al. 1997; Stephens et al. 2007b). In this system, the bla gene encoding a beta-lactamase naturally present in C. crescentus was put under control of PxylX, rendering cells phenotypically AmpR. Two types of mutations are conceivable in this system. Mutations that disrupt the operator sequence in PxylX, and mutations that inactivate the XylR repressor, as depicted in Figure 1. xylR and PxylX mutants can be distinguished based on the poor growth of the former on minimal media containing glucose as the sole carbon source, as reported before (Stephens et al. 2007b). C. crescentus cells carrying cItet give rise to spontaneous TetR mutants, and those carrying the xylbla marker give rise to spontaneous AmpR mutants, unlike the NA1000 strain. Sequencing revealed that both AmpR and TetR mutants carry mutations in the predicted targets (see below). Therefore, we successfully used forward mutational assays for this model organism.

Roles of dinB and imuC in spontaneous mutagenesis

We sought to determine the role of dinB and imuC in spontaneous mutagenesis using both markers in fluctuation assays (Figure 2). The results show that both the dinB and imuC strains show AmpR and TetR mutation rates comparable to the respective parental strains. In the case of imuC, mutation rates are indistinguishable from the parental strains ones in both markers, given the overlap in the confidence intervals. The dinB strain shows comparable AmpR mutation rates, but slightly decreased TetR mutagenesis. Nevertheless, the small difference observed (less than 2-fold) is usually not considered biologically relevant. These results confirm our previous observations using the more limited rpoB marker, which can only detect base substitutions. In those experiments, we showed that imuC does not influence the rate of RifR mutations (Martins-Pinheiro et al. 2017). Although these results indicate that both DinB and ImuABC have a limited role in spontaneous mutagenesis, we reasoned that quantitative determination of mutation rates lack the sensitivity to detect small, but biologically important, changes in the mutational signatures in cells lacking these polymerases, as exemplified by the small differences in TetR mutants observed in the dinB strain. Therefore, we proceeded to analyze the sequences of spontaneous mutations found in all loci under study.

Spontaneous mutation signatures in cI

We analyzed the sequences of TetR mutants obtained with the set of strains containing the cItet marker, and the results are represented in Figure 3. The positions of all mutations analyzed are
described in Table S2. In all strains, small indels account for a large fraction of the mutations observed, in agreement with studies in other organisms using similar markers (Schaaper et al. 1986). All these mutations are localized in homopolymeric runs in cI (data not shown). Both dinB and imuC strains show alterations in the number of such frameshifts. 1 bp deletions represent 30% of the mutations observed in the parental strain, and approximately 15% of the mutations observed in the imuC derivative. No -1 frameshifts were detected in the dinB strain among the TetR mutants analyzed. On the other hand, both dinB and imuC deficiencies lead to an increase in the number of 1 bp insertions in cI.

Previously, we have described that the spontaneous base substitution signature of C. crescentus in the rpoB gene is dominated by A:T → G:C transitions, which is different from the bias towards C:G → T:A observed in most organisms studied to date (Martins-Pinheiro et al. 2017). The sequences of mutations in cI confirm this trend, given that A:T → G:C changes outnumber C:G → T:A transitions in the wt background. Curiously, the number of C:G → T:A substitutions is increased in the dinB background, but decreased in imuC.

**Spontaneous mutation signatures in xylR**

The spectrum of spontaneous mutations in xylR is summarized in Figure 4. The positions of all mutations analyzed are described in Table S3. This gene has a very pronounced mutational hotspot, in which a cytosine insertion occurs after base 230 of the open reading frame (Figure S1). Interestingly, this hotspot does not consist of a homopolymeric run, and does not present any obvious secondary structure formation. Therefore, the basis for the presence of this hotspot is not known. The dinB strain shows a marked increase in the proportion of mutations in this hotspot.

Apart from mutations in the hotspot, the dinB strain lacks 1 bp deletions in homopolymeric runs in xylR, as seen in cI, suggesting that those are, to a large extent, generated by Pol IV activity in C. crescentus. Another feature in common between the two markers is that A:T → G:C transitions are the most frequent type of base substitution observed in the wt strain. The same is observed for dinB and imuC mutants in xylR. Both dinB and imuC deficiency cause an increase in the number of C:G → T:A mutations in xylR, a feature not observed in the cI gene for the imuC mutant, in which we observed the opposite effect. Nevertheless, dinB deficiency leads to an increase of C:G → T:A in both markers. Other differences in the patterns of base substitutions in xylR can be seen among the strain backgrounds, with the imuC strain showing more A:T → T:A and A:T → C:G transversions. Taken together, the results obtained with the two loci point to a clear role of dinB in preventing 1 bp insertions and C:G → T:A transitions, and in the formation of -1 bp frameshifts. Minor changes in the mutational spectrum can be seen in the imuC strain. This polymerase seems to have a role in preventing A:T → T:A mutations in xylR and A:T → C:G transversions in both markers.
Spontaneous mutation signatures in PxylX

We also investigated the AmpR mutations localized in PxylX in cells carrying the xylbla marker. Although the 14 bp operator sequence is a very small mutational target compared to the 1.25 kbp long xylR ORF, we observed that PxylX mutations correspond to approximately 1/4 of all AmpR mutations in cells carrying xylbla (data not shown). There is no significant variation among the three strain backgrounds, but there is a remarkable reversion of the mutational bias observed in rpoB (Martins-Pinheiro et al. 2017), cl and xylR (Figure 5A). Here, we find that C:G → T:A mutations largely outnumber A:T → G:C transitions. C:G → T:A mutations are detected in 3 independent positions within the XylR operator located in PxylX, ruling out a hotspot to account for the results (Figure 5B). Another striking point is that no frameshifts were observed among all the PxylX mutations analyzed.

Discussion

In this work, we have successfully implemented two forward markers for mutagenesis studies in C. crescentus. Using these tools, we confirmed previous observations in which a A:T → G:C mutations are the most prevalent type of base substitution observed in C. crescentus (Martins-Pinheiro et al. 2017). Curiously, this is the predominant type of mutation found in mismatch repair deficient E. coli, but not wild type cells (Lee et al. 2012). In wild type E. coli, as well as in many other organisms, C:G → A:T mutations predominate (Lee et al. 2012), and have been proposed as the universal mutational bias in bacteria (Hershberg and Petrov 2010; Hildebrand et al. 2010). These forward mutational markers will be valuable tools for future studies aiming at understanding such difference between C. crescentus and most other organisms.

Nevertheless, we spotted an interesting deviation of this A:T → G:C bias in C. crescentus. When mutations in the PxylX region are analyzed, there is a clear shift in the predominant mutation type, with C:G → T:A transitions being the most frequent base substitution. The basis for this deviation is not yet understood. We envision two not mutually excluding explanations. First, it is possible that lesions giving rise to C:G → T:A transitions, such as uracil residues formed by spontaneous cytosine deamination, are repaired more efficiently in transcribed regions compared to non-transcribed ones. Additionally, the constant binding of the XylIR repressor to the operator sequence could hinder the access repair proteins to DNA lesions and/or affect the rate of lesion formation. These two hypotheses could also help to explain the proportionally higher mutation rates in the small PxylX target.
We also analyzed the role of the error-prone DNA polymerases ImuC and DinB in spontaneous mutagenesis. *imuC* is controlled by the SOS response in *C. crescentus*, whereas *dinB* is not (Galhardo et al. 2005; da Rocha et al. 2008). Additionally, no conditions where *dinB* expression is increased has been found in high throughput studies under DNA damaging conditions (Modell et al. 2011). Therefore, to the best of our knowledge, *dinB* expression is constitutive in *C. crescentus*, unlike other bacteria such as *E. coli* and *Pseudomonas aeruginosa* (Courcelle et al. 2001; Sanders et al. 2006), but similarly to *M. tuberculosis*, where the two *dinB* orthologues are not part of the SOS regulon (Kana et al. 2010; Smollett et al. 2012). This observation is reminiscent of the data in *C. crescentus* and indicates that inducibility by DNA damage is not a universal feature of *dinB* in bacteria. Furthermore, the *M. tuberculosis* orthologues have no obvious role in DNA damage tolerance, and do not influence the rate and spectrum of spontaneous mutagenesis (Kana et al. 2010).

In *C. crescentus* we found that this polymerase plays a role in spontaneous mutagenesis, given that 1 bp deletions seem to be totally DinB-dependent. Interestingly, ImuC also plays a role in the genesis of this same type of mutation. These mutations typically arise in homopolymeric runs, as a consequence of replication slippage. DinB overexpression has been long known to lead to an increase in the number of 1bp deletions (Kim et al. 1997; Kim et al. 2001), which occur through a dNTP-stabilized misalignment (Kobayashi et al. 2002). Nevertheless, our data suggest that physiological levels of DinB promote such mutagenesis in *C. crescentus*. Future studies are needed to understand if this phenomenon happens during TLS of endogenous lesions or simply by gaining access to ongoing replication of undamaged templates. Other possibility is that DinB may be mutagenic in DNA synthesis during recombination intermediates (Pomerantz et al. 2013). Stress-induced mutagenesis in non-growing cells also has a strong *dinB*-dependence (Mckenzie et al. 2001). It could be the case that upon saturation of the cultures and cessation of growth, a *dinB*-dependent stress-induced mechanism is triggered in *C. crescentus*, contributing to the appearance of the DinB-dependent frameshifts in the fluctuation assays. Nevertheless, cultures were plated only a few hours after they reached saturation in our experimental conditions, and not after the longer periods of time required to detect stress-induced mutations (Shee et al. 2011).

Also, in both *cI* and *xylR* the absence of *dinB* leads to an increase in the proportion of C:G → T:A transitions. This may indicate a role of this constitutively expressed polymerase in maintaining the A:T → C:G bias in *C. crescentus*. Other types of mutations were found to be influenced by error-prone polymerases, such as the A:T → C:G and A:T → T:A transversions in *xylR*, both increased in the *imuC* background. In *cI*, ImuC seems to contribute to the formation of C:G → T:A transitions. The loci specificity of these observations probably reflects local sequence contexts which may either favor increased endogenous lesion formation or hinder DNA repair, providing lesion substrates for
translesion synthesis by these polymerases. This is evident in the case of xylR mutations in the imuC strain, where some of the A:T → T:A events occurred in the same position (Table S3).

Altogether, our results point to a role of DinB in the genesis of small deletions in _C. crescentus_ cells not exposed to DNA-damaging agents. This study also demonstrates the importance of detailed analysis of mutational spectra, showing that it can reveal important small-scale changes in the proportion of base substitutions across different genetic backgrounds, which cannot be assessed by mere quantification of mutation rates.

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Internet Resources

FluCalc, Web tool for Fluctuation analysis: http://flucalc.ase.tufts.edu

Supplementary material

The supplementary material will be available in the final version of the article.
Figure 1 - Rationale of the xylbla marker. The xylX gene in the xyl operon has been replaced by blaA. Nevertheless, repression by XylR in the absence of xylose renders cells phenotypically Amp$^5$. Cells can become phenotypically Amp$^R$ by loss of function mutations (M) in xylR, or by mutations in the xylR operator inside P$_{xylX}$.

Figure 2 - Tet$^R$ and Amp$^R$ mutation rates. Tet$^R$ mutation rates were determined using 66 cultures from 6 independent experiments. Amp$^R$ mutation rates were determined using 55 cultures from 5 independent experiments. Both the parental strains containing the cItet and xylbla markers (wt) and their dinB and imuC derivatives were analyzed. Mutation rates and 95% confidence intervals (represented by the error bars) were calculated using the MSS-MLE (Ma-Sandri-Sarkar Maximum Likelihood Estimator).
**Figure 3** - Distribution of the different base substitutions in *cl* in wt, *dinB* and *imuC* strains. Results are shown for NA1000 strain (wt) and mutant strains (*dinB* and *imuC*). *n* indicates the number of mutants analyzed in each strain. The different base substitutions are indicated. Del 1 bp: 1 bp deletions. Ins 1 bp: 1 bp insertions. Ins 21 bp: 21 bp insertion detected in the wt strain.

**Figure 4** - Distribution of the different base substitutions in *xylR* in wt, *dinB* and *imuC* strains. The different base substitutions are indicated. Del 1 bp: 1 bp deletions. Ins 1 bp: 1 bp insertions not located in the hotspot. Ins 1 bp hotspot: 1 bp insertions located in the hotspot. Small dels: 2-8 bp deletions. ME ins: insertion of mobile elements. *n* indicates the number of mutants analyzed in each strain.
**Figure 5 - PxylX mutations.** A: Distribution of the different base substitutions in PxylX in wt (parental strain), *dinB* and *imuC* strains. The different base substitutions are indicated. *n* indicates the number of mutants analyzed in each strain. B: A small region in the PxylX region is shown, with the XylR binding site underlined. Sequences above and below the line show the different mutations detected.
Table 1 – Bacterial strains and plasmids used in this study

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<td>Cloning vector, non-replicating in <em>C. crescentus</em></td>
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