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RESEARCH ARTICLE

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$CroS_{R391}$, an ortholog of the λ Cro repressor, plays a major role in suppressing $polV_{R391}$-dependent mutagenesis

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Abstract

When subcloned into low-copy-number expression vectors, *rumAB*, encoding $polV_{R391}$ ($RumA'_2B$), is best characterized as a potent mutator giving rise to high levels of spontaneous mutagenesis *in vivo*. This is in dramatic contrast to the poorly mutable phenotype when $polV_{R391}$ is expressed from the native 88.5 kb R391, suggesting that R391 expresses *cis*-acting factors that suppress the expression and/or the activity of $polV_{R391}$. Indeed, we recently discovered that $SetR_{R391}$, an ortholog of λ cI repressor, is a transcriptional repressor of *rumAB*. Here, we report that $CroS_{R391}$, an ortholog of λ Cro, also serves as a potent transcriptional repressor of *rumAB*. Levels of RumA are dependent upon an interplay between $SetR_{R391}$ and $CroS_{R391}$, with the greatest reduction of RumA protein levels observed in the absence of $SetR_{R391}$ and the presence of $CroS_{R391}$. Under these conditions, $CroS_{R391}$ completely abolishes the high levels of mutagenesis promoted by $polV_{R391}$ expressed from low-copy-number plasmids. Furthermore, deletion of $croS_{R391}$ on the native R391 results in a dramatic increase in mutagenesis, indicating that $CroS_{R391}$ plays a major role in suppressing $polV_{R391}$ mutagenesis *in vivo*. Inactivating mutations in $CroS_{R391}$ therefore have the distinct possibility of increasing cellular mutagenesis that could lead to the evolution of antibiotic resistance of pathogenic bacteria harboring R391.

KEYWORDS

DNA polymerase V, integrating conjugative element, mutagenesis, R391, SOS response

1 | INTRODUCTION

The accelerating emergence of drug-resistant pathogenic microorganisms is a critical global public health concern (https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance). Antibiotic over-use and prophylaxis, in both humans and livestock, for the treatment of disease-causing bacteria has led to the selection and increased prevalence of so-called "superbugs" that have often acquired resistance to multiple classes of antimicrobial compounds (Wendlandt et al., 2015). Estimates suggest a 10-fold increase in mortality rates from untreatable bacterial infections within the next 30 years (https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf). The urgency

of this impending crisis has recently led to a concerted effort to identify new synthetic compounds and natural products that might represent novel families of antibiotics (Bhattarai et al., 2020). In addition, the genetic mechanisms that give rise to antibiotic resistance, such as stress-induced mutagenesis and broad-host-range drug-resistance genetic elements (i.e., plasmids, genomic islands, and integrative and conjugative elements [ICE]), are being extensively studied as potential targets for mitigating the development of resistance.

Escherichia coli possesses five DNA polymerases, three of which, pol II (encoded by *polB*), pol IV (encoded by *dinB*), and polV (encoded by *umuDC*), are induced as part of the SOS response to stress and DNA damage (Simmons et al., 2008). Expression of these polymerases has previously been shown to contribute to the evolution of antibiotic drug resistance in many pathogenic strains of bacteria (Cirz et al., 2006a; 2006b; 2007; Cirz & Romesberg, 2006). PolV alone is responsible for up to a 100-fold increase in spontaneous mutagenesis after SOS induction (Fijalkowska et al., 1997; Sweasy et al., 1990). As a consequence, the activity of polV is subject to multiple levels of regulation (Goodman et al., 2016). In addition to transcriptional repression by the SOS repressor, LexA (Bagg et al., 1981), the UmuD protein has to undergo activated RecA (RecA*)-mediated posttranslational cleavage to UmuD′ (Burckhardt et al., 1988; Nohmi et al., 1988; Shinagawa et al., 1988), so that it can interact with UmuC (Woodgate et al., 1989) and generate polV ($UmuD'_2C$) (Tang et al., 1999). Cleavage of UmuD to UmuD′ also leads to a change in the spatial location of polV inside the cell (Robinson et al., 2015). The polVs activity is further increased through an interaction with RecA and ATP to generate the polV Mutasome (polV Mut) (Jiang et al., 2009). Last, but not least, intracellular levels of UmuD, UmuD′, and UmuC are normally kept to a minimum through targeted proteolysis (Frank et al., 1996; Gonzalez et al., 1998, 2000).

The polV orthologs are found in many strains of bacteria, as well as bacteriophage, self-transmissible R-plasmids, and ICEs (Ho et al., 1993; McLenigan et al., 1999; Pinney, 1980; Upton & Pinney, 1983). Given that these orthologs are located on mobile genetic elements and are likely to find themselves in very different genetic environments, one might expect alternate mechanisms of regulation to keep their activity in check until needed.

A good example is the LexA-regulated polV ortholog (encoded by *rumAB*) found on R391. R391 and a closely related ICE, SXT, exhibit 95% identity over 65 kb of their sequences (Hochhut et al., 2001). However, one notable difference between R391 and SXT is that *rumB* is inactivated in SXT due to the insertion of an *ISCR2* element into the gene, and as a consequence, in contrast to R391, SXT does not possess an active polV.

$SetR_{SXT}$, a λ cI-like repressor, has been shown to regulate genes involved in conjugation, integration, and excision of the SXT element (Beaber et al., 2004; Poulin-Laprade & Burrus, 2015; Poulin-Laprade et al., 2015). Previously, we examined the role of the related $SetR_{R391}$ protein for its role in regulating $polV_{R391}$ (Gonzalez et al., 2019). We found that the regulatory region of the $rumAB_{R391}$ operon contains a single site that is highly similar to the known multiple $SetR_{SXT}$ 14-bp operator sequences (Figure 1), leading us to suggest that $SetR_{R391}$ could be involved in the repression of the $rumAB_{R391}$ genes. We found that co-expression of $rumAB_{R391}$ and $setR_{R391}$ from the same low-copy plasmid reduced the levels of mutagenesis observed in a *lexA* defective SOS-induced strain indicating that $SetR_{R391}$ can regulate the $rumAB_{R391}$ genes (Gonzalez et al., 2019). Moreover, the $SetR_{R391}$ protein was shown to specifically bind to the 14-bp operator sequence that overlaps with the −35 promoter element upstream of *rumAB* (Figure 1). However, we found that plasmid pRLH421, which contains ~21.5 kb of R391, including both $setR_{R391}$ and *rumAB*, nevertheless exhibited very high levels of mutagenesis in an SOS-induced strain [*recA718 lexA51*(Def)]. We argued that, in this SOS-induced strain, the activated RecA co-protease (encoded by *recA718*) cleaves the $SetR_{R391}$ protein, in a similar fashion to LexA cleavage, inactivating $SetR_{R391}$ and allowing expression of the *rumAB* operon. Indeed, when a non-cleavable allele of $setR_{R391}$ was cloned into pRLH421, we observed a significant decrease in mutagenesis (Gonzalez et al., 2019). Additional experiments revealed, however, that intact R391 exhibits even lower levels of mutagenesis in strains in which RecA is in a constitutively and highly activated state (*recA730*), suggesting that there is at least one additional negative regulator of the $rumAB_{R391}$ genes encoded by R391 that is not affected by activated RecA (unpublished observations).

R391 also expresses a protein that is phylogenetically related to Cro-like transcriptional repressor proteins (Figure S1) (Boltner et al., 2002). While the $CroS_{R391}$ protein shows some phylogenetic divergence from related Cro-like proteins, it is 100% identical to the $CroS_{SXT}$ protein (Figure S1). Previous studies on the $CroS_{SXT}$ and $SetR_{SXT}$ proteins revealed that they both bind to four conserved operator sequences: *OL*, *O1*, *O2*, and *O3* (albeit with differing affinities) in the *croS-setR* intergenic region that regulates their own expression from divergent promoters (Poulin-Laprade & Burrus, 2015). In SXT, it is known that $CroS_{SXT}$ also regulates the $setCD_{SXT}$ genes and, similar to $SetR_{SXT}$, is involved in regulating conjugative transfer (Poulin-Laprade & Burrus, 2015; Poulin-Laprade et al., 2015). The interplay between the ICE $SetR_{SXT}$ and $CroS_{SXT}$ proteins is therefore reminiscent of the λ cI and λ Cro proteins that govern the transition between lysogenic and lytic pathways during the bacteriophage life cycle (Johnson et al., 1978; Svenningsen et al., 2005; Takeda et al., 1977).

FIGURE 1 Cartoon of the *rumAB* promoter region. A single SetR/CroS binding site is shown in blue color. This site partially overlaps with the −35 promoter element, shown in gold. A LexA binding site is shown in green, which partially overlaps with the −10 promoter element (also shown in gold). The ribosome binding site (RBS) is shown in purple, and the first two codons of RumA are shown in red

We hypothesized that like $SetR_{R391}$, $CroS_{R391}$ would function as a transcriptional regulator of the $rumAB_{R391}$ operon and thereby play a role in regulating $rumAB_{R391}$-mediated mutagenesis. To test this hypothesis, we have constructed a series of plasmids expressing $SetR_{R391}$ and/or $CroS_{R391}$ and investigated their effects on *rumAB* expression and $polV_{R391}$-dependent mutagenesis *in vivo*. We report here that $CroS_{R391}$ is a transcriptional repressor of *rumAB* and may be the major factor that suppresses $polV_{R391}$ activity on R391.

2 | RESULTS

2.1 | Comparison of $polV_{R391}$-dependent mutagenesis when *rumAB* is expressed from R391 or subcloned onto a low-copy-number vector

Our studies examined the multifaceted nature of the regulation of the R391 mutagenic response. Regulation of the mutagenic activity of $polV_{R391}$ when expressed in its native genetic environment promotes minimal levels of mutagenesis (Pinney, 1980), even in strains in which the LexA repressor is inactivated and RecA is constitutively activated (previous unpublished observations and Figure 2). However, when *rumAB* was subcloned onto a low-copy-number plasmid, there was a dramatic increase in $polV_{R391}$ mutator activity, especially in strains with mutations in *recA* (*recA718* and *recA730*) that lead to a constitutive RecA* phenotype (Ho et al., 1993) (Figure 2). Indeed, $polV_{R391}$ is the most potent polV ortholog characterized to date (Kulaeva et al., 1995; Mead et al., 2007). The fact that $polV_{R391}$ appears inactive when expressed from the native R391 indicates that there is likely to be *cis*-acting factor(s) expressed from R391 that normally act to suppress the potent mutator activity of $polV_{R391}$.

2.2 | $CroS_{R391}$ plays a major role in suppressing $polV_{R391}$-dependent mutagenesis

The *rumAB* operon was originally subcloned in 1993 into the low-copy vector pGB2 (Churchward et al., 1984) as a partial *Eco*RI digest of R391 (Ho et al., 1993). R391 is normally chromosomally located and integrated into the 5′ end of *prfC* (Hochhut et al., 2001). However, the 21.5-kb insert cloned into pGB2 to generate pRLH421 is clearly of episomal origin, since it contains both 5′ and 3′ ends of the linear R391. Unfortunately, sequence analysis of the insert in pRLH421 (Genbank: U13633) reveals that the $croS_{R391}$ gene is truncated at an internal *Eco*RI site in the gene (Figure S2). We suspected that the *croS*_ΔC truncation could explain our disparate mutagenesis results with pRLH421 (high levels), versus intact R391 (low levels), if $CroS_{R391}$ does indeed repress the $rumAB_{R391}$ genes. To test this notion, we constructed a series of pRLH421-derived plasmids with combinations of wild-type $croS_{R391}$ and $setR_{R391}$, and/or deletions of $croS_{R391}$ and $setR_{R391}$ while still retaining the *rumAB* operon (Table 1) (Figure S2).

The various plasmid iterations of $croS_{R391}$ and $setR_{R391}$ were transformed into MVG114 [Δ*umuDC596*::*ermGT*, *lexA51*(Def), *recA718*, and *hisG4*(*Oc*)] in order to analyze RumAB-dependent spontaneous mutagenesis utilizing the histidine reversion assay. In the *lexA51*(Def) background, the RecA718 protein is in a partially activated state (RecA*) (McCall et al., 1987) and promotes significant levels of polV-dependent spontaneous mutagenesis in the absence of DNA damage (Sweasy et al., 1990). As with our earlier findings (Ho et al., 1993), pRLH421 gave very high levels of mutagenesis (Figure 3). Similarly, both the $\Delta croS_{R391}/setR^{+}_{R391}$ construct (pJM1355) and the double $\Delta croS_{R391}/\Delta setR_{R391}$ construct (pJM1359) also gave high levels of mutagenesis revealing that the *rumAB* operon is not appreciably downregulated in the *recA718 lexA51*(Def) background. However, in strains harboring plasmid constructs that express $CroS_{R391}$ the level of mutagenesis is significantly reduced. While the levels of mutagenesis with the $croS^{+}_{R391}/setR^{+}_{R391}$ construct (pJM1356) are reduced 2.7-fold (cf. pJM1355) to 4.2-fold (cf. pRLH421), the $croS^{+}_{R391}/\Delta setR_{R391}$ construct (pJM1360) results in a 50- to 75-fold reduction in mutagenesis (Figure 3).

2.3 | $CroS_{R391}$ repression can operate *in trans* and is specific for the *rumAB* promoter

To demonstrate that the regulation of *rumAB*-dependent mutagenesis was specifically due to $CroS_{R391}$ and not some other factor encoded in the R391 DNA cloned in the pRLH421 derivatives described above, we deleted ~20.3 kb from *Sca*I to *Sma*I of the R391 DNA leaving only the various $croS_{R391}$ and $setR_{R391}$ operons (Figure S2). The pJM1378 (Table 1), a pCC1 derivative (Epicenter/Genscript), carrying the $rumAB_{R391}$ operon, including the *rumAB* promoter, was transformed alone or in combination with pJM1365 ($\Delta croS_{R391}/setR^{+}_{R391}$), pJM1366 ($croS^{+}_{R391}/setR^{+}_{R391}$), pJM1367 ($\Delta croS_{R391}/\Delta setR_{R391}$), and pJM1368 ($croS^{+}_{R391}/\Delta setR_{R391}$) into MVG114 [Δ*umuDC596*::*ermGT*, *lexA51*(Def), *recA718*, and *hisG4*(*Oc*)]. Again, utilizing the histidine reversion mutagenesis assay, we found that *rumAB*-dependent mutagenesis was lower when expressing both wild-type $CroS_{R391}$ and $SetR_{R391}$ (pJM1366) corresponding to our above finding with plasmid pJM1356 ($croS^{+}_{R391}/setR^{+}_{R391}$) (Figures 4a and S3). In contrast, when $setR_{R391}$ is deleted such that only $CroS_{R391}$ is expressed (pJM1368), *rumAB*-dependent mutagenesis is virtually eliminated (Figures 4a and S3), again in agreement with the results with pJM1360 ($croS^{+}_{R391}/\Delta setR_{R391}$). Furthermore, we found that in all strains where there is no $CroS_{R391}$ expressed (pJM1378 alone or with pJM1365, or pJM1367), there is a very high level of spontaneous mutagenesis. These results confirm that regulation of *rumAB*-dependent mutagenesis occurs during an interplay between $SetR_{R391}$ and $CroS_{R391}$ and that this regulation can operate in *trans*.

Knowing that there is a single $SetR_{R391}$ binding site upstream of the $rumAB_{R391}$ operon (Figure 1) (Gonzalez et al., 2019) and that the four SetR binding sites in the *setR-croS* intergenic region of the SXT element are also bound by $CroS_{SXT}$ (Poulin-Laprade & Burrus, 2015), we wanted to show that the $CroS_{R391}$ repression of

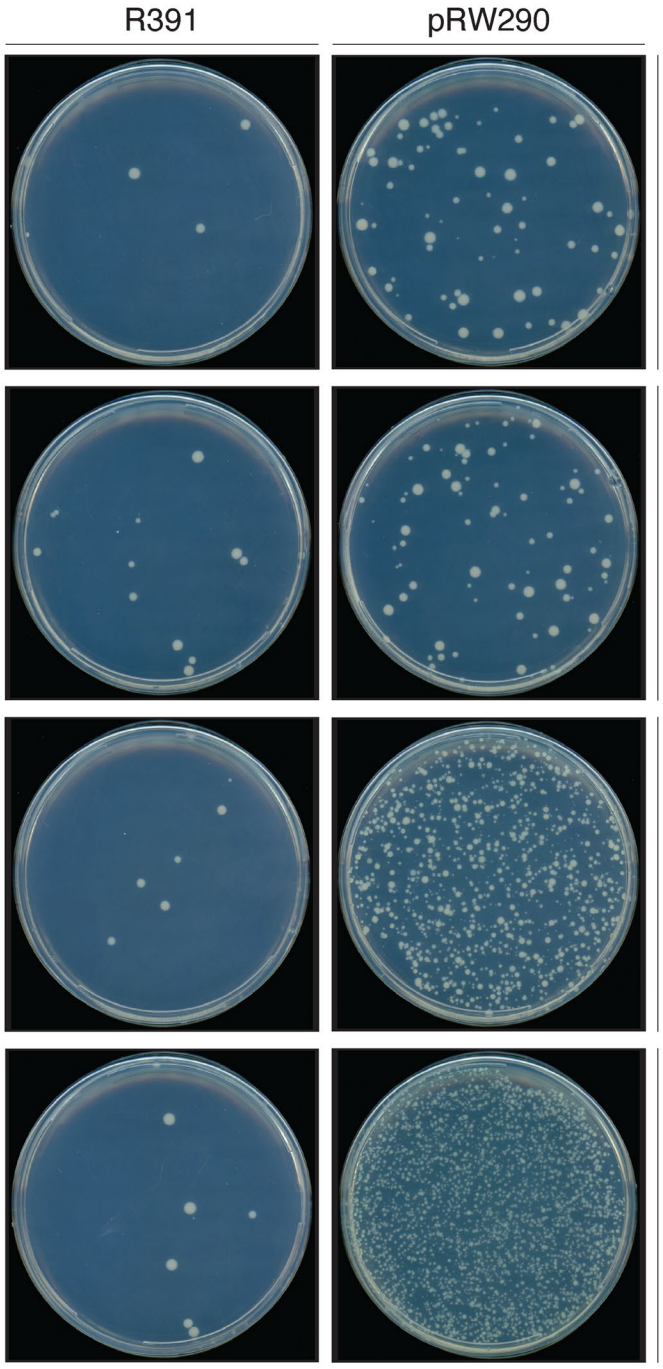

FIGURE 2 Spontaneous mutagenesis promoted by R391, or pRW290, in different genetic backgrounds. Cells were plated on minimal low histidine agar plates as described in Section 4.3: *Qualitative analysis of spontaneous reversion of the hisG4*(Oc) *allele*. His$^+$ revertants appear as creamy white colonies against the dark background behind the agar plate. As observed, the 88.5-kb R391 promotes low levels of spontaneous mutagenesis in all genetic backgrounds. This is in contrast to pRW290, which only expresses the *rumAB*$_{R391}$ operon from a low-copy-number vector. Although the *rumAB* operon is subject to transcriptional regulation by the LexA repressor, there is little difference in mutagenesis between *lexA*$^+$ (RW120) and *lexA51*(Def) (RW546) strains. Mutagenesis increases significantly when RecA is partially activated for co-protease functions (*recA718*; MVG114) or fully activated for co-protease functions (*recA730*; RW578)

rumAB$_{R391}$ was dependent on the *rumAB* promoter region. We therefore replaced the *rumAB*$_{R391}$ promoter region in pJM1378 with the promoter region of the *E. coli recA* gene, to create plasmid pJM1467 (*recA-promoter*::*rumAB*$_{R391}$) (Table 1). As before, pJM1467 was transformed alone or in combination with pJM1365 (Δ*croS*$_{R391}$/*setR*$^+_{R391}$), pJM1366 (*croS*$^+_{R391}$/*setR*$^+_{R391}$), pJM1367 (Δ*croS*$_{R391}$/Δ*setR*$_{R391}$), and pJM1368 (*croS*$^+_{R391}$/Δ*setR*$_{R391}$) into MVG114, and histidine reversion mutagenesis was performed (Figures 5a and S3). Most plasmid combinations gave uniformly high levels of mutagenesis. The exception was pJM1467 together with pJM1368 (*croS*$^+_{R391}$/Δ*setR*$_{R391}$) that gave ~85% of the level of mutagenesis observed with the other plasmid combinations. It should be emphasized that this is in dramatic contrast to when RumAB$_{R391}$ is expressed from the native SetR/CroS binding site-containing *rumAB* promoter, where CroS$_{R391}$ expressed from pJM1368 eliminated virtually all RumAB-dependent mutagenesis (cf. Figures 4a, 5a, and S3).

2.4 | CroS$_{R391}$ regulation of RumA$_{R391}$ protein level is specific to the *rumAB*$_{R391}$ promoter

In order to demonstrate that the CroS$_{R391}$ protein regulates mutagenesis by repressing the *rumAB* genes, we performed western blot analysis using rabbit anti-CroS$_{R391}$ (this study) and anti-RumA$_{R391}$ antibodies.

TABLE 1 Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
R391	Integrated into *prfC*	(Ho et al., 1993)
R391Δ*croS*	Integrated into *prfC* and with a deletion of $croS_{R391}$	This study
pRW290	Low copy number, Spc^R, ~2-kb fragment of R391 expressing *rumAB*	(Szekeres et al., 1996)
pRLH421	Low-copy-number, Spc^R, ~21.5-kb fragment of R391	Genbank:U13633 (Gonzalez et al., 2019)
pJM1355	Low-copy-number, Spc^R, ~21.5-kb fragment of R391 with $\Delta croS_{R391}$ $setR^+_{R391}$	This study
pJM1356	Low-copy-number, Spc^R, ~21.5-kb fragment of R391with $croS^+_{R391}$ $setR^+_{R391}$	This study
pJM1359	Low-copy-number, Spc^R, ~21.5-kb fragment of R391 with $\Delta croS_{R391}$ $\Delta setR_{R391}$	This study
pJM1360	Low-copy-number, Spc^R, ~21.5-kb fragment of R391 with $croS^+_{R391}$ $\Delta setR_{R391}$	This study
pJM1365	Low-copy-number, Spc^R, with $\Delta croS_{R391}$ $setR^+_{R391}$	This study
pJM1366	Low-copy-number, Spc^R, with $croS^+_{R391}$ $setR^+_{R391}$	This study
pJM1367	Low-copy-number, Spc^R, with $\Delta croS_{R391}$ $\Delta setR_{R391}$	This study
pJM1368	Low-copy-number, Spc^R, with $croS^+_{R391}$ $\Delta setR_{R391}$	This study
pCC1Bac™	Single-copy-number, Cm^R	Genscript
pJM1378	pCC1-based, Cm^R, with $rumAB_{R391}$	This study
pJM1467	pCC1-based, Cm^R, with *recA-promoter*::$rumAB_{R391}$	This study
pRed/ET	Plasmid encoding the *red* gene cluster (*red*γβα) and *rec*A Tet^R	(Wang et al., 2006)
pFLPe	Plasmid encoding the enhanced FLP recombinase, Amp^R	Gen-H

Whole cell protein extracts were made from MVG114 strains harboring pJM1378 (*rumAB*) alone or in combination with pJM1365 ($\Delta croS_{R391}/setR^+_{R391}$), pJM1366 ($croS^+_{R391}/setR^+_{R391}$), pJM1367 ($\Delta croS_{R391}/\Delta setR_{R391}$), or pJM1368 ($croS^+_{R391}/\Delta setR_{R391}$). As expected, only the strains harboring the pJM1366 ($croS^+_{R391}/setR^+_{R391}$) and pJM1368 ($croS^+_{R391}/\Delta setR_{R391}$) constructs expressed any $CroS_{R391}$ protein (Figure 4b), and the level of CroS was identical in the presence or absence of SetR, suggesting that the phenotypic differences observed with the two plasmids (Figure 4a) are dependent on the presence of the SetR protein and not the level of CroS protein. When compared to strains lacking CroS, the levels of $RumA_{R391}/RumA'_{R391}$ proteins are significantly reduced in the strains harboring the pJM1366 ($croS^+_{R391}/setR^+_{R391}$) and pJM1368 ($croS^+_{R391}/\Delta setR_{R391}$) constructs (Figure 4c). Moreover, in the strain harboring pJM1368, which expresses only $CroS_{R391}$, the level of $RumA_{R391}$ protein is less than in the strain harboring pJM1366, despite expressing the same amount of CroS (Figure 4b), and the $RumA'_{R391}$ protein is close to the limits of detection (Figure 4c). These findings demonstrate that the $CroS_{R391}$ protein does indeed regulate the expression of the $RumA_{R391}$ protein and that its regulation can be modulated in the presence of $SetR_{R391}$. In addition, in the absence of the $SetR_{R391}$ protein (pJM1368), $CroS_{R391}$ protein may also have some inhibitory effect on RecA-mediated cleavage of $RumA_{R391}$ to $RumA'_{R391}$ (see below for more discussion).

Next, we wanted to show that the $CroS_{R391}$ regulation of RumA protein expression is specific to the *rumAB* promoter region. Plasmid pJM1467 (*recA-prom*::$rumAB_{R391}$), which expresses $RumAB_{R391}$ from the *E. coli recA* promoter, was co-transformed into MVG114 along with pJM1365 ($\Delta croS_{R391}/setR^+_{R391}$), pJM1366 ($croS^+_{R391}/setR^+_{R391}$), pJM1367 ($\Delta croS_{R391}/\Delta setR_{R391}$), or pJM1368 ($croS^+_{R391}/\Delta setR_{R391}$). Western blot analysis using the anti-RumA antibodies was performed (Figure 5b). We found that the levels of $RumA_{R391}/RumA'_{R391}$ proteins are enhanced when expressed from the *E. coli recA* promoter as compared to $RumA_{R391}/RumA'_{R391}$ protein expressed from the native $rumAB_{R391}$ promoter. However, unlike the previous results, the levels of $RumA_{R391}$ protein expressed from the *recA* promoter are not affected by the presence of the $CroS_{R391}$ protein (pJM1366 and pJM1368) indicating that $CroS_{R391}$ transcriptional repression is specific to the $rumAB_{R391}$ promoter. Again, in the absence of the $SetR_{R391}$ protein (pJM1368), we observed a reduction in cleavage of $RumA_{R391}$ to $RumA'_{R391}$ (Figure 5). We believe that the reduction in RumA cleavage is due to an indirect inhibitory effect of $CroS_{R391}$ on the spontaneous generation of RecA* in the *recA718* strain, since significantly more RumA cleavage was observed in the same *recA718* strain exposed to the DNA damaging agent, Mitomycin C, or in the highly proficient RecA*-forming *recA730* strain (± Mitomycin C) (Figure S4).

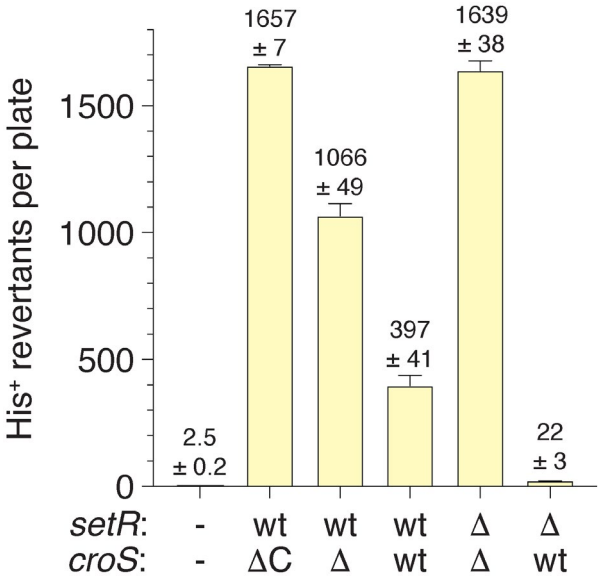

FIGURE 3 RumAB$_{R391}$-dependent mutagenesis is regulated by *croS*$_{R391}$. The histidine reversion assay was performed on the *E. coli* strain MVG114 or MVG114 strains transformed with pRLH421 or various *croS*$_{R391}$ or *setR*$_{R391}$ wild-type or deletion combinations. (wt/ΔC) represents MVG114 transformed with the plasmid pRLH421 that contains ~21.5 kb of R391 and harbors a C-terminal deletion of the *croS*$_{R391}$ gene. MVG114 was transformed with plasmids pJM1355, pJM1356, pJM1359 and pJM1360, and the *croS*$_{R391}$ or *setR*$_{R391}$ genotypes are indicated. Error bars indicate standard error of the mean (*SEM*)

2.5 | Expression of RumA/RumA′ in a *recA*$^+$ *lexA*$^+$ strain expressing SetR ± and CroS ± after antibiotic-induced SOS induction

Based on the known interplay between CroS and SetR (Poulin-Laprade & Burrus, 2015; Poulin-Laprade et al., 2015), we hypothesized that *rumAB* would be the most repressed upon conditions that activate R391 transfer, namely at low SetR concentrations and high CroS levels, after damage- or stress-induced induction of the SOS response. We were therefore interested in assaying expression of RumA in a wild-type *recA*$^+$ *lexA*$^+$ strain (RW520) harboring the various *setR-croS* plasmids, which also encode the *rumAB*$_{R391}$ operon (pJM1355, pJM1356, pJM1359, and pJM1360), in which the SOS response was stress induced by treatment with the antibiotic, Ciprofloxacin, for up to 3 hr (Figure 6). Ciprofloxacin-mediated induction of the SOS response was followed by western blot analysis of the LexA-regulated RecA and RumA proteins. With all plasmid combinations, a strong induction of RecA was observed after treatment with Ciprofloxacin, indicating that the chromosomally encoded LexA repressor had been inactivated *in vivo* (Figure 6). The lack of any effect of the various *setR-croS* plasmids on RecA expression also indicates that *recA* is not negatively regulated by either SetR or CroS. In contrast, the timing of the induction of RumA$_{R391}$ was dependent upon the presence, or absence, of SetR or CroS (Figure 6). For example, in strains harboring pJM1359 (Δ*croS*$_{R391}$/Δ*setR*$_{R391}$), where *rumA* is only regulated by LexA, there

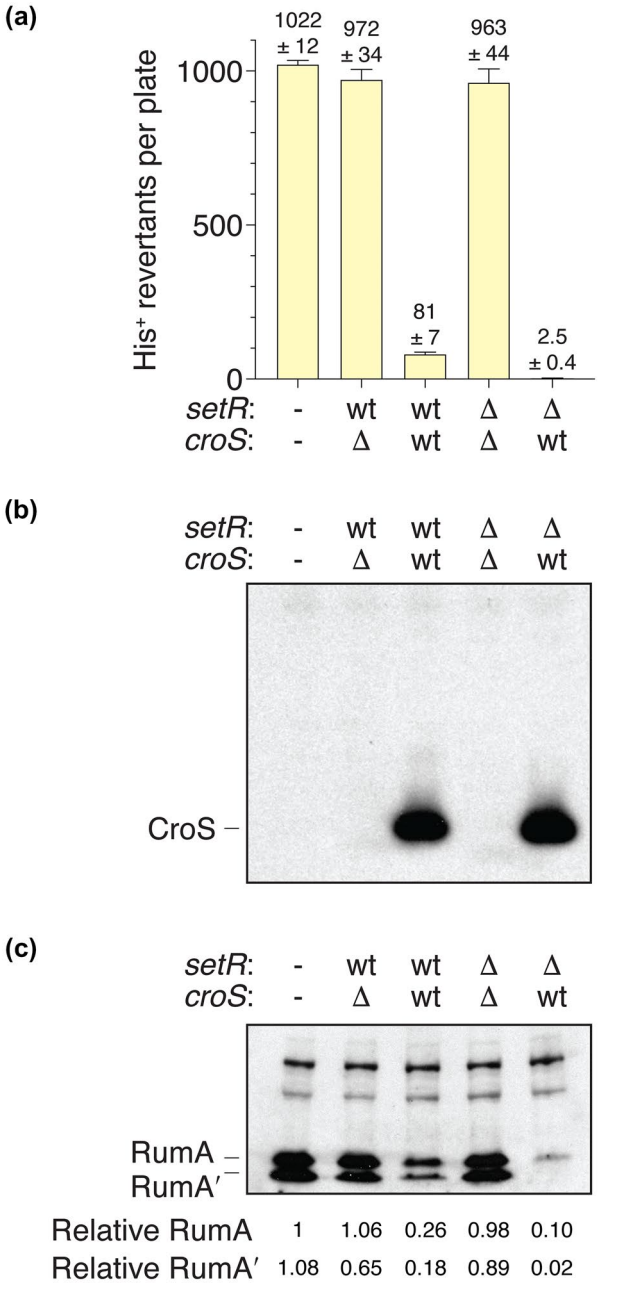

FIGURE 4 CroS$_{R391}$ *trans*-regulation of RumAB$_{R391}$-dependent mutagenesis. (a) Spontaneous histidine reversion mutagenesis assays utilizing MVG114 strains harboring pJM1378 alone (−/−), a pCC1 derivative (copy-control plasmid) carrying the *rumAB*$_{R391}$ operon, were transformed with low-copy pGB2 derivatives (pJM1365, pJM1366, pJM1367, and pJM1368) carrying various iterations of the *croS*$_{R391}$/*setR*$_{R391}$ operon (Table 1). The genotypes of *croS*$_{R391}$ or *setR*$_{R391}$, either wild-type or deleted, are indicated. (b) Western blot analysis using an anti-CroS antibody indicating that only strains harboring a plasmid with a wild-type *croS*$_{R391}$ gene express any CroS protein. (c) Western blot using an anti-RumA antibody indicating that strains that express the CroS protein have significantly reduced levels of the RumA protein. However, strains that express only SetR show no reduction in the level of RumA protein. Numbers reported for the levels of RumA and RumA′ are relative to RumA in track 1

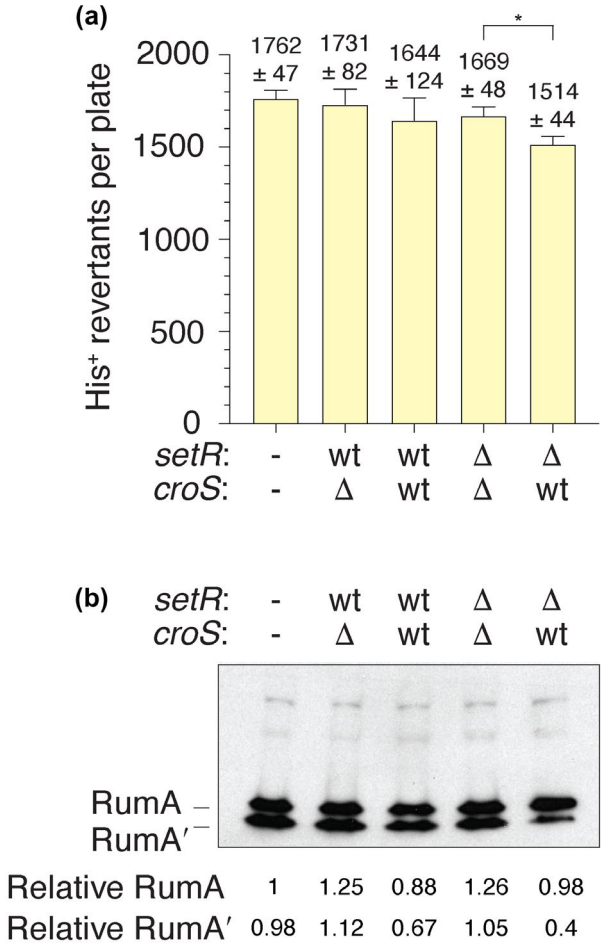

FIGURE 5 $CroS_{R391}$ regulation of $RumAB_{R391}$-dependent mutagenesis is dependent on the $rumAB_{R391}$ promoter region. (a) Spontaneous histidine reversion mutagenesis assays utilizing MVG114 strains harboring pJM1467 (−/−), a pCC1 derivative carrying the $rumAB_{R391}$ operon under the control of the *recA* promoter, were transformed with low-copy pGB2 derivatives (pJM1365, pJM1366, pJM1367, and pJM1368) carrying various iterations of the $croS_{R391}$-$setR_{R391}$ operon (Table 1). The genotypes of $croS_{R391}$ or $setR_{R391}$, either wild-type or deleted, are indicated. The histogram illustrates the mean colony count for each indicated strain ($n = 5$). Error bars represent the standard error of the mean (*SEM*). An unpaired two-tailed *t* test was used to compare the mean colony counts for the Δ*setR* Δ*croS* and the Δ*setR croS*$^+$ strains. $^* = p < .05$. (b) Western blot using an anti-RumA antibody indicating that the level of RumA expressed from the *E. coli recA* promoter does not change appreciably in the presence, or absence, of SetR or CroS. Numbers reported for the expression levels of RumA and RumA′ are relative to RumA in the left-hand lane

was a time-dependent induction of RumA, which peaked around 1 hr after Ciprofloxacin treatment, followed by conversion of RumA to RumA′ 2- to 3-hr posttreatment. By comparison, the peak of RumA induction in the presence of pJM1355 ($\Delta croS_{R391}/setR^+_{R391}$) was around 2 hr, suggesting that the presence of SetR delays RumA expression by an hour. However, there was no effect on the conversion of RumA to RumA′ that nevertheless occurred 2–3 hr posttreatment. (Figure 6). In contrast, no Ciprofloxacin-induced expression of RumA was observed in the presence of pJM1360 ($croS^+_{R391}/\Delta setR_{R391}$), consistent with our earlier observations (Figure 4c). Interestingly, in the presence of pJM1356 (expressing both $croS^+_{R391}/setR^+_{R391}$), expression of RumA peaked at 1 hr post-Ciprofloxacin treatment; from that point on, RumA levels decrease and by 3 hr, they are barely detectable. Such observations can readily be explained by the interplay and hierarchy of the three transcriptional repressors. We know that LexA is cleaved just a few minutes after DNA damage, so as to induce the 40+ protein SOS regulon (Fernández de Henestrosa et al., 2000). In contrast, SetR, which we have previously shown to be cleaved much slower than LexA *in vitro* (Gonzalez et al., 2019), would be expected to be cleaved and inactivated by RecA* *in vivo* around the 1- to 2-hr point, which would then allow $CroS_{R391}$ sole access to the SetR/CroS binding site in the *rumAB* promoter, thereby effectively eliminating residual expression of the RumA protein. The fact that RumA is detected in the $croS^+_{R391}/setR^+_{R391}$ strain, but not in the $croS^+_{R391}/\Delta setR_{R391}$ strain, implies that $SetR_{R391}$ normally competes with $CroS_{R391}$ *in vivo* and to some degree blocks its access to the SetR-CroS binding site in the *rumAB* promoter. Such a scenario explains why $polV_{R391}$, encoded by the stably integrated R391, promotes such low levels of mutagenesis after DNA damage, or in constitutively activated RecA* strains, where both LexA and SetR repressors are inactivated and *rumAB* is repressed solely by CroS (Figure 2).

2.6 | Deletion of $croS_{R391}$ on R391 leads to enhanced mutagenesis

Since R391 stably integrates into the *E. coli* chromosome at the 5′ end of the *prfC* gene (Hochhut et al., 2001), we were able to construct a $croS_{R391}$ deletion mutant in the MVG114 genetic background and compare mutagenesis between the R391 and the R391Δ*croS* strains. First, we examined mutagenesis using *a galK2*(Oc) reversion papillation assay in which orange-red Gal$^+$ mutant "papillae" grow up within bacterial colonies plated on MacConkey-galactose agar media. Representative colonies from the R391 and R391Δ*croS* strains are shown in Figure 7. The R391 strains gave 0–3 Gal$^+$ mutant papillae per colony, whereas the R391Δ*croS* gave 30–50 Gal$^+$ mutant papillae per colony. Second, we utilized a rifampicin mutagenesis assay in which cells are plated on LB agar plates containing 100 μg ml^{-1} rifampicin. R391 Δ*croS* strains exhibited a 10-fold increase in rifampicin resistant mutagenesis, as compared to the strains harboring the wild-type R391 (Figure 8). Both of these results demonstrate that the CroS protein, expressed from its native locus within an intact R391, downregulates *rumAB* and tightly controls mutagenesis in strains carrying the R391 element.

3 | DISCUSSION

3.1 | Unprecedented transcriptional regulation of R391 encoded *rumAB*

Previous studies have shown that chromosomally encoded *E. coli* polV is tightly regulated via a combination of transcriptional, post-translational, and spatial regulation (Goodman et al., 2016). It is

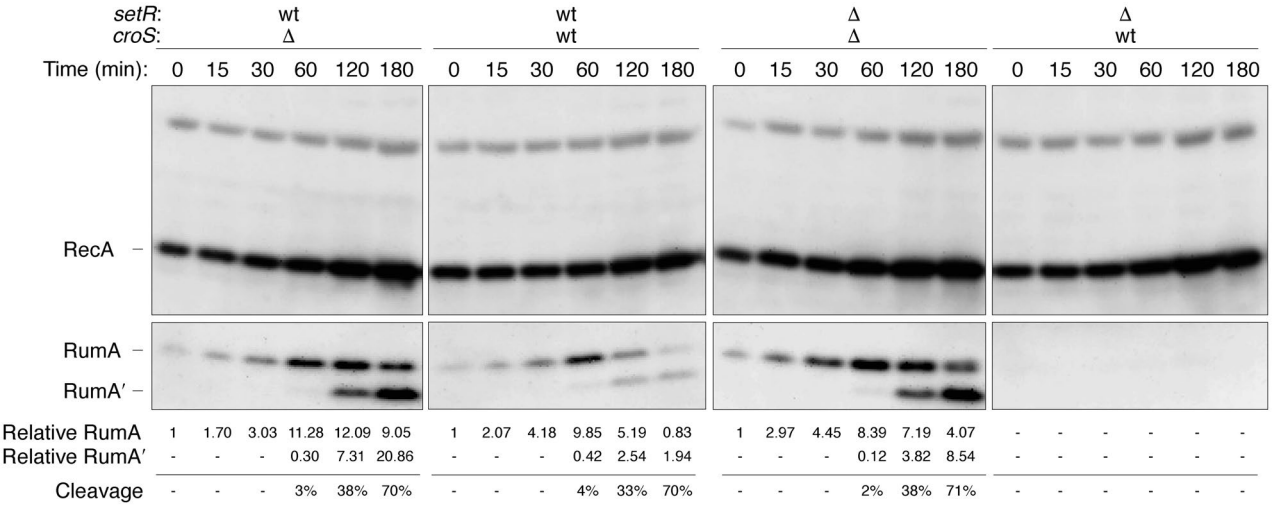

FIGURE 6 Expression of RecA, RumA, and RumA′ in wild-type *recA*$^+$ *lexA*$^+$ cells after exposure to the SOS-inducing antibiotic, Ciprofloxacin. Western blot analysis was performed on whole-cell protein extracts from RW520 (*recA*$^+$ *lexA*$^+$) harboring pRLH421 derivatives (pJM1355, pJM1356, pJM1359, or pJM1360), with various *croS*$_{R391}$ or *setR*$_{R391}$ wild type, or deletion combinations as indicated. To induce the SOS response, cells were treated with 30 ng ml^{-1} Ciprofloxacin for various times, as indicated in the figure. Levels of RecA and RumA/RumA′ were detected using affinity purified polyclonal rabbit antibodies to RecA and RumA proteins. The number reported for the level of RumA or RumA' is relative to a cross-reacting band in the same track

therefore likely that polV orthologs are also subject to strict regulation. Indeed, since the mid-1990s, when the R391 *rumAB* operon was sequenced, it has been known that *rumAB*-encoded polV$_{R391}$ is regulated by the LexA transcriptional repressor (Kulaeva et al., 1995). Recently, we reported that the *rumAB* operon is also regulated by the λ cI-like transcriptional repressor, SetR$_{R391}$ (Gonzalez et al., 2019). Here, we provide *in vivo* data that are compelling and consistent with CroS$_{R391}$ as acting as a third and potentially the most potent transcriptional regulator of the *rumAB* operon. For example, when expressed in *cis*- or *trans*- with *rumAB*, CroS$_{R391}$ completely inhibits its polV$_{R391}$-dependent mutagenesis (Figures 3 and 4). The lack of polV$_{R391}$ mutagenesis *in vivo* is attributed to extremely low-level expression of RumA (Figure 4) and RumB (unpublished observations) from its native promoter in the presence of CroS$_{R391}$. The most reasonable explanation for such a phenotype is the unhindered access that CroS$_{R391}$ has to the single SetR-CroS binding site in the promoter region of the *rumAB* operon (Figure 1), where it acts as a strong transcriptional repressor of the *rumAB* operon. Interestingly, expression of RumA actually increases when CroS$_{R391}$ and SetR$_{R391}$ are co-expressed (Figures 4 and 6), implying that SetR$_{R391}$ may at least partially block CroS$_{R391}$ access and binding to the SetR-CroS binding site, thereby preventing it from acting as a potent transcriptional repressor. Indeed, expression of RumA in a wild-type strain after antibiotic-induced SOS induction appears to result from an interplay of all three transcriptional repressors—LexA, SetR$_{R391}$, and CroS$_{R391}$—such that extremely low levels of polV$_{R391}$ are only present, even after full induction of the SOS response (Figure 6).

The polV$_{R391}$ is a potent mutator DNA polymerase when uncoupled from its normal regulatory pathways (Figure 2). Its unprecedented regulation by three separate transcriptional repressors, two of which (LexA and SetR) are cleaved and inactivated after DNA damage, therefore only allows for the very limited expression of the highly error-prone DNA polymerase (Figure 6), before the third (non-cleavable) repressor (CroS), curtails RumAB expression, and provides a mechanism whereby the cell returns to a resting state, with low levels of cellular mutagenesis.

3.2 | Inactivation of *croS*$_{R391}$ increases the potential of enhanced mutagenesis

Deletion of *croS*$_{R391}$ on the intact R391 stably integrated into the *E. coli* genome allowed us to evaluate the effect of the CroS$_{R391}$ protein on repression of the *rumAB*$_{R391}$ operon and mutagenesis from R391 in its native locus. Utilizing both a qualitative *galK2*(Oc) reversion papillation assay (Figure 7) and a quantitative assay rifampicin resistance assay (Figure 8), we found that mutagenesis is significantly increased in MVG114 strains harboring R391 Δ*croS* as compared with strains harboring wild-type R391. These results confirm our assertion that the CroS$_{R391}$ protein is the major R391-encoded negative regulator of the *rumAB*$_{R391}$ operon and indicate that there are likely no additional *rumAB*$_{R391}$ regulators encoded on the full-length R391.

R391 is widely distributed in enterobacteriaceae that are opportunistic pathogens that cause a variety of infections in humans (Bie et al., 2017; Fang et al., 2018; Kong et al., 2020; Slattery et al., 2020; Song et al., 2013). Given that CroS$_{R391}$ appears to be the "master regulator" to switch off *rumAB* expression, naturally occurring inactivating mutations in *croS* therefore have the potential of increasing the development and proliferation of polV$_{R391}$-dependent antibiotic resistance in a wide range of pathogenic microorganisms.

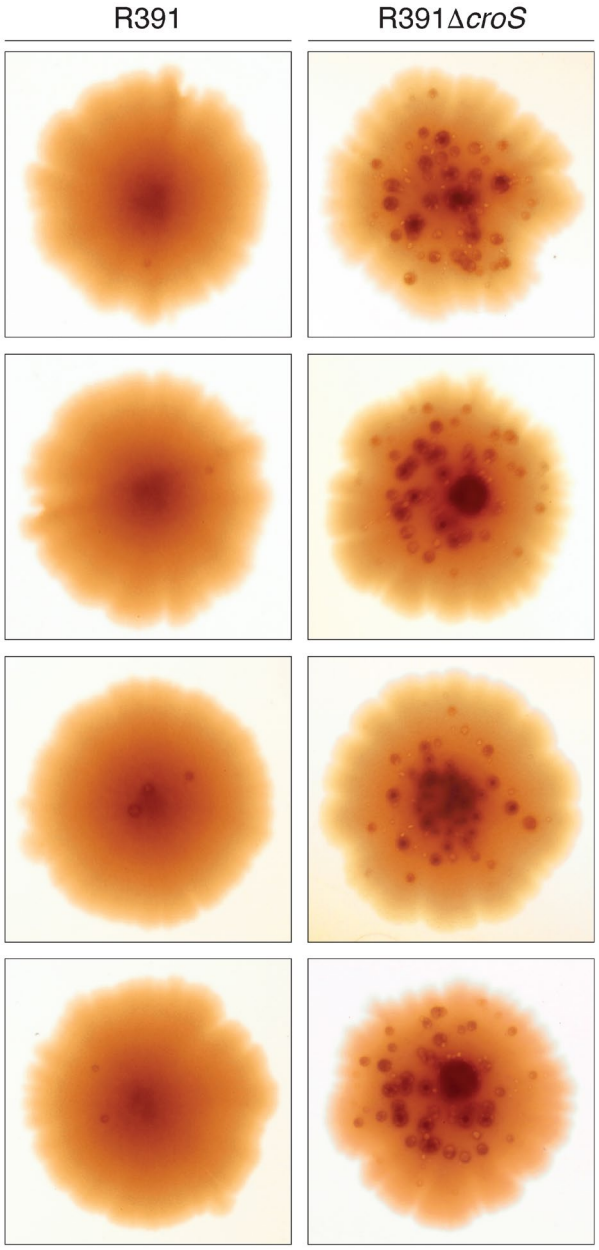

FIGURE 7 The *galK2*(Oc) reversion papillation assay of R391 or R391Δ*croS* strains. R391 or R391Δ*croS* were moved into MVG114 by conjugal transfer. Cells were plated onto MacConkey-galactose agar media and grown for 8 days at 37°C. Pictures of representative colonies from each strain were taken showing the appearance of Gal+ papillae indicating the level of mutagenesis occurring within the colonies. The MVG114/R391Δ*croS* colonies contain approximately 10–50 times the number of revertant papillae, when compared to the MVG114/R391 colonies

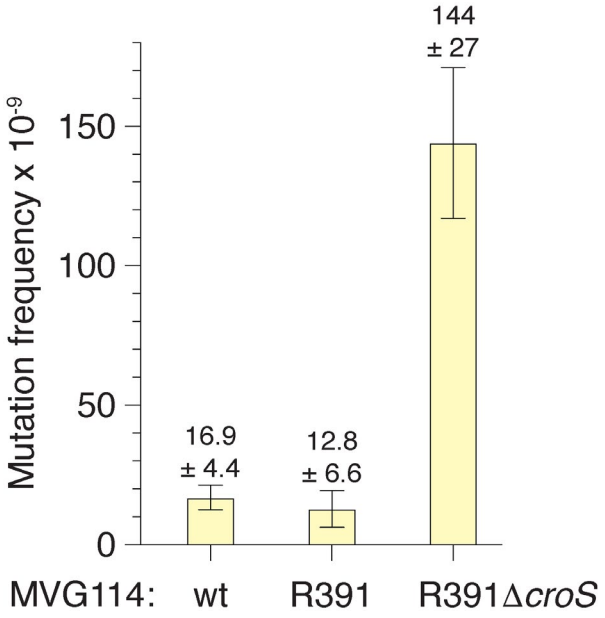

FIGURE 8 Rifampicin mutagenesis in R391 and R391Δ*croS* strains. MVG114, MVG114/R391, and MVG114/R391Δ*croS* cultures were started from with approximately 1,000 cells, or less, and grown overnight to stationary phase. Cells were plated onto LB agar plates containing rifampicin to select for rifampicin-resistant mutants. Appropriate dilutions were plated to LB agar plates to determine viable counts, and the frequency of mutagenesis to rifampicin resistance was calculated. Error bars represent the standard error of the mean (*SEM*). The MVG114/R391Δ*croS* strain exhibits an ~10-fold higher frequency of mutagenesis to rifampicin resistance than the MVG114/R391 strain

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and plasmids

Plasmids are listed in Table 1. Bacterial strains are listed in Table 2.

Previously, R391 DNA (formerly known as the IncJ plasmid, R391) was isolated in its unintegrated episomal form in an *E. coli* strain harboring the *recA718* allele (RW96) (Ho et al., 1993). This episomal R391 DNA was partially digested with *Eco*RI, and an ~21.5-kb fragment was cloned into the low-copy-number vector pGB2 (Churchward et al., 1984) to generate pRLH421 (Genbank U13633) (Gonzalez et al., 2019). Unfortunately, the pRLH421 plasmid contains a truncation of the $croS_{R391}$ gene in the divergent operon with $setR_{R391}$ at an internal *Eco*RI within the $croS_{R391}$ gene. To reconstruct the $croS_{R391}$ gene, a fragment, designated "croS complete NarI-PmeI-Bsu36I," was synthesized (Genscript) that includes from the *Nar*I site to the *Eco*RI site of pGB2, the complete $croS_{R391}$ open reading frame, the upstream promoter sequences, and a *Bsu*36I site replacing the start of the $setR_{R391}$ gene. The *croS* complete fragment was subcloned into pRLH421 from the *Nar*I (in pGB2) to *Pme*I (in the promoter region) to create an intact $croS^{+}_{R391}$ $setR^{+}_{R391}$ operon (pJM1356). Further, the $croS_{R391}$ complete fragment was subcloned into pRLH421 from the *Nar*I to *Bsu*36I (in $setR_{R391}$) to create a $croS^{+}_{R391}/\Delta setR_{R391}$ operon within only 222 bp of the 3′ end of the $setR_{R391}$ gene (pJM1360). Another fragment designated "croS deletion NarI-PmeI-Bsu36I," was synthesized (Genscript) from the *Nar*I site to the first five bases of the *Eco*RI site of pGB2, the promoter sequences upstream of the $croS_{R391}$ start ATG and a *Bsu*36I site replacing the start of the $setR_{R391}$ gene. The *croS* deletion fragment was subcloned into pRLH421 from the *Nar*I to *Pme*I to create a $\Delta croS_{R391}/setR^{+}_{R391}$ operon (pJM1355). Further, the $croS_{R391}$ deletion fragment was subcloned into pRLH421 from the *Nar*I to *Bsu*36I to create a $\Delta croS_{R391}/\Delta setR_{R391}$ operon (pJM1359).

TABLE 2 *E. coli* strains used in this study

Strain	Relevant genotype	Source or reference
RW120[a]	Δ*umuDC595*::*cat hisG4*(Oc) *galK2*(Oc)	(Ho et al., 1993)
RW546[a]	Δ*umuDC595*::*cat lexA51*(Def) *hisG4*(Oc) *galK2*(Oc)	(Fernández de Henestrosa et al., 2000)
RW578[a]	Δ*umuDC595*::*cat lexA51*(Def) *recA730* *hisG4*(Oc) *galK2*(Oc)	(Mead et al., 2007)
RW520[a]	Δ*umuDC596*::*ermGT hisG4*(Oc) *galK2*(Oc)	LGI stocks
RW584[a]	Δ*umuDC596*::*ermGT lexA51*(Def) *recA730* *hisG4*(Oc) *galK2*(Oc)	(McDonald et al., 2012)
MVG114[a]	Δ*umuDC596*::*ermGT lexA51*(Def) *recA718* *hisG4*(Oc) *galK2*(Oc)	(Gonzalez et al., 2019)
MVG114[a]	R391	This study
RW1766[b]	Δ*dinB61*::*ble*	This study
MVG114[a]	R391 Δ*croS*	This study

[a]Full genotype: *thr-1 araD139* Δ*(gpt-proA)62 lacY1 tsx-33 glnV44 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211*.

[b]Δ*dinB61*::*ble* derivative of MG1655 ($F^- \lambda^-$ *rph-1*).

TABLE 3 Oligonucleotides used in this study

Name	Sequence	Source
P1[a]	CTCGCTTTTGTGTTTACGTAAAATGCTGACCATTATTTGCCCTTAATCCTAAATTAACCCTCACTAAAGGGCGG	BioSpring
P2[a]	CAGTTTAAACTGTAACATTCAATCTTGTAACAGTTATTATTGTAACAGGAGACGGTTAGCACGGAGTTCATTAGGGCTC	Biospring
cp01[b]	GCGATGCCATACCAATGAAGTCG	BioSpring
cp02[b]	CAACGTAACCAATCGGCAGTCG	BioSpring
cp03[b]	CCAGTGACTTCACGCCACTCC	BioSpring
cp04[b]	CAGGATCAACAACGATCACTGCC	BioSpring

[a]Used in the generation of linear *knock out* cassette.

[b]Used in the amplification of the modified region and subsequent DNA sequencing.

To create plasmids that carry just these four iterations of the $croS_{R391}/setR_{R391}$ operon, the plasmids pJM1355, pJM1356, pJM1359, and pJM1360 were digested with *Sca*I and *Sma*I and re-ligated, which deletes ~20.3 kb of R391, leaving only the $croS_{R391}/setR_{R391}$ region including about 200 bases downstream of the end of the $setR_{R391}$ gene. These plasmids are designated pJM1365 ($\Delta croS_{R391}/setR^+_{R391}$), pJM1366 ($croS^+_{R391}/setR^+_{R391}$), pJM1367 ($\Delta croS_{R391}/\Delta setR_{R391}$), and pJM1368 ($croS^+_{R391}/\Delta setR_{R391}$).

The *rumAB* operon and the *rumAB* promoter region were cloned into the copy control plasmid, pCC1 (Genscript), to generate pJM1378. In addition, the *rumAB* operon fused to the *E. coli recA* promoter region was cloned into pCC1 to generate pJM1467 (*recA-prom*::*rumAB*) (Genscript). These low-copy pCC1Bac plasmids are compatible with the pGB2-based $croS_{R391}/setR_{R391}$ plasmids described above, such that the various iterations of the $croS_{R391}/setR_{R391}$ operon can be co-expressed with the *rumAB* operon.

4.2 | Generation of R391Δ*croS*

RW1766/R391 was constructed by inoculating an individual colony of RW1766 and RW120/R391 into 5-ml LB and incubating overnight at 37°C without shaking. The next morning, the culture was streaked on to plates containing Zeocin (25 μg ml^{-1}) and Kanamycin (50 μg ml^{-1}) to select for isolates of RW1766 that had acquired R391 via bacterial conjugation.

The marker-less removal of the *croS* gene from the R391 element of *E. coli* RW1766/R391 was performed according to Zhang et al. (1998) (see Figure S5). In a first recombination step, a linear *knock out* cassette, generated with primers listed in Table 3, was used to replace the *croS* gene via Red/ET recombination by using plasmid pRed/ET. This resulted in a chloramphenicol-resistant intermediate strain with the genotype Δ*croS*::FRT-Cm^R-FRT (Figure S5). In a second recombination step, the selection marker was removed in a FLP recombinase-mediated fashion, leaving a single FRT site at the former *croS* locus (genotype: Δ*croS*::FRT). The intermediate and final clones were analyzed by Sanger sequencing using primers listed in Table 3 covering the complete modified and adjacent regions.

New strains containing wild-type R391 or R391Δ*croS* were made by conjugal transfer of the ICE from RW1766 (Table 2) to the desired recipient strain by selecting on plates containing Streptomycin (100 μg ml^{-1}) and Kanamycin (50 μg ml^{-1}).

4.3 | Qualitative analysis of spontaneous reversion of the *hisG4*(Oc) allele

The *E. coli* strain MVG114 was transformed with various plasmid constructs expressing $CroS_{R391}$, $SetR_{R391}$, and RumAB, either alone or in various iterations (Table 1). To assay for reversion of the *hisG4(Oc)* allele, three to five isolates of each strain were grown overnight at 37°C in LB medium containing the appropriate antibiotic(s). Five hundred microliters of the cultures were centrifuged, and the pellets were resuspended in an equal volume of SM buffer. One hundred microliters of the resuspended pellets were spread on each of the five low-histidine minimal plates (Davis and Mingioli minimal agar plates; Davis & Mingioli, 1950): plus glucose (0.4% wt/vol); agar (1.0% wt/vol); proline, threonine, valine, leucine, and isoleucine (all at 100 μg ml^{-1}); thiamine (0.25 μg ml^{-1}); and histidine (1 μg ml^{-1}). After incubating the plates for 4 days at 37°C, the His^+ mutant colonies were counted and averaged between the independent cultures and standard error of the mean calculated.

4.4 | *The galK2*(Oc) reversion papillation assay

The *galK2*(Oc) papillation is a visual reversion mutagenesis assay that was previously used to identify genes from R-plasmids that encode orthologs of the *E. coli umuDC* genes including *rumAB* from R391 (Ho et al., 1993). Briefly, ~ 50–75 *E. coli* cells harboring the *galK2*(Oc) allele are plated onto MacConkey-galactose agar media and grown for 8 days at 37°C. A number of small orange-red Gal^+ papillae that grow up within a bacterial colony are noted from multiple colonies. Comparison of the number of papillae allows the assessment of the level of mutagenesis in the MVG114 strains harboring R391 versus MVG114 harboring R391Δ*croS*.

4.5 | Rifampicin mutagenesis assay

Selection for rifampicin resistance is another generally used mutagenesis assay. Base-pair substitution mutations arising within the central 202 bp of the *rpoB* gene can give rise to resistance to the rifampicin antibiotic. Five milliliter cultures of MVG114/R391 and MVG114/R391Δ*croS* were started, in triplicate, from an initial inoculum containing ~1,000 viable cells and grown for 24 hr at 37°C. One hundred microliters of these cultures were spread on five LB agar plates containing 100 μg ml^{-1} rifampicin. In addition, these cultures were serially diluted, and appropriate volumes were plated to LB agar plates to determine viable cell counts. Subsequently, frequencies of rifampicin mutations arising within the cultures were calculated.

4.6 | Western blot analysis of $CroS_{R391}$, $RumA_{R391}$, and RecA proteins

E. coli cultures were grown in Luria-Bertani media at 37°C until exponential phase (OD_{600} ~0.5). For the experiments shown in Figure 6, Ciprofloxacin (30 ng ml^{-1}) was added to the culture and cells were harvested by centrifugation at subsequent time points (as indicated in Figure 6). For all other experiments, undamaged cells were harvested at an OD_{600} ~0.5. The cell pellet was resuspended in NuPage LDS sample buffer (Novex) and freeze-thawed to produce the whole cell extracts. Cell extracts were electrophoresed on NuPage 4%–12% Bis-Tris gels (Novex). Proteins were transferred to an Invitrolon polyvinylidene fluoride membrane (Novex) that was probed with appropriate dilutions of affinity purified rabbit anti-$CroS_{R391}$, anti-$RumA_{R391}$, or anti-*E. coli* RecA antibodies and subsequently probed with an appropriate dilution of Goat Anti-Rabbit IgG (H+L)-AP Conjugate (Bio-Rad). Using the CDP-Star chemiluminescent assay (Tropix), the $CroS_{R391}$, $RumA_{R391}/A'_{R391}$, or RecA proteins were visualized on Carestream Biomax XAR Film after various exposure times. Digital images were also captured using an Alpha Innotech FluorChem HD2. These images were then imported as .tif files into LI-COR Biosciences Image Studio Lite software, where band density was quantified using the Data Analysis tool. Relative protein levels for bands of interest were calculated by normalizing band density to that of nonspecific bands within each lane and then expressed relative to a cross-reacting reference band.

4.7 | Overexpression and purification of CroS

The gene encoding R391 *croS* was codon optimized for expression in *E. coli* and chemically synthesized (Genscript) as a 309 bp *Nde*I-*Pst*I fragment and cloned into pUC57 (Genscript). The *Nde*I-*Pst*I fragment was subsequently subcloned into the same sites of pCF2. pCF2 is a medium copy plasmid that expresses Isopropyl ß-D-1-thiogalactopyranoside-inducible glutathione-*S* transferase (GST). The construct also contains a PreScission (GE Healthcare) protease site immediately downstream of the GST protein and upstream of the unique *Nde*I site. When the target gene is cloned into the *Nde*I site of pCF2, a GST-fusion protein is generated with a PreScission site immediately upstream of the target protein. CroS was initially purified as a GST-CroS fusion protein as a custom service by scientists at Eurofins, as previously described (Poulin-Laprade & Burrus, 2015), and the GST-affinity tag subsequently removed after the PreScission protease treatment (Eurofins).

4.8 | CroS antibodies

Polyclonal antibodies to the purified CroS protein were raised in rabbits as a custom service (Covance) and affinity purified. These antibodies are highly specific with very few cross-reacting bands in western blots of *E. coli* extracts lacking CroS.

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assistance in generating the figures shown herein, as well as quantifying the levels of RumA and RumA′ reported in Figures 4–6, and S5.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with the content of this article.

AUTHOR CONTRIBUTIONS

Conceptualization: JPM, MG, and RW
Funding Acquisition: MG and RW
Investigation: JPM, DRQ, AV, ARM, JR, MS, MG, and RW
Writing-Original Draft: JPM and RW
Writing-Review and Editing: JPM, DRQ, AV, ARM, JR, MS, MG, and RW.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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SUPPORTING INFORMATION

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