Determinants of the cytotoxicity of PrrC anticodon nuclease and its amelioration by tRNA repair

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ABSTRACT

Breakage of $tRNA^{Lys(UUU)}$ by the *Escherichia coli* anticodon nuclease PrrC (*Eco*PrrC) underlies a host antiviral response to phage T4 infection that is ultimately thwarted by a virus-encoded RNA repair system. PrrC homologs are prevalent in other bacteria, but their activities and substrates are not defined. We find that induced expression of *Eco*PrrC is toxic in *Saccharomyces cerevisiae* and *E. coli*, whereas the *Neisseria meningitidis* PrrC (*Nme*PrrC) is not. PrrCs consist of an N-terminal NTPase module and a C-terminal nuclease module. Domain swaps identified the *Eco*PrrC nuclease domain as decisive for toxicity when linked to either the *Eco* or *Nme* NTPase. Indeed, a single arginine-to-tryptophan change in the *Nme*PrrC nuclease domain (R316W) educed a gain-of-function and rendered *Nme*PrrC toxic to yeast, with genetic evidence for $tRNA^{Lys(UUU)}$ being the relevant target. The reciprocal Trp-to-Arg change in *Eco*PrrC (W335R) abolished its toxicity. Further mutagenesis of the *Eco*PrrC nuclease domain highlighted an ensemble of 15 essential residues and distinguished between hypomorphic alleles and potential nuclease-nulls. We report that the RNA repair phase of the bacterial virus-host dynamic is also portable to yeast, where coexpression of the T4 enzymes Pnkp and Rnl1 ameliorated the toxicity of *Nme*PrrC-R316W. Plant tRNA ligase AtRNL also countered *Nme*PrrC-R316W toxicity, in a manner that depended on AtRNL's 5′-kinase and ligase functions.

Keywords: RNA ligase; polynucleotide kinase-phosphatase; ribotoxin; tRNA breakage; wobble uridine modification

INTRODUCTION

The *Escherichia coli* tRNA anticodon nuclease PrrC (*Eco*PrrC) mediates an RNA-damaging innate immune response to bacteriophage T4 infection (Kaufmann 2000). The normally latent *Eco*PrrC nuclease is switched on by the virus-encoded Stp peptide synthesized early during T4 infection (Amitsur et al. 1989, 1992; Penner et al. 1995). The activated form of *Eco*PrrC incises the $tRNA^{Lys(UUU)}$ anticodon loop at a single site 5′ of the wobble uridine, leaving 2′,3′ cyclic phosphate and 5′-OH ends at the break. Ensuing depletion of functional $tRNA^{Lys}$ blocks the synthesis of T4 late proteins and prevents spread of the virus through the bacterial population. However, phage T4 thwarts the host cell's defense strategy by encoding a tRNA repair system, consisting of polynucleotide kinase-phosphatase (Pnkp) and RNA ligase 1 (Rnl1), that heals and seals the broken tRNA ends (Amitsur et al. 1987).

*Eco*PrrC consists of two domains: an N-terminal nucleoside triphosphate phosphohydrolase (NTPase) module (aa 1–264) related to the ABC transporter NTPase family and a distinctive C-terminal ribonuclease module (aa 265–396) that has no apparent similarity to any known nuclease or tRNA binding protein (Kaufmann 2000; Blanga-Kanfi et al. 2006). PrrC homologs are present in the proteomes of many other bacteria, though their biological activities and RNA targets are uncharted. We reported recently that the ribotoxicity of bacterial PrrC is portable to eukarya. Specifically, we found that induced expression of *Eco*PrrC in budding yeast cells is fungicidal, signifying that *Eco*PrrC is toxic in a eukaryon in the absence of any other bacterial or viral proteins (Meineke et al. 2011). Testing for rescue of toxicity by increased tRNA gene dosage implicated $tRNA^{Lys(UUU)}$ as an *Eco*PrrC target in yeast. An extensive survey of the effects of alanine and conservative mutations on *Eco*PrrC toxicity in yeast identified 22 essential residues in the NTPase domain and 11 in the nuclease domain and delineated structure-function relationships at each essential position (Meineke et al. 2011). Overexpressing PrrCs with inactivating mutations in the NTPase active site ameliorated the toxicity of wild-type *Eco*PrrC; these dominant

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negative effects were not observed with PrrCs containing inactivating mutations in the nuclease domain. Our findings, building on the elegant studies of *Eco*PrrC by Gabi Kaufmann and colleagues (Meidler et al. 1999; Jiang et al. 2001, 2002; Amitsur et al. 2003; Blanga-Kanfi et al. 2006), support a model in which *Eco*PrrC toxicity is contingent on head-to-tail dimerization of the ABC-like NTPase domains and the consequent formation of two composite NTP phosphohydrolase active sites, which in turn activates the nuclease domains in *cis*, only one of which needs be functional.

Remarkably, not all bacterial PrrCs are created equal with respect to their activity in yeast, e.g., *Streptococcus mutans* PrrC (*Smu*PrrC) is toxic in yeast, whereas *Neisseria meningitidis* (*Nme*PrrC) is benign (Meineke et al. 2011). The failure of *Nme*PrrC to arrest yeast growth was surprising to us, insofar as the nontoxic *Nme*PrrC protein has a significantly higher degree of amino acid identity (57%) with the *Eco*PrrC polypeptide than does the toxic *Smu*PrrC (42%). It is conceivable that (1) *Nme*PrrC is nontoxic in yeast because it lacks RNase activity; (2) *Nme*PrrC is a bonafide ribotoxin, but its target is not present in budding yeast (or is present but not essential for yeast growth); or (3) *Nme*PrrC requires additional proteins (or activating metabolites) from the cognate bacterium to manifest its RNase functions.

In the present study, we explored this issue by studying a series of chimeric PrrCs. Domain swaps established that the *Eco*PrrC nuclease module is decisive for yeast toxicity when linked to either the *Eco* or *Nme* NTPase domain. Inspection of the primary structure differences between the nuclease domains of toxic (*Eco* and *Smu*) and nontoxic (*Nme*) PrrCs highlighted potential candidate toxicity determinants that we queried by mutating individual side chains in *Nme*PrrC to their counterparts in *Eco*PrrC. We thereby identified a single amino acid change in *Nme*PrrC (arginine to tryptophan) that elicited a gain-of-function and rendered it toxic to yeast. The reciprocal Trp to Arg change at the corresponding residue in *Eco*PrrC ablated its toxicity in yeast. From the results of tRNA rescue experiments and the effects of genetic manipulation of the wobble uridine modification on the activity of the ''enabled'' *Nme*PrrC-R316W mutant, we surmised that $tRNA^{Lys(UUU)}$ is the relevant target for the *Nme*PrrC-R316W ribotoxin.

We extended these findings by showing that the RNA repair phase of the bacterial tRNA restriction-repair host-virus dynamic is also portable to yeast, where coexpression of the T4 enzymes Pnkp and Rnl1 ameliorated the toxicity of *Nme*PrrC-R316W. Expression of plant tRNA ligase in yeast also countered *Nme*PrrC-R316W toxicity. Our findings, in conjunction with earlier studies (Nandakumar et al. 2008), offer proof of principle for the ability of RNA repair to modulate the effects of programmed tRNA damage in eukarya.

RESULTS

Domain swaps between *Eco*PrrC and *Nme*PrrC implicate the nuclease domain as the source of species-variation in PrrC toxicity

The *E. coli* and *N. meningitidis prrC* genes were introduced into yeast on *CEN* plasmids under the control of a glucose-repressed/galactose-inducible *GAL1* promoter. *Eco*PrrC induction inhibited yeast growth on agar medium containing galactose, whereas *Nme*PrrC induction had no effect on cell growth (Fig. 1). An inference from these results is that *Eco*PrrC can incise essential target RNAs in yeast, but *Nme*PrrC cannot.

Turning to the question of why *Nme*PrrC is nontoxic, we performed reciprocal domain swap experiments, in which the N-terminal NTPase domain of *Eco*PrrC (aa 1–264) was fused to the C-terminal nuclease domain of *Nme*PrrC (aa 246–380) and the *Nme*PrrC NTPase domain (aa 1–245) was joined to the *Eco*PrrC nuclease module (aa 265–396) (Fig. 1). The N-*Nme*/*Eco*-C chimera was clearly toxic in yeast, while the N-*Eco*/*Nme*-C hybrid was not (Fig. 1). However, the very faint growth of the *Nme*/*Eco*PrrC-expressing yeast cells on galactose agar seen with the more concentrated cell spottings (Fig. 1) suggested that the *Nme*/*Eco* hybrid is a genetic hypomorph vis à vis *Eco*PrrC (see below). We conclude that the *Eco*PrrC nuclease module is decisive for yeast toxicity when linked to either the *Eco* or *Nme* NTPase domain.

To see if the differential toxicity of bacterial PrrCs also obtains in a bacterium, we tested the effects of induced expression of the *Eco, Smu,* and *Nme* PrrCs and the *Eco*/*Nme* and *Nme*/*Eco* chimeras, on the growth of *E. coli*. The respective *prrC* genes were introduced into *E. coli* Top10 cells on pBAD plasmids under the control an arabinose-inducible promoter. Serial dilutions of *E. coli* pBAD-*prrC*

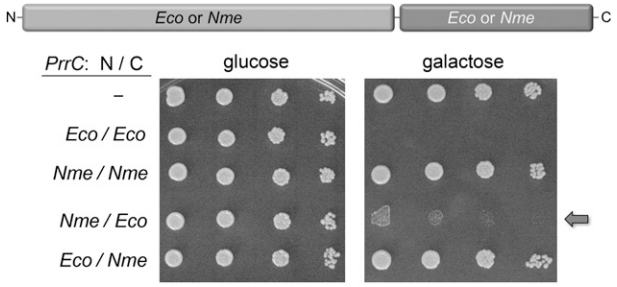

FIGURE 1. Domain swaps between toxic *Eco*PrrC and nontoxic *Nme*PrrC. Bacterial PrrC proteins consist of an N-terminal NTPase domain fused to a C-terminal nuclease domain, as shown. *Eco*PrrC and *Nme*PrrC, and domain-swapped PrrCs (N-*Eco*/*Nme*-C and N-*Nme*/*Eco*-C), were tested for their effects on the growth of *S. cerevisiae*. Serial fivefold dilutions of yeast cells bearing a *CEN* plasmid encoding the indicated galactose-regulated *prrC* gene or an empty *CEN* vector (–) were spotted on –Leu agar plates containing glucose or galactose as specified.

cultures grown in LB medium were plated on LB agar (*prrC* expression repressed) or LB agar with 0.2% arabinose (*prrC* expression induced). The results showed that *Eco*PrrC and *Smu*PrrC were toxic to *E. coli*, whereas *Nme*PrrC was not (Fig. 2). The *Nme*/*Eco* hybrid inhibited growth of *E. coli* on arabinose agar, albeit not as profoundly as wild-type *Eco*PrrC, as indicated by the tiny colony size of *Nme*/*Eco*PrrC-expressing bacteria (Fig. 2). In contrast, arabinose induction of the *Eco*/*Nme*PrrC hybrid had no effect on *E. coli* growth (Fig. 2). We surmise from the results shown in Figures 1 and 2 that it is not simply the case that the eukaryal milieu masks an intrinsic ribotoxin activity of *Nme*PrrC and the *Eco*/*Nme* hybrid. Rather, it seems that members of the PrrC family differ with respect to their biological activity, which could reflect distinctive RNA target specificities and/or reliance on unique species-specific coactivators. It is pertinent to note that, whereas *Eco*PrrC targets $tRNA^{Lys(UUU)}$ in *E. coli* and yeast, the imputed ribotoxicity and possible RNA targets of *Nme*PrrC are tabula rasa, even in *Neisseria*.

We next examined the effects of transient expression of the toxic *Eco* and *Smu* PrrCs on *E. coli* survival. The *Eco*PrrC and *Smu*PrrC expression plasmids had no effect on the rate of bacterial growth in liquid medium lacking arabinose, i.e., compared to the growth of control bacteria carrying the empty vector (Supplemental Fig. S1A). In contrast, the growth of bacteria carrying the *Eco* and *Smu* PrrC plasmids was arrested by 3 to 4 h after transfer to arabinose-containing medium, an effect not seen with bacteria carrying the empty vector (Supplemental Fig. S1A). By analyzing bacterial survival after transient arabinose induction in liquid medium and return to control medium, we found that *Eco*PrrC expression was bacteriostatic, i.e., the number of viable bacteria in the culture was stable for the 5-h interval of arabinose exposure. (Supplemental Fig. S1B). In contrast, *Smu*PrrC was bactericidal, eliciting a 240-fold decrement in the viable cell count by 3 h of SmuPrrC induction (Supplemental Fig. S1B). We noted progressive recovery of viability at 4 and 5 h post-induction of *Smu*PrrC, suggesting the outgrowth of survivors. The instructive point here is that the cytostatic effect of *Eco*PrrC expression in *E. coli* contrasts with its cytocidal properties in budding yeast (Meineke et al. 2011), as opposed to *Smu*PrrC expression, which is cytocidal in bacteria and yeast.

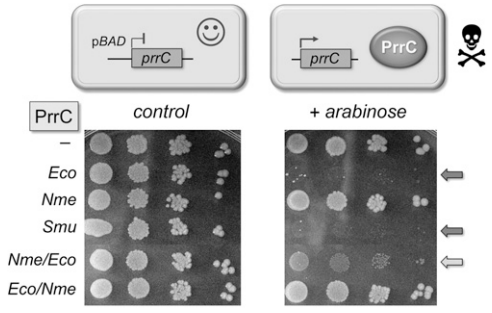

FIGURE 2. The nuclease domain is an exchangeable determinant of PrrC toxicity in *E. coli*. Serial dilutions of *E. coli* cells bearing a pBAD plasmid encoding the indicated arabinose-regulated *prrC* gene or an empty vector were spotted on LB-ampicillin agar plates with or without arabinose. When grown on control medium lacking arabinose, *prrC* expression is switched off, and the bacteria grow normally. When grown on medium containing 0.2% arabinose, the *prrC* expression is turned on, and if the PrrC ribotoxin is active, bacterial growth is arrested (absence of colonies) or slowed (presence of tiny colonies).

Species-specific toxicity determinants in the PrrC nuclease domain

To vet the hypothesis that the PrrC nuclease domain harbors key determinants of RNA target specificity, we attempted to coax the nontoxic *Nme*PrrC to become toxic in yeast. Our search for gain-of-function mutations was guided by alignment of the nuclease domains of the *Eco*, *Smu*, and *Nme* PrrCs, which highlighted seven ''deviant'' amino acids in nontoxic *Nme*PrrC that we changed to the ''consensus'' equivalents present in the toxic *Eco*PrrC and *Smu*PrrC proteins, individually or as a pairwise change in vicinal residues (Fig. 3A). We found that five of the *Nme*PrrC mutants (N309S, E322D, Q354K, D366N, and E288A-N289D) remained nontoxic in yeast. In contrast, the *Nme*PrrC R316W mutant was enabled by a single amino acid substitution to arrest the growth of yeast cells on medium containing galactose (Fig. 3B). Thus, a gain-of-function was achieved. In a similar vein, we were able to convert the nontoxic *Eco*/*Nme* PrrC chimera (Fig. 1) into an active ribotoxin in yeast by the equivalent Arg-to-Trp mutation in its *Nme*-derived nuclease domain (data not shown).

If the identity of this amino acid as tryptophan is decisive as a toxicity determinant in PrrC, then we might expect mutation of Trp335 in *Eco*PrrC to diminish or eliminate its ribotoxicity. Indeed, changing Trp335 to arginine (to mimic the side chain in *Nme*PrrC) abolished the toxicity of *Eco*PrrC in yeast (Fig. 3C). Similar loss-of-function effects were elicited by changing Trp335 to alanine (which truncates the side chain at the β-carbon) or by conservative substitutions with other γ-branched aromatic amino acids (tyrosine, phenylalanine, or histidine) (Fig. 3C). Thus, tryptophan is strictly essential at this position for the ribotoxicity of *Eco*PrrC in yeast.

We compared the severity of the yeast growth arrest triggered by the two gain-of-function mutants—*Nme*R316W and *Eco/Nme*RW—to that of the active *Nme/Eco* chimeric PrrC. By analyzing yeast survival after transient galactose induction and return to glucose, we found that *Eco/NmeRW* expression was fungicidal; the number of viable cells in the yeast culture decreased by a factor of 20 after 15 h of induction (Supplemental Fig. S2). In contrast, expression of the *Nme*R316W and *Nme/Eco* PrrC proteins was effectively

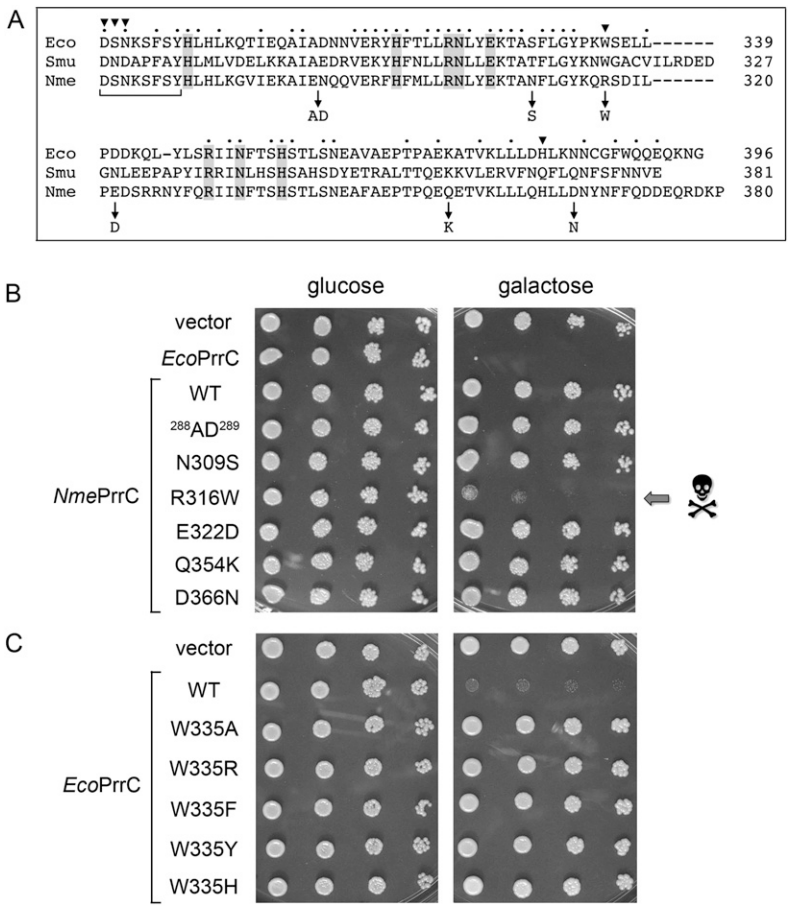

FIGURE 3. A gain-of-function mutation renders *Nme*PrrC toxic to yeast. (*A*) The amino acid sequence of the nuclease domain of *Eco*PrrC is aligned to the homologous segments of *Smu*PrrC and *Nme*PrrC. Positions of side-chain identity/similarity in all three proteins are indicated by • *above* the alignment. The eight conserved residues defined previously as essential for yeast toxicity are shaded gray. Positions of side chain variation between the nontoxic *Nme*PrrC and the toxic *Eco* and *Smu* PrrCs are indicated by arrows *below* the alignment, which specify the *Nme*PrrC mutations tested for gain-of-toxicity in yeast. The LARP motif is demarcated by the bracket *below* the sequences. *Eco*PrrC residues subjected to mutational analysis in the present study are indicated by ▼. (*B*) Serial fivefold dilutions of yeast cells bearing a *CEN* plasmid encoding the indicated galactose-regulated *prrC* gene or an empty *CEN* vector were spotted on –Leu agar plates containing glucose or galactose as specified. (*C*) Toxicity tests for wild-type *Eco*PrrC and the indicated W335 mutants are shown.

cytostatic, i.e., viable cell counts increased less than threefold over 15 h in galactose-containing medium (Supplemental Fig. S2).

Overexpression of yeast tRNA$^{Lys(UUU)}$ blunts the toxicity of *Nme*R316W

If an intracellular ribotoxin exerts its effect by breaking a specific cellular RNA target, then one might expect to reverse the toxicity by overexpressing the RNA target (Jablonowski et al. 2006). We found that a multicopy 2μ plasmid carrying the yeast gene for tRNA$^{Lys(UUU)}$ protected yeast cells from the toxic effects of the gain-of-function mutant *Nme*R316W (Fig. 4, top panel). In contrast, 2μ plasmids bearing genes encoding either the isoacceptor tRNA$^{Lys(CUU)}$ or various other yeast tRNAs (tRNA^{Glu}, tRNA^{Gln}, tRNA^{Arg}, tRNA^{Tyr}, tRNA^{Leu}, or tRNA^{Gly}) had no effect on *Nme*R316W toxicity (data not shown). This instructive result indicated that tRNA$^{Lys(UUU)}$ is a target of *Nme*R316W in yeast. The same tRNA rescue profile was seen previously for two toxic hypomorphs of *Eco*PrrC (Meineke et al. 2011). We surmise that a latent tRNA$^{Lys(UUU)}$ anticodon nuclease activity of *Nme*PrrC is revived by the R316W mutation.

Influence of wobble uridine modifications on the toxicity of *Nme*R316W

*Eco*PrrC incises bacterial tRNA$^{Lys(UUU)}$ at a single phosphodiester 5′ of the modified wobble base mnm^{5}s^{2}U (5-methylaminomethyl-2-thiouridine) (Jiang et al. 2001). The mnm^{5}U wobble modification does not exist in eukaryal tRNAs, which have mcm^{5}s^{2}U (5-methoxycarbonylmethyl-2-thiouridine) instead (Fig. 4). Some tRNA anticodon nucleases rely on the modified wobble base as a target specificity determinant. For example, *K. lactis* γ-toxin requires the mcm^{5}U modification in its tRNA^{Glu} target, such that yeast *elp3*Δ and *trm9*Δ mutants, which either fail to modify the C5 atom or fail to add the terminal methyl group (Fig. 4), are resistant to γ-toxin's effects (Lu et al. 2005, 2008; Jablonowski and Schaffrath 2007). Here we found that *Nme*R316W was toxic to *elp3*Δ cells and that this toxicity was reversed by overexpressing tRNA$^{Lys(UUU)}$ (Fig. 4, middle panel), which signifies that *Nme*R316W can target tRNA with a wobble uridine with no modifications at the C5 atom. In contrast, *Nme*R316W did not prevent growth of the *trm9*Δ cells on galactose; rather it had only a slight effect on growth, as gauged by colony size compared to the vector control, and this slight effect was reversed by 2μ tRNA$^{Lys(UUU)}$ (Fig. 4, bottom panel). We noted similar effects of the yeast *elp3*Δ and *trm9*Δ mutations on the ribotoxin activities of two *Eco*PrrC hypomorphs: S219T and C386A (Meineke et al. 2011). Our findings suggest that *Nme*R316W has gained both the activity and target specificity of *Eco*PrrC in yeast, albeit at the level of a hypomorphic *Eco*PrrC variant.

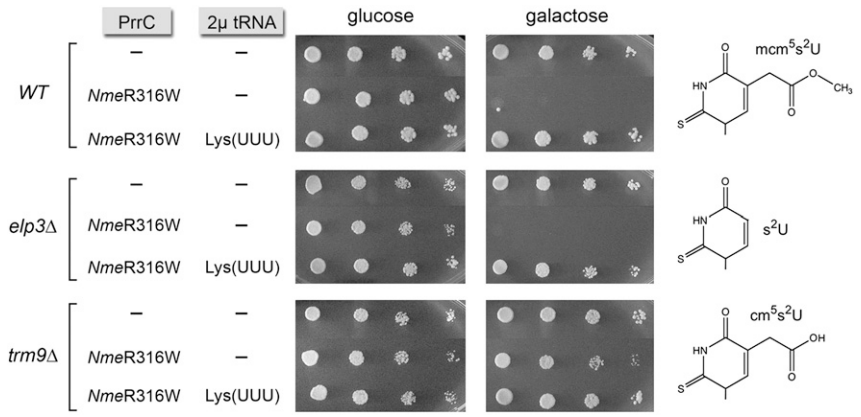

FIGURE 4. Rescue of *Nme*PrrC-R316W toxicity by 2μ $tRNA^{Lys(UUU)}$ and effect of wobble U modification on toxicity. Serial fivefold dilutions of wild-type (WT), *elp3*Δ, and *trm9*Δ yeast cells bearing a *CEN* plasmid encoding galactose-regulated *Nme*PrrC-R316W or the empty *CEN* vector (–) plus a 2μ plasmid carrying the $tRNA^{Lys(UUU)}$ gene or an empty 2μ vector (–) were spotted on –Leu–Ura agar plates containing glucose or galactose. The structures of the wobble uridine modifications found in $tRNA^{Lys(UUU)}$ of wild-type yeast (mcm^5s^2U), and yeast mutants *elp3*Δ (s^2U; 2-thiouridine) and *trm9*Δ (cm^5s^2U; 5-carboxymethyl-2-thiouridine), are shown at *right*.

Heterologous RNA repair enzymes ameliorate the toxicity of *Nme*R316W

Yeast cells are susceptible to tRNA ribotoxins because the endogenous yeast tRNA ligase is unable to rectify the break in the anticodon loop of the tRNAs targeted by the ribotoxin. However, expression of plant or phage T4 tRNA repair enzymes protect yeast from growth arrest by *K. lactis* γ-toxin because they are able to reverse the damage inflicted in the anticodon loop of $tRNA^{Glu(UUC)}$ (Nandakumar et al. 2008). Given that the native function of the T4 RNA repair system is to neutralize the PrrC-driven antiviral response (Amitsur et al. 1987), it was of interest to us to see whether importing phage and plant tRNA repair enzymes into yeast might protect a eukaryon against PrrC's toxicity. Our initial experiments showed that neither plant tRNA ligase (AtRNL) nor T4 Rnl1+Pnkp were able to overcome galactose-induced *Eco*PrrC growth arrest (data not shown). We considered two potential explanations for the negative outcome: (1) that the heterologous repair enzymes were inherently unable to fix the PrrC-induced damage to yeast $tRNA^{Lys(UUU)}$; or (2) that the level of tRNA incision activity of *Eco*PrrC after galactose induction was too vigorous to be offset by the activities of the heterologous repair systems, i.e., the RNA repair system loses an uphill battle against relentless tRNA cleavage. In the latter case, we might expect that dialing back on the strength of the PrrC activity, by expressing a hypomorphic version of the ribotoxin, might tip the dynamic in favor of RNA repair. Indeed, this is what we observed when the *Nme*R316W variant was induced in yeast cells expressing heterologous repair enzymes (Fig. 5).

In this experiment, we introduced *CEN* plasmids expressing Rnl1 and Pnkp into a yeast strain bearing the galactose-regulated *Nme*R316W expression plasmid and then tested growth under toxin-off and toxin-on conditions. For comparison, we also tested the effects of overexpressing the yeast tRNA ligase Trl1 (by introducing a 2μ plasmid bearing *TRL1* under the control of a constitutive yeast *TPI1* promoter) and the plant tRNA ligase AtRNL (delivered on a 2μ plasmid and driven by the *TPI1* promoter). Whereas control cells and 2μ *TRL1* cells did not thrive on medium containing galactose, the phage tRNA repair system and plant AtRNL allowed cell growth (Fig. 5). It is apparent from the colony size that the phage tRNA repair system is more salutary than AtRNL and that neither repair system restored growth on galactose to the level of control cells that lack the *Nme*R316W expression cassette (Fig. 5). This result underscores the theme (Nandakumar et al. 2008) that a repair-based cure of ribotoxicity may be incomplete in the face of constitutive RNA damage.

The observation that plant tRNA ligase rescues cells from *Nme*PrrC-R316W growth arrest while yeast tRNA ligase does not suggests that there are intrinsic differences in the ability of the plant and yeast systems to repair the broken $tRNA^{Lys(UUU)}$ anticodon loop. To probe the roles of the

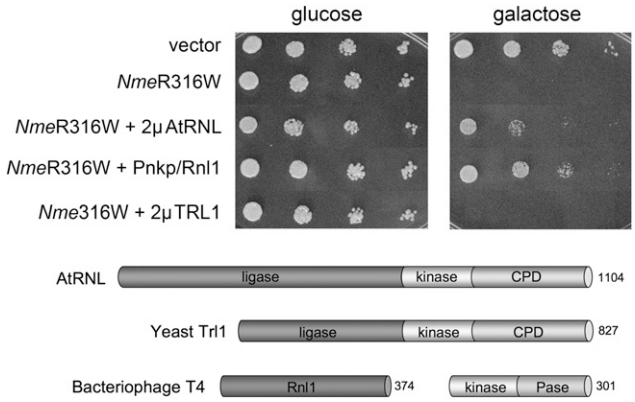

FIGURE 5. Rescue of *Nme*PrrC-R316W toxicity by tRNA repair enzymes. (*Top* panel) Growth of yeast cells bearing a *CEN* plasmid encoding galactose-regulated *Nme*PrrC-R316W or the empty *CEN* vector (–) and either a 2μ *TPI1-AtRNL* plasmid, a 2μ *TPI1-TRL1* plasmid, or a *CEN* plasmid expressing T4 Pnkp and Rnl1 as specified was assessed by spotting serial fivefold dilutions to minimal synthetic agar medium containing glucose or galactose. (*Bottom* panel) The tRNA ligases of plant (AtRNL) and yeast (Trl1) are composed of three discrete catalytic domains: an N-terminal ligase module; a central 5′-OH polynucleotide kinase module; and a C-terminal RNA 2′,3′ cyclic phosphodiesterase (CPD) module. The phage T4 tRNA repair system consists of separate sealing (Rnl1) and healing (Pnkp) enzymes.

three catalytic activities of AtRNL, we tested a collection of mutant AtRNL alleles bearing lethal alanine mutations in the active sites that specifically ablate the ligase (K152A or E326A), kinase (S701A or D726A), or CPD (T1001A or H1060A) functions (Fig. 6; Wang et al. 2006). The wild-type, kinase-dead, ligase-dead, and CPD-dead AtRNL proteins were expressed from 2μ plasmids in yeast cells bearing the *Nme*R316W plasmid and tested in parallel for growth on glucose and galactose media (Fig. 6). None of the AtRNL-Ala mutations affected growth on glucose because tRNA splicing activity is provided by the endogenous Trl1 enzyme. However, these mutations had disparate effects on yeast growth on galactose, depending on which catalytic activity was affected. The CPD activity of AtRNL was not required to confer *Nme*R316W resistance, insofar as the CPD-dead alleles could rescue growth on galactose, albeit not as effectively as wild-type AtRNL (Fig. 6). We surmise that the endogenous level of yeast Trl1 CPD suffices to heal the 2′,3′ cyclic phosphate end of broken $tRNA^{Lys}$. In contrast, the two ligase-dead and the two kinase-dead AtRNL mutants failed to protect against *Nme*R316W (Fig. 6). Thus, the ligase and 5′ kinase activities of AtRNL are essential for *Nme*R316W resistance.

These enzymatic requirements for AtRNL repair of PrrC damage in vivo are different from what was observed for AtRNL rescue of $tRNA^{Glu}$ damage by *K. lactis* γ-toxin. Protection against γ-toxin required the ligase activity but was unaffected by the kinase-dead mutations (Nandakumar et al. 2008). We envision that these differences might be attributable to the fact that PrrC and γ-toxin incise the anticodon loop on opposite sides of the mcm^5s^2U wobble nucleoside in their respective tRNA targets. γ-toxin cleaves on the 3′ side of the wobble nucleoside to form a mcm^5s^2U–2′,3′-cyclic phosphate end that, when hydrolyzed by the CPD activity of Trl1 or AtRNL, will yield an mcm^5s^2U–3′OH, 2′-PO_4 end. It was proposed that (1) the Trl1 ligase domain is hindered from sealing γ-toxin-incised $tRNA^{Glu}$ by the presence of the bulky mcm^5s^2U base at the 3′-OH, 2′-PO_4 end; (2) the unmodified 5′-OH nucleoside at the γ-toxin incision site in $tRNA^{Glu}$ can be phosphorylated by either the Trl1 or AtRNL kinase domains, accounting for the ability of kinase-dead AtRNL to rescue growth on galactose; and (3) the capacity of AtRNL to rectify γ-toxin damage is a unique property of its ligase domain, which is apparently adept at sealing the broken tRNA with a bulky mcm^5s^2U base at the 3′-OH, 2′-PO_4 end (Nandakumar et al. 2008). Extending that line of reasoning to PrrC, which incises on the 5′ side of the wobble nucleoside to yield an unmodified 2′,3′ cyclic phosphate terminus and a 5′-OH mcm^5s^2U terminus, leads to the following speculations: (1) The Trl1 kinase module is ineffective at phosphorylating the bulky 5′-OH mcm^5s^2U nucleoside, whereas the AtRNL kinase is competent to do so; and (2) the Trl1 ligase domain is ineffective at sealing the healed 5′-PO_4 end with a bulky mcm^5s^2U nucleoside, whereas the AtRNL ligase is competent to do so. Nothing is known as yet concerning the structural features of the yeast and plant ligases that dictate their differential healing and sealing of base-modified RNA breaks.

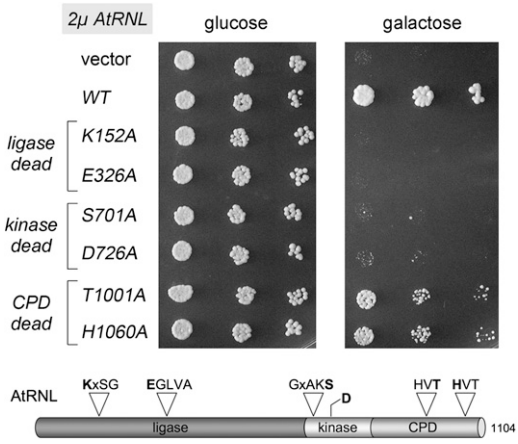

FIGURE 6. RNA sealing and 5′ healing activities of AtRNL dictate resistance to PrrC. (*Top* panel) Growth of yeast cells bearing a *CEN* plasmid encoding galactose-regulated *Nme*PrrC-R316W or the empty *CEN* vector (–) and either an empty 2μ *HIS3* vector or a 2μ *HIS3 TPI1-AtRNL* plasmid encoding wild-type plant tRNA ligase or the indicated ligase-dead, kinase-dead, or CPD-dead mutant was assessed by spotting serial fivefold dilutions to minimal synthetic agar medium containing glucose or galactose. The galactose plate was photographed after 4 d of incubation at 30°C. (*Bottom* panel) AtRNL mutants. The positions of the covalent adenylylation motif (KxxG) and the metal-binding motif (EGxxx) at the ligase active site, the P-loop motif GxxK(S/T) at the kinase active site, and the two HxT motifs that comprise the CPD active site are depicted *above* the AtRNL polypeptide. The sites of enzyme-inactivating alanine mutations in the AtRNL active sites are highlighted in bold.

Further mutational analysis of the *Eco*PrrC nuclease domain

Our previous study of the effects of alanine and conservative mutations on *Eco*PrrC toxicity in yeast identified 11 essential residues in the C-terminal nuclease domain. Here, we identified Trp335 as an additional essential constituent of *Eco*PrrC (Fig. 3C). To extend the structure-function analysis of the nuclease domain, we initiated a new round of alanine and conservative mutagenesis, focusing mainly on a putative lysine anticodon recognizing peptide (LARP) motif, ^{284}KYGDSNKSFSY^{294}, that had been the subject of studies by the Kaufmann lab (Klaiman et al. 2007). The LARP motif (denoted by bracket in Fig. 3A), mutations of which affect the tRNA substrate preference of *Eco*PrrC (Jiang et al. 2001, 2002), is found only in a subset of PrrC proteins (Klaiman et al. 2007; Davidov and Kaufmann 2008). It is speculated that LARP is a determinant of the target specificity of those PrrC proteins that contain the motif. However, LARP may not be the decisive factor with respect to yeast toxicity of bacterial PrrCs, insofar as the *Eco*PrrC LARP is not conserved (only 3/11 identical residues) in *Smu*PrrC, which is toxic in yeast. We reported previously

that two alanine mutations in the *Eco*PrrC LARP motif (at Ser291 and Ser293, which are conserved in *Nme*PrrC) (Fig. 3A) had no effect on cytotoxicity in yeast (Meineke et al. 2011).

Here we introduced alanine in lieu of *Eco*PrrC LARP motif residues Asp287, Ser288, and Asn289, and also at the distal residue His381 (Fig. 3A, targeted positions denoted by ▼). The mutant alleles were inserted into *CEN* plasmids under *GAL*-control. Tests of yeast growth on glucose and galactose showed that the D287A, S288A, and H381A mutants were nontoxic, whereas N289A retained toxicity (Fig. 7; and data not shown). Substituting His381 conservatively with glutamine and asparagine also rendered *Eco*PrrC nontoxic in yeast (data not shown). Thus, His381 joins four other histidines in the nuclease domain (His295, His297, His315, and His356) as strictly essential for *Eco*PrrC toxicity in yeast (Meineke et al. 2011). Because His381 is replaced by glutamine in the toxic *Smu*PrrC protein (Fig. 3A), we suspect that His381 is not acting as general acid-base catalyst of RNA transesterification. Replacing the essential LARP motif residue Ser288 with asparagine restored toxicity to *Eco*PrrC (data not shown); note that asparagine is naturally present at the equivalent position of *Smu*PrrC. These findings suggest that the hydrogen bonding capacity of Ser288 is pertinent for PrrC activity in yeast.

We were especially interested in structure-activity relations at the essential Asp287, in light of prior suggestions

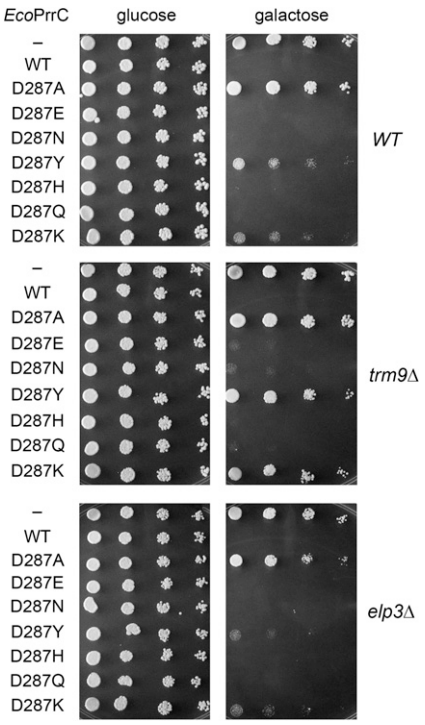

FIGURE 7. Mutational analysis of *Eco*PrrC Asp287. Serial fivefold dilutions of wild-type (WT), *elp3*Δ, and *trm9*Δ yeast cells bearing a *CEN* plasmid encoding galactose-regulated *Eco*PrrCs as specified were spotted on –Leu agar plates containing glucose or galactose.

that this residue is a target specificity determinant (Meidler et al. 1999; Jiang et al. 2001, 2002). Thus, we replaced Asp287 conservatively with glutamate and asparagine and nonconservatively with tyrosine, histidine, glutamine, and lysine (Fig. 7). The D287E, D287N, D287H, and D287Q mutants displayed full galactose-dependent toxicity in yeast (Fig. 7, top panel), suggesting that hydrogen-bonding might be the key property of this side chain. Certainly, the results exclude a strict requirement for negative charge at this position. On the other hand, the D287Y and D287K mutants were partially inhibitory to yeast growth, insofar as the yeast cells expressing these variants grew on galactose agar but formed tiny colonies compared to D287A-expressing cells or the vector control (Fig. 7, top panel). We surmise that charge inversion (in D287K) or increased side chain bulk (in D287Y) at this position exert negative effects on *Eco*PrrC function in yeast.

The Kaufmann lab had shown that missense mutations at Asp287 alter the tRNA cleavage preferences of PrrC in vivo when expressed in *E. coli* and/or in vitro, e.g., such that particular Asp287 mutants are either more or less fastidious regarding the impact of wobble base modifications (Jiang et al. 2001, 2002). In this light, we compared the effects of induced expression of Asp287A mutants in yeast cells that have the wild-type mcm^5s^2U wobble modification versus *elp3*Δ and *trm9*Δ cells that lack all or part of the mcm^5 moiety (Fig. 7). The salient findings were as follows: (1) PrrC mutants D287Y and D897K were less toxic in *trm9*Δ cells than in wild-type or *elp3*Δ cells; and (2) other nonalanine Asp287 mutants retained toxicity in all three strain backgrounds (Fig. 7). The findings fortify our inferences from the analysis of *Nme*R316W toxicity (Fig. 4) that hypomorphic PrrC variants have diminished ability to inflict damage at an incompletely modified cm^5s^2U wobble nucleoside.

Restoration of the toxicity of a subset of PrrC nuclease domain mutants by increased gene dosage

We have now identified a total of 15 residues in the nuclease domain of *Eco*PrrC that are essential for toxicity in yeast. This module has no discernible primary structure similarity to any known ribonucleases or tRNA-binding proteins, which makes it difficult to guess which essential residues might be directly involved in catalysis versus substrate recognition, PrrC folding/stability, etc. As discussed above, the mutational analysis readily identified PrrC hypomorphs that retained toxicity in yeast when expressed from a *CEN* plasmid but were either amenable to alleviation of toxicity by co-expression of RNA repair enzymes (unlike wild-type PrrC) or were affected in their toxicity by the status of the wobble uridine modification (also unlike wild-type PrrC). Other PrrC hypomorphs (e.g., D287K) were simply less inhibitory to yeast growth than wild-type PrrC when expressed from a *CEN* plasmid.

Taking this one step further, we reasoned that (1) the collection of 15 PrrC-Ala mutants in the nuclease domain that were deemed nontoxic when expressed from *CEN* plasmids might include additional, more severely affected hypomorphs; (2) some of these putative hypomorphs might regain their toxicity when expressed from a multicopy 2μ plasmid; and (3) recovery of toxicity by overexpression of a particular mutant would weigh against the mutated residue being strictly essential for catalysis.

To evaluate this scenario, we transferred the 15 $_{GAL}$-*prrC-Ala* expression cassettes to 2μ plasmids, introduced them into *Saccharomyces cerevisiae*, and tested the transformants for galactose-dependent toxicity (Table 1). Two of the originally nontoxic mutants regained full toxicity at high gene dosage (scored as ++ in Table 1): These were H297A and K299A. Three other mutants—S288A in the LARP motif, N352A, and His381A—regained partial activity (scored as + in Table 1). We surmise that these six side chains are unlikely to be directly catalytic. We presume that the set of nine EcoPrrC-Ala mutants that did not regain toxicity at high gene dosage encompasses bonafide constituents of the nuclease active site. Among the candidate active site residues are three strictly essential and conserved histidines (His295, His315, His356), two strictly essential and conserved arginines (Arg320 and Arg349), and one strictly essential and conserved glutamate (Glu324). The Kaufmann lab had proposed that Arg320, Glu324, and His356 comprise a catalytic triad that they implicate in chemical catalysis of transesterification at the wobble nucleotide to generate 2′,3′ cyclic phosphate and 5′-OH product strands (Blanga-Kanfi et al. 2006). Our results are consistent with their model but raise the prospect that additional residues might play a catalytic role. Of course, a definitive interpretation of the mutational data awaits an atomic structure of the nuclease domain.

TABLE 1. High gene dosage can restore toxicity of certain defective PrrC nuclease domain mutants

2μ PrrC	Toxicity on galactose
D267A	–
D287A	–
S288A	+
H295A	–
H297A	++
K299A	++
H315A	–
R320A	–
N321A	+
E324A	–
W335A	–
R349A	–
N352A	+
H356A	–
H381A	+

DISCUSSION

Programmed tRNA damage by site-specific endoribonucleases is a shared feature of cellular stress responses and self-nonself discrimination in a wide range of prokaryal and eukaryal taxa. tRNA anticodon breakage results in inhibition of protein synthesis, either by depletion of the pool of specific tRNA isoacceptors or by a mechanism by which the broken tRNA fragments per se can have a signaling role without significantly depleting the pool of the tRNA target (Thompson and Parker 2009; Ivanov et al. 2011 and references therein). *E. coli* PrrC was the first example of an intracellular tRNA restriction endonuclease. The activity of PrrC is normally suppressed by its association with its cognate "antitoxin," a type I DNA restriction-modification enzyme (*Eco*prrI) encoded by neighboring ORFs in the *prr* operon (Levitz et al. 1990; Tyndall et al. 1994). tRNA ribotoxins have distinctive target specificities as follows: *E. coli* PrrC for $tRNA^{Lys(UUU)}$ (Amitsur et al. 1987; Jiang et al. 2001, 2002); colicin E5 for Tyr, His, Asn, and Asp tRNAs (Ogawa et al. 1999); colicin D for $tRNA^{Arg}$ (Tomita et al. 2000); enterobacterial VapC for $tRNA^{fmet}$ (Winther and Gerdes 2011); *K. lactis* γ-toxin for $tRNA^{Glu(UUC)}$ (Lu et al. 2005, 2008); and *Pichia acaciae* toxin for $tRNA^{Gln(UUG)}$ (Klassen et al. 2008). The findings that the cytotoxic effects of *Eco*PrrC, colicin E5, and colicin D are portable to budding yeast (Ogawa et al. 2009; Shigematsu et al. 2009; Meineke et al. 2011) attest that eukaryal tRNAs are vulnerable to attack by bacterial anticodon nucleases. Suppression of *EcoPrrC* toxicity in yeast by overexpression of $tRNA^{Lys(UUU)}$ indicates that PrrC exerts toxicity via the homologous tRNA substrate in bacteria and eukarya, notwithstanding their differences in $tRNA^{Lys}$ anticodon modifications.

PrrC homologs are dispersed widely among bacterial taxa, but virtually nothing is known about the biological functions and target specificities of these PrrC proteins. Nonetheless, our initial assumption was that all bacterial PrrC proteins are RNA endonucleases. Thus, it was surprising that *Nme*PrrC, which is among the closest homologs of *Eco*PrrC, displayed no toxicity when expressed in yeast, even though the more distantly related *Smu*PrrC protein was fungicidal. The lack of toxicity of *Nme*PrrC in *E. coli* and the results of our domain-swap experiments, in which the source of the PrrC nuclease domain emerged as the decisive factor for toxicity in yeast or *E. coli*, raised the prospect that either (1) *Nme*PrrC is an active ribotoxin, but its RNA target is not present in *E. coli* or yeast; or (2) *Nme*PrrC is not an active ribotoxin. With the former model in mind, we attempted to convert the nontoxic *Nme*PrrC into a toxic derivative by screening for missense gain-of-function mutants. Remarkably, this succeeded with a single nucleotide change, of an AGG codon to a TGG codon, that replaced an Arg in *Nme*PrrC with a Trp residue found at the equivalent position of *Eco*PrrC. Because the gain-of-function *Nme*R316W mutant exerted its toxicity via $tRNA^{Lys(UUU)}$ and because any mutation of the tryptophan

abolished *Eco*PrrC toxicity, we thought we might have identified an essential species-specific determinant of PrrC target specificity.

A simpler alternative scenario was suggested to us by Gabi Kaufmann, in which *Nme*PrrC has mutated to a nontoxic variant under selection pressure. The pressure arises because the *prr* operon of *N. meningitidis* MC58 (the source of the *Nme*PrrC gene) has degenerated by the acquisition of premature stops and frameshifts in the NMB0831 gene that would otherwise encode the HsdS(PrrB) subunit of the restriction-modification complex that keeps *E. coli* PrrC in an inactive state. The *Neisseria* operon also has a gene encoding an IS30-family transposase inserted into the ORF that would otherwise encode the HsdR(PrrD) subunit of the restriction-modification complex. These genetic changes would seem to ablate the antitoxin that normally exerts a brake on PrrC's nuclease. In order to survive this loss, the bacterium can be expected to acquire an inactivating mutation in the PrrC anticodon nuclease. We agree with this evolutionary sequence as the likely explanation for the lack of toxicity of *Nme*PrrC. What is remarkable is that the toxicity of *Nme*PrrC can be reconstituted by a single missense change. A key lesson from these experiments is that not all PrrC homologs can be presumed to have anticodon nuclease activity. The combination of yeast toxicity assays and domain swaps affords a useful genetic strategy to assess ribotoxicity and species variations in PrrC biological activity, especially with PrrCs encoded by taxa that are not tractable genetically.

tRNA repair as an antidote to ribotoxic tRNA damage is a well-established component of the virus-host dynamic during T4 infection of *prr*$^+$ *E. coli* (Amitsur et al. 1987). We had shown previously that RNA repair enzymes can also protect yeast against growth arrest caused by *K. lactis* γ-toxin (Nandakumar et al. 2008). Here we extend the paradigm of RNA repair to PrrC-mediated eukaryal cytotoxicity, which can be ameliorated by either the phage T4 RNA repair system (Pnkp plus Rnl1) or plant AtRNL. In light of evidence that tRNA damage can trigger strong cellular responses, including inhibition of protein synthesis, nonlethal growth arrest, and cell death, we envision that RNA repair might play a role in tuning the severity of RNA damage or in recovering from its effects.

MATERIALS AND METHODS

Yeast expression plasmids

Yeast *CEN LEU2* plasmids containing the *Eco*PrrC, *Smu*PrrC, or *Nme*PrrC ORFs under the transcriptional control of a *GAL1* promoter were described previously (Meineke et al. 2011). Domain swaps and missense mutations were introduced in the *prrC* genes by two-stage overlap extension PCR with fusogenic or mutagenic primers. The *prrC* ORF was sequenced in each case to verify the intended hybrid junctions or coding change and exclude the acquisition of unwanted coding changes during amplification and cloning. EcoRI/SalI fragments containing $_{GAL1}$-*prrC-Ala* expression cassettes were excised from the respective *CEN* plasmids and inserted into the multicopy yeast plasmid pRS423 (2μ *HIS3*). Yeast 2μ *URA3* plasmids bearing yeast tRNA genes were as described (Jablonowski et al. 2006; Meineke et al. 2011). RNA repair plasmids pRS423-TPI1-AtRNL (2μ *HIS3*) and pRS423-TPI1-TRL1 (2μ *HIS3*) carry the plant and yeast tRNA ligase genes, respectively, under the transcriptional control of the yeast *TPI1* promoter. RNA repair plasmid pRS413-Pnkp/Rnl1 (*CEN HIS3*) vector expresses phage T4 Pnkp under the control of the yeast *SLU7* promoter and T4 Rnl1 under the control of the yeast *TPI1* promoter.

Arabinose-inducible PrrC expression plasmids

The *Eco*PrrC, *Smu*PrrC, *Nme*PrrC, and chimeric PrrC open reading frames were amplified by PCR from their respective yeast *CEN* plasmids using a sense-strand primer that introduced an NheI site immediately 5′ of the translation start codon. The PCR products were digested with NheI and SalI and inserted between the corresponding restriction sites of the bacterial expression plasmid pBAD18. The *prrC* ORF was sequenced in each case to verify the intended coding sequence.

PrrC yeast toxicity assays

Yeast cells were transformed with PrrC plasmid DNAs, and transformants were selected on appropriate minimal synthetic media on 2% (w/v) Bacto agar plates. Toxicity of the plasmid-encoded PrrC proteins was gauged as described (Meineke et al. 2011). Cells derived from single transformants were grown at 30°C in liquid culture in selective media containing 2% glucose. The cultures were adjusted to A_{600} of 0.1 and then diluted in water in serial fivefold decrements. Aliquots (3 μL) of the dilutions were then spotted in parallel on selective agar plates containing either 2% glucose or 2% galactose. The plates were photographed after incubation at 30°C for 2 d (glucose) or 3 d (galactose) unless specified otherwise.

PrrC bacterial toxicity assays

Top10 cells (*araABD*$^-$, Invitrogen) were transformed with pBAD-PrrC plasmids. Cells derived from single ampicillin-resistant colonies were grown in LB medium containing 200 μg/mL ampicillin for 4 h at 37°C. The cultures were adjusted to attain A_{600} of 0.025 and then diluted in 20-fold decrements in water. Aliquots (3 μL) of the dilutions were spotted in parallel on LB agar plates containing 100 μg/mL ampicillin with or without 0.2% L-arabinose. The plates were photographed after incubation for 24 h at 37°C.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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