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An SOS-Regulated Type 2 Toxin-Antitoxin System▽

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The *Escherichia coli* chromosome encodes seven demonstrated type 2 toxin-antitoxin (TA) systems: cassettes of two or three cotranscribed genes, one encoding a stable toxin protein that can cause cell stasis or death, another encoding a labile antitoxin protein, and sometimes a third regulatory protein. We demonstrate that the *yafNO* genes constitute an additional chromosomal type 2 TA system that is upregulated during the SOS DNA damage response. The *yafNOP* genes are part of the *dinB* operon, of which *dinB* underlies stress-induced mutagenesis mechanisms. *yafN* was identified as a putative antitoxin by homology to known antitoxins, implicating *yafO* (and/or *yafP*) as a putative toxin. Using phage-mediated cotransduction assays for linkage disruption, we show first that *yafN* is an essential gene and second that it is essential only when *yafO* is present. Third, *yafP* is not a necessary part of either the toxin or the antitoxin. Fourth, although DinB is required, the *yafNOP* genes are not required for stress-induced mutagenesis in the *Escherichia coli* Lac assay. These results imply that *yafN* encodes an antitoxin that protects cells against a *yafO*-encoded toxin and show a protein-based TA system upregulated by the SOS response.

Toxin-antitoxin (TA) systems are modules in bacterial genomes that can cause growth arrest and/or programmed cell death in cells harboring them (1, 18, 19, 54). Type 1 TA systems consist of an RNA antitoxin and a protein toxin, in which the RNA antitoxin inhibits translation of the toxin mRNA. Type 2 TA systems typically consist of two genes in an operon, transcriptionally and translationally coupled, in which, usually, the upstream gene encodes a labile antitoxin protein and the downstream gene encodes a stable toxin protein (Fig. 1A). Continuous transcription of the operon, and thus continuous transcription of the antitoxin, ensures protection from the effects of the toxin. Interruption in transcription of the operon tips the balance in favor of the toxin because antitoxin is no longer made and is rapidly degraded.

TA systems were originally identified on plasmids as "plasmid-addiction modules" (34) that maintain the plasmid in the host cell. Failure of cells to inherit the plasmid results in rapid loss of the labile antitoxin, unmasking of the stable preexisting toxin, and death of the cell in a process known as postsegregational killing (13, 38).

More recently, TA systems have been discovered in the *Escherichia coli* chromosome, raising the question of their function there (1, 19). Proposed functions for chromosomal TA systems include roles in nutritional stress response (8), protection from phages (27), formation of "persister" cells that resist antibiotics (32), selfish genetic elements (38, 54), and antiaddiction modules that allow bacteria to resist plasmid addiction (54). Several type 2 chromosomal TA systems have been demonstrated in *E. coli*, including *relBE*, *mazEF*, *dinJ-yafQ*, *prlF-yhaV*, *yefM-yoeB*, *chpBI-chpBK*, and *hipAB* (1, 3, 19, 20, 39, 45, 50). Of these, *relBE* and *mazEF* exert their toxic effects during controlled responses such as during amino acid starvation (8, 28). Additionally, *mazF* kills cells in response to DNA-damaging and oxidative stresses (28). One type 2 (*dinJ-yafQ*) (45) and three characterized type 1 (*hok*, *symER*, and *tisAB-istr1*) (31, 46, 55) TA systems were previously reported to be controlled by the SOS response to DNA damage. However, *dinJ-yafQ* appears not to be SOS regulated. Although a consensus LexA binding site was identified upstream of the *dinJ-yafQ* operon (35) and LexA bound to this site in one study (56) but not in another (14), LexA does not regulate this operon in vivo upon exposure to mitomycin C (14) or UV light (11). The role of stress responses in regulating TA systems is an interesting problem. Here we provide evidence for the first *E. coli* chromosomal type 2 TA system under SOS response control, *yafNO*.

yafNOP was originally identified as a putative TA system by homology of *yafN* to known antitoxins, implicating *yafO* (and/or *yafP*) as a putative toxin(s) (19). *yafNOP* lies downstream of *dinB* in the *dinB* operon (43) (Fig. 1B), which is upregulated midway through the SOS DNA damage response (11). DinB is a central player in stress-induced mutagenesis mechanisms in *E. coli* (9, 17, 42), *Salmonella enterica* (48), *Pseudomonas putida* (53), and *Bacillus subtilis* (52). The proximity of *yafNOP* to *dinB* piqued our interest in these genes. Previously, Brown and Shaw demonstrated in classical TA system dissection that when *yafO* was overexpressed growth inhibition occurred (5). No additional work regarding either *yafN* or *yafP* was conducted (5). In contrast to this and to the notion that *yafNOP* was a TA system was the published *yafN* deletion strain of the Keio *E. coli* knockout collection, which should have been inviable if *yafN* were the antitoxin (2). Given these conflicting results, we reinvestigated *yafNOP*. Here, we show

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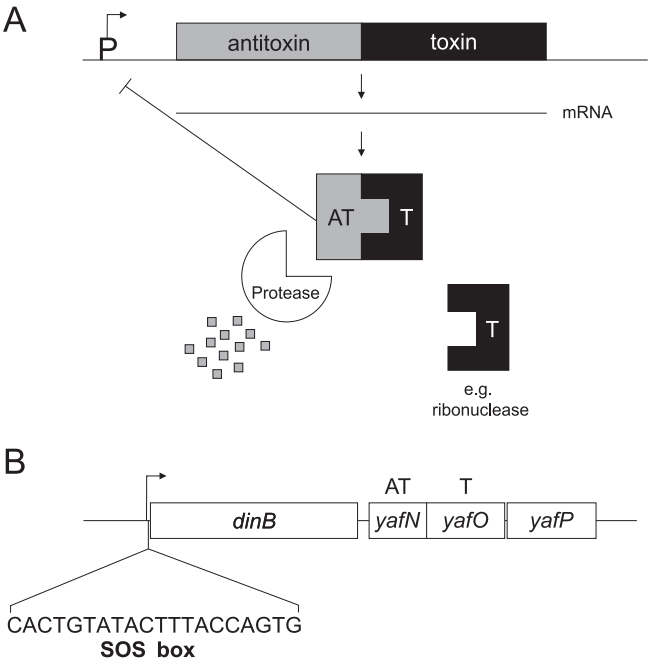


FIG. 1. Type 2 TA system organization and the *dinB* operon. (A) Type 2 TA system organization. Type 2 TA systems are usually two gene operons in which the antitoxin (upstream) and toxin (downstream) genes are cotranscribed/translated. The antitoxin protein is degraded more rapidly than the toxin, such that the cell requires continuous transcription/translation to avoid stasis/death induced by the toxin. (B) The *E. coli dinB* operon. The *yafNOP* genes, encoding a putative TA system, lie downstream of *dinB* in the operon. The operon is upregulated by the SOS DNA damage response (SOS box shown). *yafN*, encoding the putative antitoxin, is 297 bp; *yafO*, encoding the putative toxin, is 399 bp; and *yafP*, of unknown function, is 453 bp in length. Some strains used in this study (derivatives of SMR4562 or FC40) contain an additional copy of the *dinB* operon via a duplication of the operon in the F′ episome. T, toxin; AT, antitoxin.

that *yafN* and *yafO* (not *yafP*) constitute a TA system and explore its possible function in the cell.

MATERIALS AND METHODS

Media, antibiotics, and growth conditions. The media used included Luria-Bertani-Herskowitz (LBH; 1% tryptone, 0.5% NaCl, 0.5% yeast extract, 2 μg/ml thymine), tryptone broth (TB; 1% tryptone, 0.5% NaCl), and BBL plates (0.5% NaCl, 1% BBL Trypticase peptone solidified with 1.5% agar). K-glucose consists of 2× M9 (44) with the following additions: 7.5% Casamino Acids, 5 mM $MgSO_4$, 0.05% NaCl, 0.2% glucose, 10 μg/ml vitamin B_1, 3 μM $FeCl_3$, and 50 μM $CaCl_2$. Plating culture broth is TB with the following additions: 0.2% maltose, 5 mM $MgSO_4$, 10 μg/ml thymine, and 10 μg/ml vitamin B_1. Carbon sources glucose, maltose, glycerol, and lactose were all used at 0.1% unless otherwise stated. The antibiotics used included tetracycline (Tet; 10 μg/ml, 3.33 μg/ml when sodium citrate [CIT] is present), kanamycin (Kan; 30 μg/ml), chloramphenicol (25 μg/ml), bleomycin (various concentrations, specified in the text), mitomycin C (various concentrations, specified in the text), and rifampin (100 μg/ml). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 40 μg/ml) was also used. The buffers used included TM (Tris buffer + $MgSO_4$, pH 7.5) and M9 (44). Bacteria were grown at 32°C or 37°C, as stated for each experiment.

Strains and new alleles. *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. New deletion alleles created for this study are shown in Table 2, and the PCR primers used to construct the new deletions by short-homology recombination methods (12) are shown in Table 3. Phage P1-mediated transductions were performed as described previously (44). To construct λ lysogens, stationary-phase TB cultures of nonlysogens were diluted 200× into plating culture broth, shaken for 6 hours at 32°C, pelleted, and resuspended in TM at a 1:4 dilution. Phage were added at a multiplicity of infection of 10 per cell, allowed to sit at room temperature for 10 minutes, diluted 10× with TB, shaken for 2 hours at 32°C, and then plated onto MacConkey-maltose plates seeded with 10^8 to 10^9 λ*c*I and 10^8 to 10^9 λ*cIh80* phage to kill nonlysogens, and red (maltose-positive) colonies were picked, purified, and verified. λ*cIh80* adsorbs via an alternate receptor and therefore kills nonlysogens with mutated maltose uptake receptors, which are resistant to wild-type λ adsorption.

Cloning of *yafN* into pBAD24. *yafN* was amplified from strain FC36 using primers *yafN*-F-KpnI (AAAAAAGGTACCATTCTGGTGTGCATTATTATG) and *yafN*-R-HindIII (AAAAAAAAGTTCTTATTCCTTAAAGTCATTG) with Platinum Pfx polymerase (Invitrogen) and cloned into pCR-BluntII-Topo-Kan^r using Invitrogen's Zero Blunt Topo PCR cloning kit. *yafN* was excised from the cloning plasmid with KpnI and XhoI and ligated into pBAD24 using T4 DNA ligase (Invitrogen), creating the plasmid pLS1. Insertion of *yafN* into pBAD24 was confirmed by sequencing (SeqWright, Houston, TX).

Quantitative phage P1-mediated cotransduction assays. Phage P1-mediated cotransductions were performed based on the Miller protocol (44) with the following modifications. Transductants selected on LBH-Tet with 20 mM CIT were then patched in 100-colony grids onto LBH-Kan-CIT (and LBH-chloramphenicol-CIT for Δ*yafN* Δ*yafP* cotransduction) to screen for recovery of markers of interest. Plates were monitored over several days of incubation for possible slowly growing colonies.

Sequencing of JW0222 *yafO*. The *yafO* gene of strain JW0222 was amplified using PCR primers *yafN*-F-KpnI and *yafP*ExtR (AGTTTGTGGAATCAGAAA ACG). PCR product was purified with Qiagen's QIAquick PCR purification kit and sequenced using the above-mentioned primers (SeqWright, Houston, TX).

Mutagenesis and DNA damage sensitivity assays. Stress-induced mutagenesis assays were performed according to the method of Harris et al. (25). For UV light kill curves, cultures were grown to early/mid-log phase (optical density at 600 nm, 0.1 to 0.3) in LBH and then spread onto LBH agar plates, exposed to the indicated UV light dose, incubated in the dark at 37°C for 16 h, and scored for colonies. For bleomycin and mitomycin sensitivity assays, saturated LBH cultures were diluted and grown to mid-log phase (optical density at 600 nm, 0.3 to 0.6) and spotted onto fresh drug-containing plates at indicated dilutions. Bleomycin was dissolved in 0.9% NaCl. Mitomycin C was dissolved in sterile H_2O (final pH, 6.0), per the manufacturer's recommendation. Plates were incubated for 16 h at 32°C and scored for CFU.

Lambda burst size assay from UV-induced lysogens. Saturated LBH cultures of *E. coli* λ lysogens were diluted in K-glucose and grown to 1.5×10^8 cells/ml. An aliquot of cells was removed, lysed with lysozyme and chloroform, and submitted to plaque assay to quantify phage that was spontaneously induced from the prophage state. Cells were UV irradiated (50 J/m^2) to induce the lambda prophage to lytic growth and shaken at ≥300 rpm for 1.5 h at 37°C in darkness. Lysozyme (50 mg/ml) and chloroform were added to lyse cells and release intracellular phage, debris were pelleted, and the supernatants were assayed for PFU. Phage titers were calculated and used to determine burst size (PFU/cell, corrected for preexisting phage).

Flow cytometry and cell sorting. Flow cytometry analyses and fluorescence-activated cell sorting (FACS) were performed per the method in reference 47 with the following modifications. Log-phase cells were analyzed using the BD FACSAria cell sorter (BD Biosciences). Data shown are the means of five experiments (one culture/experiment for experiments 1 to 4 and two cultures/experiment for experiment 5). At least 10^6 nongreen and 8×10^3 (typically $>10^4$) green cells were sorted per culture. Sorting purity controls were performed before each experiment (per the method in reference 47). Propidium iodide (PI) staining was performed per the method in reference 47.

RESULTS

***yafN* is essential in the presence of *yafO*.** To test whether *yafNOP* is a functional TA system, we examined whether the putative antitoxin gene, *yafN*, is an essential gene but only in the presence of a functional putative toxin gene, *yafO*. As diagrammed in Fig. 2A, using a cotransduction assay, we transduced *E. coli* cells with phage P1 that had been grown on cells carrying the *zae-502*::Tn*10* Tet resistance (Tet^r) gene partially linked with a chromosomal Δ*yafN*::Kan deletion, conferring Kan resistance (Kan^r), or other mutation of interest. The P1 donor cells carried a *yafN*$^+$-expressing plasmid to allow their viability (if essential) and prevent the accumulation/

TABLE 1. *E. coli* strains, plasmids, and lambda phage used in this study[a]

Strain, phage, or plasmid	Relevant genotype	Reference(s) or source
Strains		
BT340	DH5α(pCP20)	7 (via CGSC)
BW25113(pKD46)	pKD46	12 (via CGSC)
BW25141(pKD3)	pKD3	12 (via CGSC)
BW25141(pKD13)	pKD13	12 (via CGSC)
CAG18436	MG1655 *zae-502*::Tn*10*	51
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*ΔM15	21 (via W. Bridger [Edmonton, Alberta, Canada])
FC36	Δ(*lac-proAB*)$_{XIII}$ *thi ara* Rif^r	6
FC40	Δ(*lac-proAB*)$_{XIII}$ *thi ara* Rif^r [F′ *proAB*$^+$ *lacI33*Ω*lacZ*]	6
JW0222	Δ*yafN*::FRTKanFRT	2
MG1655	Wild type	4
N2731	*recG258*::Tn*10* mini-Kan	36 (via R. Kolodner [San Diego, CA])
SMR4562	Genotype same as that of FC40, independent construction	41
SMR5833	SMR4562(pKD46)	43
SMR5889	SMR4562 Δ*dinB50*::FRT [F′ Δ*dinB50*::FRT]	43
SMR6068	SMR4562 Δ*yafN11*::FRTKanFRT [F′ *yaf*$^+$]	SMR5833 × DNA of Δ*yafN*::FRTKanFRT amplified from pKD13 [from BW25141 (pKD13)], location screened by mating
SMR6074	SMR4562 [F′ Δ*yafO14*::FRTKanFRT]	SMR5833 × DNA of Δ*yafO*::FRTKanFRT amplified from pKD13 [from BW25141 (pKD13)], location screened by mating
SMR6076	FC36 Δ*yafO14*::FRTKanFRT	FC36 × P1(SMR6074)
SMR6080	SMR4562 Δ*yafP18*::FRTKanFRT [F′ *yaf*$^+$]	SMR5833 × DNA of Δ*yafP*::FRTKanFRT amplified from pKD13 [from BW25141 (pKD13)], location screened by mating
SMR6082	FC36 Δ*yafP18*::FRTKanFRT	FC36 × P1(SMR6080)
SMR6221	FC36 Δ*yafP20*::FRT	SMR6082 × pCP20 (from BT340)
SMR6233	MG1655(pKD46)	MG1655 × pKD46 [from BW25113(pKD46)]
SMR6353	MG1655 Δ(*yafN-yafP*)*776*::FRTKanFRT	SMR6233 × DNA of Δ(*yafN-yafP*)::FRTKanFRT from pKD13 [from BW25141(pKD13)]
SMR6669	MG1655 Δ*att*λ::P$_{sulA}$Ω*gfp-mut2*	26, 40
SMR7491	SMR4562 Δ(*yafN-yafP*)*602* [F′ Δ(*yafN-yafP*)*602*]	43
SMR10285	SMR4562 Δ(*yafN-yafO*)*779*::FRTKanFRT [F′ *yaf*$^+$]	SMR5833 × DNA of Δ(*yafN-yafO*)::FRTKanFRT amplified from pKD13 [from BW25141 (pKD13)]
SMR10287	FC36 Δ(*yafN-yafO*)*779*::FRTKanFRT	FC36 × P1(SMR10285)
SMR10291	SMR4562 Δ*yafN11*::FRTKanFRT *zae-502*::Tn*10* [F′ *yaf*$^+$]	SMR6068 × P1(CAG18436)
SMR10483	FC36 Δ(*yafN-yafO*)*779*::FRTKanFRT *zae-502*::Tn*10*	SMR10287 × P1(CAG18436)
SMR10943	FC36(pBAD24)	FC36 × pBAD24 (from ATCC 87399)
SMR10946	DH5α(pLS1)	DH5α × pLS1 (initial plasmid construction)
SMR10948	FC36(pLS1)	FC36 × pLS1 (from SMR10946)
SMR10953	SMR4562 Δ*yafP18*::FRTKanFRT *zae-502*::Tn*10* [F′ *yaf*$^+$]	SMR6080 × P1(CAG18436)
SMR10988	MG1655 Δ*att*λ::P$_{sulA}$Ω*gfp-mut2* Δ(*yafN-yafP*)*776*::FRTKanFRT	SMR6669 × P1(SMR6353)
SMR10990	MG1655 *recG258*::Tn*10* mini-Kan	MG1655 × P1(N2731)
SMR10995	SMR4562 Δ*yafN11*::FRTKanFRT [F′ *yaf*$^+$] [pKD46]	SMR6068 × pKD46 [from BW25113(pKD46)]
SMR11008	SMR4562 Δ*yafN11*::FRTKanFRT Δ*yafP19*::FRT*cat*FRT *zae-502*::Tn*10* [F′ *yaf*$^+$]	SMR11168 × P1(CAG18436)
SMR11168	SMR4562 Δ*yafN11*::FRTKanFRT Δ*yafP19*::FRT*cat*FRT [F′ *yaf*$^+$]	SMR10995 × DNA of Δ*yafP*::FRT*cat*FRT amplified from pKD3 [from BW25141(pKD3)]
SMR11228	MG1655(λ)	MG1655 × λSR108
SMR11229	MG1655(λ) Δ(*yafN-yafP*)*776*::FRTKanFRT	SMR11228 × P1(SMR6353)
SMR11231	MG1655(λ) *recG258*::Tn*10* mini-Kan	SMR11228 × P1(N2731)
Phage lambda		
λSR108	λ wild type	F. Stahl (Oregon)
Plasmids		
pBAD24	Plasmid containing arabinose-inducible promoter P$_{BAD}$, Amp^r	23 (via ATCC 87399)
pLS1	Plasmid containing *yafN* under the control of P$_{BAD}$	This work
pKD3	*cat*-containing plasmid, template for PCR	12
pKD13	*kan*-containing plasmid, template for PCR	12
pCP20	*FLP*-containing plasmid, used to excise drug markers flanked by FRT sequences	12
pKD46	Red recombinase expression plasmid	12

[a] FRT, FLP recombination target; CGSC, *E. coli* Genetic Stock Center (Yale University); ATCC, American Type Culture Collection.

TABLE 2. Description of new deletion alleles

Strain	New allele	Wild-type gene length (bp)	Deletion coordinates (distance from gene translation start)	Replacement cassette[a]
SMR6068	Δ*yafN11*::FRTKanFRT	294	+13 to +279	FLPable Kan
SMR6074	Δ*yafO14*::FRTKanFRT	399	+13 to +384	FLPable Kan
SMR6080	Δ*yafP18*::FRTKanFRT	453	+16 to +438	FLPable Kan
SMR6221	Δ*yafP20*::FRT	453	+16 to +438	FRT scar
SMR10285	Δ(*yafN-yafO*)*779*::FRTKanFRT	See individual genes above	+13 (*yafN*) to +384 (*yafO*)	FLPable Kan
SMR11168	Δ*yafP19*::FRT*cat*FRT	453	+40 to +416	FLPable *cat*

[a] For each replacement cassette, the sequence of the inserted DNA begins with ATCC (Kan cassette) or TCATA (*cat* cassette) and ends with CTACA (Kan cassette) or TACAC (*cat* cassette). Sequences are listed 5′ to 3′. FRT, FLP recombination target.

selection of extragenic suppressor mutations that would allow a cell with a mutated essential gene to grow. We initially selected for the neutral Tet^r marker and then screened colonies for the Kan-replaced gene of interest (either *yafN*, *yafNO*, *yafP*, or *yafN* and *yafP* simultaneously). The expected frequency of cotransduction of these two markers (Kan^r and Tet^r) is approximately 30% based on their physical distance. Cotransduction frequencies of ~30% imply that the gene of interest is not essential, whereas cotransduction frequencies that are significantly lower imply reduced viability of transductants that received the deletion, that is, that the gene deleted is essential.

We found that Δ*yafN*::Kan *zae-502*::Tn*10* cells were cotransduced in only 0.16% ± 0.16% of Tet^r transductants (Fig. 2B). These data imply that *yafN* is an essential gene. Supporting this interpretation, we found that loss of the chromosomal copy of *yafN* by transduction could be achieved efficiently (23% ± 2.7% Δ*yafN*::Kan *zae-502*::Tn*10* cotransductants) in recipient cells carrying a *yafN*-containing plasmid (pLS1) (Fig. 2B) but not in cells carrying only the empty plasmid vector (Fig. 2B). We conclude that *yafN* is an essential gene.

If the *yafNO*(*P*) genes were a TA system, then *yafN* would be expected to be essential only when the putative toxin, YafO, was present. We found that when Δ(*yafN-yafO*)::Kan *zae-502*::Tn*10* cells were transduced, the frequency of cotransduction of the two markers with Tet^r was 29% ± 5% (Fig. 2C). These data show that *yafN* is essential only if YafO is functional and so support the interpretation that *yafN* and *yafO* are a TA system.

In contrast with our results, there is a published *yafN* deletion strain, JW0222, of the *E. coli* Keio deletion collection (2). We hypothesized that to be viable, this strain might carry a spontaneous mutation inactivating *yafO* or alternatively a duplication of the operon in which only one of two copies of *yafN* was deleted. Such mutants are likely to have been responsible for the 0.16% frequency of cotransduction seen in the Δ*yafN*::Kan *zae-502*::Tn*10* cotransduction (Fig. 2B). We sequenced the *yafO* gene of strain JW0222 and report that the *yafO* gene contains a single base pair deletion of a guanine at bp 7, which, because of the frameshift, creates an early stop codon (TAA) at bp 61 of the 399-bp *yafO* gene (Fig. 2D). The resultant YafO protein is prematurely truncated and likely to be nonfunctional.

YafP is neither a toxin nor an antitoxin component. We sought to understand whether *yafP* was integral to the function of either the antitoxin or the toxin. If *yafP* was an integral part of the antitoxin, it, like *yafN*, would be essential for viability in the presence of *yafO*. We found that Δ*yafP*::Kan *zae-502*::Tn*10* is cotransduced efficiently at 20% ± 1.8% into a strain without an additional plasmid-borne *yafP* gene and at 26% ± 2% into strains harboring an extra *yafP* gene (Fig. 3A). Thus, *yafP* is not essential in the presence of *yafO* and therefore YafP is not an integral part of the antitoxin.

If *yafP* was an integral part of the toxin, then the loss of *yafP* would be expected to allow efficient cotransduction of Δ*yafN*::Kan with the linked Tet^r marker because *yafN* would no longer be required for viability. We found that the frequency of cotransduction of the double deletion Δ*yafN*::Kan Δ*yafP*::*cat* with the linked Tet^r marker was <0.33% in cells with no extra copy of the *yafN* gene but was efficient in cells carrying the *yafN* expression plasmid pLS1 (Fig. 3B), indicating that *yafN* re-

TABLE 3. Linear replacement gene deletion primers

Strain	Linear replacement primer name	Sequence[a]	Template
SMR6068	*yafN*wL	tgtatattctggtgtgcattattatgagggtatcactgtatgcatcgaattATTCCGGGGATCCGTCGACC	pKD13
	*yafN*wR	gctgtaagttgcaggcgaataagttttgttttgaatacccgcatccttattccttaaagtcTGTAGGCTGGAGCTGCTTC	
SMR6074	*yafO*wL	tatgacggatgatgatttcaatgactttaaggaataaggatgcgggtattcATTCCGGGGATCCGTCGACC	pKD13
	*yafO*wR	atagtttcttatttgtatgttattcataatataaattcaaaaacgcatgcgTGTAGGCTGGAGCTGCTTC	
SMR6080/6221	*yafP*wL	gcagaagcgtttcgcatgcgtttttgaatttatattatgaataacatacaaATTCCGGGGATCCGTCGACC	pKD13
	*yafP*wR	ataccaggcgggcgttatttttcattgcaagctggatttaatgttgcggtttTGTAGGCTGGAGCTGCTTC	
SMR6353	*yafN*wL	tgtatattctggtgtgcattattatgagggtatcactgtatgcatcgaattATTCCGGGGATCCGTCGACC	pKD13
	*yafP*wR	ataccaggcgggcgttattttcattgcaagctggatttaatgttgcggtttTGTAGGCTGGAGCTGCTTC	
SMR10285	*yafN*wL	tgtatattctggtgtgcattattatgagggtatcactgtatgcatcgaattATTCCGGGGATCCGTCGACC	pKD13
	*yafO*wR	atagtttcttatttgtatgttattcataatataaattcaaaaacgcatgcgTGTAGGCTGGAGCTGCTTC	
SMR11168	*yafP*::CAT-L	atgaataacatacaaataagaaactatcagcctggcgatTCATATGAATATCCTCCTTAG	pKD3
	yafP::CAT-R	atgttgcggtttatatcgcatataaaaattagtaaacGTGTAGGCTGGAGCTGCTTC	

[a] Lowercase letters refer to chromosomal sequence; uppercase letters refer to template sequence. Sequences are listed 5′ to 3′.

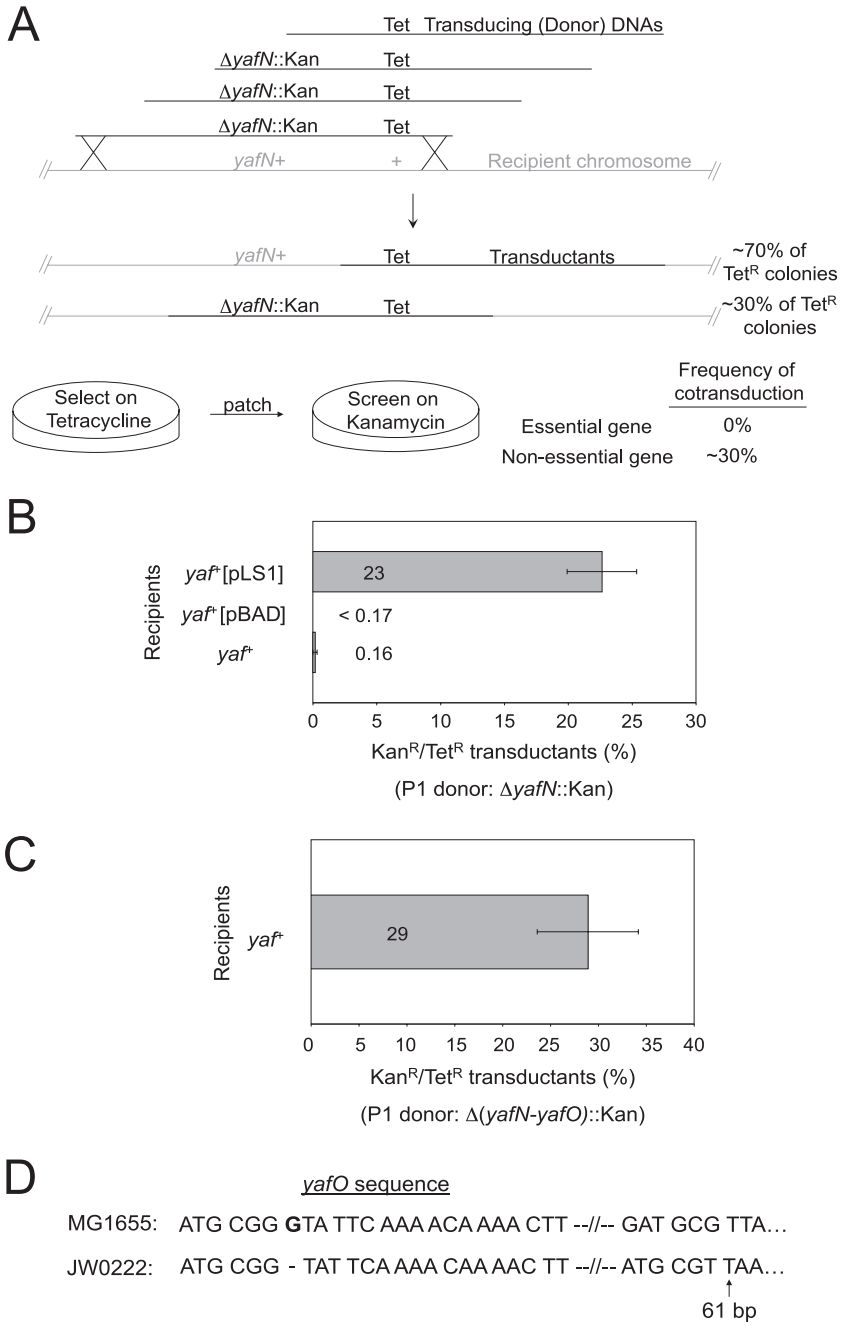


FIG. 2. Cotransduction assay for quantitative determination of inviability of various mutant strains. (A) The assay design. Phage P1 grown on strains containing gene knockouts of interest (Kan^r) with known linkage to a nonlethal Tet^r marker, *zae-502*::Tn*10*, were transduced into recipient cells and selected on Tet. Colonies were patched to Kan plates to determine the frequency of cotransduction of the markers, to determine whether the gene of interest is essential. If a gene is essential, the frequency of cotransduction of the Kan^r gene deletion among Tet^r transductants will be lower than that predicted by the distance between the markers. (B) *yafN* is essential. The Δ*yafN*::Kan deletion is cotransduced efficiently with *zae-502*::Tn*10* into cells harboring plasmid pLS1, which expresses *yafN*, but not into cells carrying no plasmid or the vector only. P1 donor: Δ*yafN*::Kan *zae-502*::Tn*10* (SMR10291). Recipient cells: *yaf*$^+$ (FC36), *yaf*$^+$[pBAD] (SMR10943), or *yaf*$^+$[pLS1] (SMR10948). Average ± 1 SEM of three experiments. (C) *yafN* is essential only in the presence of *yafO*. Δ(*yafN-yafO*)::Kan *zae-502*::Tn*10* cotransduction (P1 donor: SMR10483) into *yaf*$^+$ strain FC36. Average ± 1 SEM of three experiments. (D) The *yafO* gene is mutated in *yafN* deletion strain JW0222 of the *E. coli* gene knockout Keio collection. Sequencing of the JW0222 *yafO* gene revealed a −1-bp deletion at bp 7 that results in a premature stop codon at bp 61 of the 399-bp gene.

mains essential even when *yafP* is deleted. To address the possible concern that lingering toxin in the recipient cell (present prior to transduction of Δ*yafP*) might cause inviability after cotransduction, an additional cotransduction was performed into a Δ*yafP* recipient strain. The frequency of cotransductants remained <0.33% (Fig. 3B). We conclude that *yafN* remains essential despite the absence of *yafP*. Therefore, YafP is not a necessary part of the toxin.

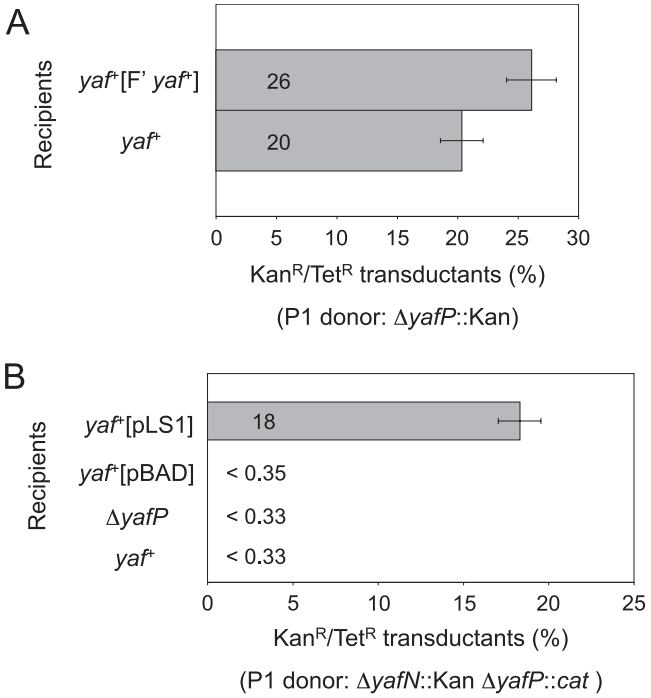


FIG. 3. YafP is not a necessary component of either toxin or antitoxin. (A) *yafP* is not essential for viability and thus not part of the antitoxin. Δ*yafP*::Kan is cotransduced efficiently with *zae-502*::Tn*10* (P1 donor: SMR10953) into recipient cells: *yaf*$^+$ (FC36) or *yaf*$^+$[F′ *yaf*$^+$] (SMR4562). Average ± SEM of three experiments. (B) Loss of *yafP* does not allow recovery of Δ*yafN* cotransductants as would be expected if *yafP* were required for toxin function. Cotransduction with P1 donor Δ*yafN*::Kan Δ*yafP*::*cat zae-502*::Tn*10* (SMR11008) into recipient cells: *yaf*$^+$ (FC36), Δ*yafP* (SMR6221), *yaf*$^+$[pBAD] (SMR10943), or *yaf*$^+$[pLS1] (SMR10948). Average ± SEM of three experiments.

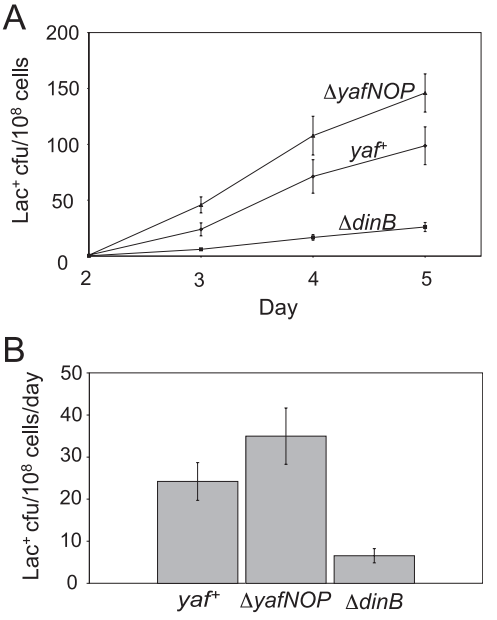


FIG. 4. The *yafNOP* genes are not required for stress-induced mutagenesis. Lac assay strains were starved on lactose for several days and monitored each day for Lac$^+$ reversion mutant colonies according to the method in reference 25. (A) Representative experiment. Lac$^+$ revertants per 10^8 cells plotted over time. Average ± SEM of five cultures. (B) Summary of mutation rates from multiple experiments. Mutation rates, calculated according to the method in reference 37, are Lac$^+$ revertants per 10^8 cells per day on days 3 to 5. Mean ± SEM from five experiments. The *yaf*$^+$ strain is SMR4562, the Δ*yafNOP* strain is SMR7491, and the Δ*dinB* strain is SMR5889.

No role in stress-induced mutagenesis. Because *dinB*, the first gene in the *dinB-yafNOP* operon, is a key player in stress-induced point mutagenesis in the *E. coli* Lac system (17, 42) and one could imagine roles for a TA system in the process, we tested for possible involvement of *yafNOP* in stress-induced *lac* reversion. We observed that the *yafNOP* strain showed slightly but not significantly higher stress-induced mutagenesis than did the *yaf*$^+$ strain, unlike *dinB*, which is required (Fig. 4A and B). Thus, the *yafNOP* genes are not required for stress-induced mutagenesis. The *yafNOP* genes were shown previously to contribute only slightly to generation-dependent mutagenesis (43).

The SOS DNA damage response. In hopes of discovering a possible role for *yafNOP* during the SOS response, we explored several assays in which DNA damage affects cell survival to test whether *yafNO* might contribute to survival or loss-of-survival phenotypes. We used several different DNA damage-inducing agents including UV light, bleomycin, and mitomycin C to induce the SOS response. We saw no significant difference between Δ*yafNOP* and *yaf*$^+$ cells in survival of UV light (Fig. 5A); bleomycin, a double-strand break-inducing agent (29) (Fig. 5B); or DNA cross-linking agent mitomycin C (30) (Fig. 5C).

A previous report suggested that the MazEF TA system protects cells against phage P1 infection (27). We found no evidence that *yafNOP* might serve as a protective unit against *E. coli* phage λ. First, the burst size of a λ lytic infection was not different when *yaf*$^+$ cells were infected than when Δ*yafNOP* cells were infected; the ratio of *yaf*$^+$ to Δ*yafNOP* was 1.4 ± 0.6 (mean ± standard error of the mean [SEM] of three experiments). Next, to test whether the *yafNOP* genes either promote or prevent the progression of λ during induction from lysogenic state, a transition controlled by the SOS response (49), we spotted various dilutions of λ lysogens of *E. coli yafNOP*$^+$ or Δ*yafNOP* cells onto different concentrations of either bleomycin- or mitomycin C-containing plates. By inducing the SOS response via DNA-damaging drugs, we simultaneously induced the λ prophage to become lytic, leading to far more severe killing in the lysogens (Fig. 6A and B) than the nonlysogens (Fig. 5B and C), indicating that the major mode of killing in lysogens was by prophage induction. However, there was no difference between the survival of Δ*yafNOP* lysogens and that of *yaf*$^+$ lysogens (Fig. 6A and B).

SOS-induced senescence is not caused by *yafNO*. Previously, Pennington and Rosenberg found that ~65% of cells that undergo spontaneous SOS induction were unable to form colonies, despite the fact that nearly all of the SOS-induced cells were viable, as determined by their ability to exclude the dye PI (47), suggesting that they were in a senescence-like state (47). To test whether this senescence-like state might result from the toxic (and possibly bacteriostatic) *yafO*, we repeated the experiment using FACS to sort *yaf*$^+$ and Δ*yafNOP* reporter strains carrying the chromosomal *gfp* gene controlled by the

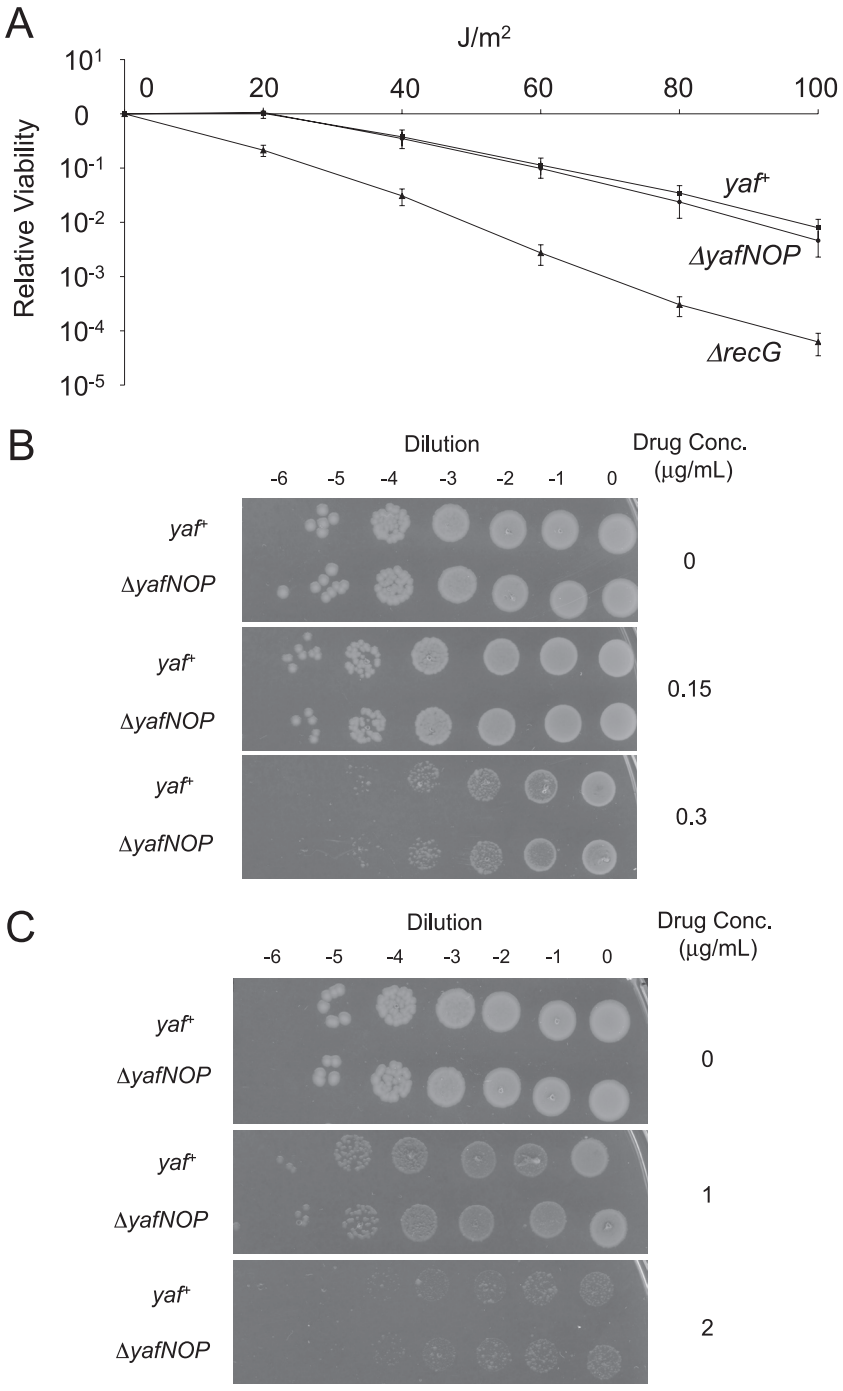


FIG. 5. Sensitivity of nonlysogens to DNA-damaging agents. (A) UV light kill curve. Log-phase cells were UV irradiated and monitored for colony formation. Average ± 1 SEM of three experiments shown. (B) Nonlysogen bleomycin kill plates. Log-phase cells were plated onto bleomycin-containing plates and monitored for viability. Bleomycin creates both single- and double-strand breaks in DNA (29). A Δ*recG* strain monitored concurrently showed sensitivity (not shown). Results shown in panels B and C are representative of two experiments. (C) Nonlysogen mitomycin C kill plates. Log-phase cells were plated onto mitomycin C-containing plates (various concentrations) and monitored for viability. Mitomycin C is a powerful DNA interstrand cross-linker (30). A Δ*recG* strain monitored concurrently showed sensitivity (not shown). For all panels, the *yaf*$^+$ strain is MG1655, the Δ*yafNOP* strain is SMR6353, and the Δ*recG* strain is SMR10990. Log dilutions are indicated.

SOS-inducible *sulA* promoter. These were sorted into spontaneously SOS-induced green and SOS-uninduced nongreen subpopulations, which compose ~1% and ~99% of the cell population, respectively (reference 47 and this study). We found that there was little difference in colony-forming abilities of the spontaneously SOS-induced green populations. We observed that 38% ± 12% of *yafNOP*$^+$ SOS-induced green cells formed colonies (35% ± 9% of green cells forming colonies

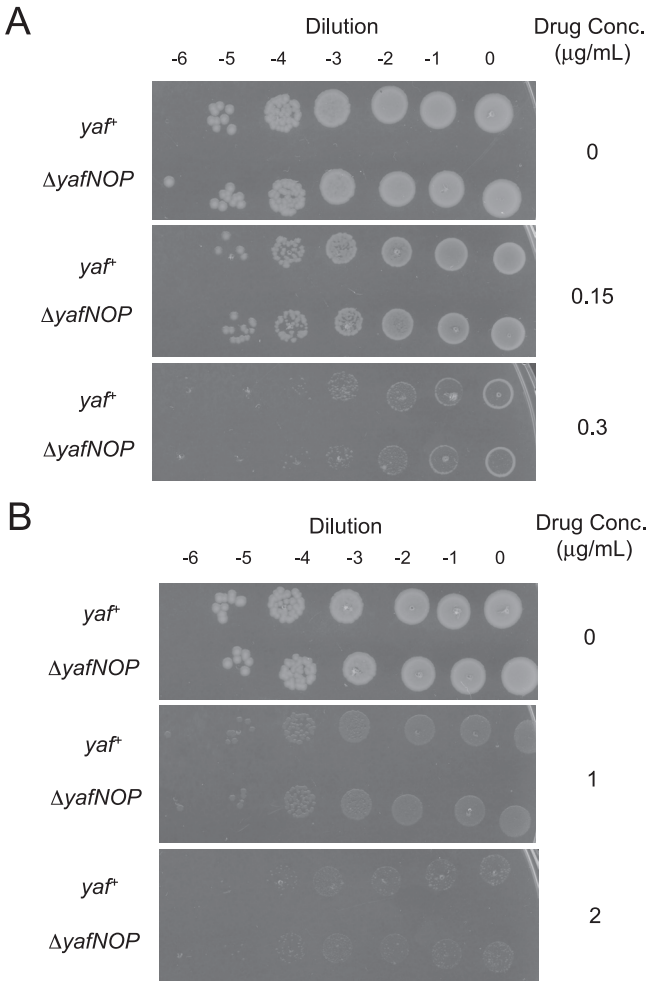


FIG. 6. YafNOP does not affect killing by prophage induction by DNA-damaging agents. DNA damage induces the SOS response and activates the λ lytic cycle, causing killing by prophage induction, as can be seen by the greater sensitivity to DNA-damaging agents of lysogens (this figure) than of nonlysogens (Fig. 5). YafNOP does not affect this killing by prophage induction. (A) Lambda lysogen bleomycin kill plates. Log-phase *E. coli* (λ) lysogens were plated onto bleomycin-containing plates (various concentrations) and monitored for viability. A Δ*recG* strain monitored concurrently showed sensitivity (not shown). (B) Lambda lysogen mitomycin C kill plates. Log-phase *E. coli* λ lysogens were plated onto mitomycin C-containing plates (various concentrations) and monitored for viability. A Δ*recG* strain monitored concurrently showed sensitivity (not shown). For both panels, the *yaf*$^+$ strain is SMR11228, the Δ*yafNOP* strain is SMR11229, and the Δ*recG* strain is SMR11231. Results shown are representative of two experiments.

normalized by 94% ± 10% of nongreen cells forming colonies to control for FACS-induced effects on colony formation) (Fig. 7). Similarly, 26% ± 5% of Δ*yafNOP* green cells formed colonies (25% ± 5% of green cells forming colonies normalized by 95% ± 2% of nongreen cells forming colonies) (Fig. 7). Also, both strains' cells were nearly all viable, with 3.6% ± 1.4% of *yafNOP*$^+$ green cells being PI$^+$ (dead) versus 4.5% ± 1.4% of Δ*yafNOP* green cells being PI$^+$. Thus, the numbers of cells in a senescence-like state of being alive (PI$^-$) but unable to form colonies were not different between *yafNOP*$^+$ and Δ*yafNOP* cells (Fig. 7).

DISCUSSION

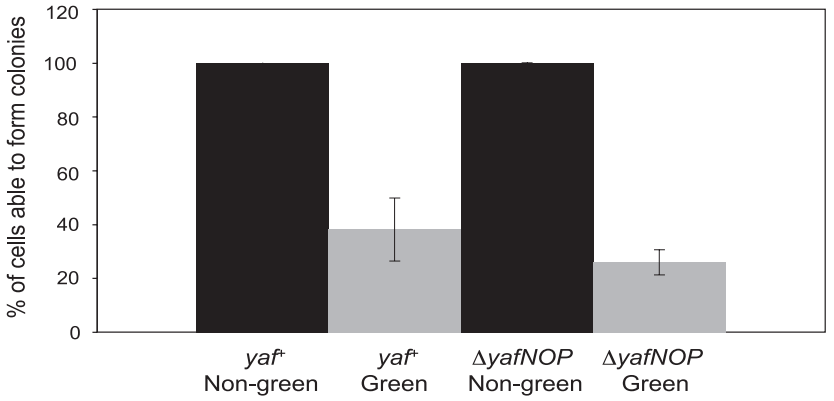
We have shown that the *yafN* gene of the *dinB-yafN-yafO-yafP* operon (43) is essential for viability only in the presence of a functional *yafO* gene (Fig. 2B and C), providing strong evidence that these two genes constitute the antitoxin and toxin genes, respectively, of a type 2 TA pair. Whereas previous in vivo demonstrations of type 2 TA systems have used the method of separate cloning of each gene into differentially inducible plasmids and showing that the toxin induces cell stasis when solely expressed, but not when the antitoxin is also expressed (1, 50), we used an alternative genetic approach that allows us to rule out possible effects specific to overexpression of either the toxin or the antitoxin. We can therefore conclude that YafO exerts its toxic effect, and YafN can quell that effect, when each gene is expressed at normal levels from its native promoter, in single copy in its normal chromosomal position.

Typically, chromosomal TA systems consist of two genes in an operon, the toxin and the antitoxin. Although there is precedent for a TA system with a third gene element, *mazEFG* (22), we found that *yafP*, the third *yaf* gene in the operon, is not an integral component of either the toxin or the antitoxin (Fig. 3A and B). This conclusion does not preclude the possibility that YafP might play a regulatory role in the YafNO TA system which we have not detected, as *mazG* does for *mazEF* (22).

The possible function of a TA system controlled by the SOS response is an interesting problem. We found that unlike *dinB* (42), the first gene in the operon (43), the *yafNOP* genes do not contribute to stress-induced mutagenesis significantly (Fig. 4), a process that requires SOS-induced levels of *dinB*, but not SOS-induced levels of any other SOS-controlled component (17). Other SOS-controlled genes such as *recA* (6, 24) and *ruvA* and *ruvB* (15, 25) are required for stress-induced Lac mutagenesis, we now appreciate, at their constitutive levels of expression, not at induced levels (17). The results presented here rule out a requirement for YafNOP in stress-induced mutagenesis even at their constitutive expression levels. Previously, YafNOP had little effect on spontaneous generation-dependent mutagenesis in nonstressed growing cells (43).

We also did not detect effects of *yafNOP* on survival of cells following various SOS-inducing treatments, for nonlysogens (Fig. 5A to C) and also for cells harboring a wild-type lambda prophage, which is induced leading to cell lysis when the SOS response is activated (Fig. 6A and B). Finally, although we could recapitulate the previous results of Pennington and Rosenberg, showing that many spontaneously SOS-induced green fluorescent cells (bearing an SOS-controlled chromosomal *gfp* reporter gene) are apparently viable but unable to form colonies when recovered by FACS (47), we found that *yafNOP* is not responsible for their senescence-like state (Fig. 7).

What might be the function of an SOS-controlled TA system? For TA systems, the toxic effects ensue when the operon's expression is decreased, so we would expect possible effects of YafO to be manifested as cells recover from an SOS response and return to normal after DNA repair, even though these genes are expressed mid-range in the SOS response (11). Perhaps YafO induces a transient cell stasis upon recovery. This might function to extend the cell cycle checkpoint caused by



PI⁻ (%)	99	96	99	96
PI⁺ (%)	0.85	3.6	0.7	4.5
± (%)	0.23	1.4	0.3	1.4

FIG. 7. The *yafNOP* genes were not responsible for the senescence-like state of spontaneously SOS-induced cells. Strains with a chromosomal *gfp* gene controlled by the SOS-inducible *sulA* promoter were sorted into SOS-induced green and SOS-uninduced nongreen populations by FACS and plated for CFU. The decreased ability of SOS-induced green cells to form colonies was not reversed by the Δ*yafNOP* mutation. Percentage of live/dead cells in each population was determined by PI staining and flow cytometry to quantify dead cells, which take up the dye. The percentage of cells in a senescence-like state, excluding PI but unable to form colonies, was not different for *yaf*$^+$ (SMR6669) and Δ*yafNOP* (SMR10988) cells. Average ± SEM of five experiments.

SOS-induced expression of the SulA inhibitor of cell division (16). Although we did not find effects of YafNO on cells after SOS induction, we cannot rule out the possibility of an important role in SOS recovery that our assays might not have detected. Alternatively or in addition, *dinB*, and presumably the rest of its operon, is also upregulated slightly by the RpoS general stress response (33). Perhaps YafO plays a role in promoting cell stasis upon recovery from the RpoS response. Additionally, although the *yafNO* genes are transcribed from the upstream SOS-controlled (and RpoS-controlled) *dinB* promoter (43), there is in vitro evidence that an additional promoter may exist immediately upstream of *yafN*, possibly creating an SOS (or RpoS)-independent *yafNOP* operon (57). If so, *yafNOP* might act outside the contexts of the SOS (or RpoS) response.

A recent report shows that DinB and also another SOS-inducible DNA polymerase, Pol V, interact directly with the NusA transcription and antitermination factor (10). The authors suggest that NusA might direct the translesion DNA synthesis activity of DinB to sites of active transcription. Similarly, we can imagine that DinB might affect NusA-dependent transcription termination, which might then provide another level of SOS control of gene expression (negative or positive). Perhaps YafNO or YafP functions in such a process, and perhaps the toxic effect of YafO is related to a transcription-termination-specific effect.

While the manuscript was being prepared, another group reported that the YafO protein is an RNase (58). Their results support our conclusions and provide a mechanism for the toxic action of the YafO toxin. Important next steps toward understanding the *yafNO* TA system include defining when as well as on what targets the toxin acts.

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