A Novel Family of *Escherichia coli* Toxin-Antitoxin Gene Pairs

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Bacterial toxin-antitoxin protein pairs (TA pairs) encode a toxin protein, which poisons cells by binding and inhibiting an essential enzyme, and an antitoxin protein, which binds the toxin and restores viability. We took an approach that did not rely on sequence homology to search for unidentified TA pairs in the genome of *Escherichia coli* K-12. Of 32 candidate genes tested, ectopic expression of 6 caused growth inhibition. In this report, we focus on the initial characterization of *yeeV*, *ykfI*, and *ypjF*, a novel family of toxin proteins. Coexpression of the gene upstream of each toxin restored the growth rate to that of the uninduced strain. Unexpectedly, we could not detect in vivo protein-protein interactions between the new toxin and antitoxin pairs. Instead, the antitoxins appeared to function by causing a large reduction in the level of cellular toxin protein.

Bacterial toxin-antitoxin pairs (TA pairs) consist of a stable toxin protein that can cause cell death by disrupting an essential cellular process, coupled with a labile antitoxin protein that can bind to and block activity of the toxin (16, 18). Also known as addiction modules, TA pairs were first identified on plasmids and characterized for their role in postsegregational killing (39). After cell division, daughter cells that do not inherit a copy of a plasmid expressing an addiction module can no longer produce antitoxin protein. Hence, following rapid degradation of residual antitoxin protein, the remaining excess toxin protein is free to bind and inhibit the cellular target, providing a selection mechanism for plasmid maintenance in the population.

More recently, several chromosomal genes similar to plasmid-borne addiction modules have been identified (19, 21, 26, 29). The cellular function of these chromosomally encoded TA pairs has not been clearly defined. Two models have been suggested by studies of proteins involved in the *Escherichia coli* stringent response (11). One hypothesis, stemming from investigation of *mazE*/*mazF* (*chpAI*/*chpAK*), a pair of genes located in the *relA* operon, is that genomic TA pairs also function as addiction modules (2). Global gene expression is down regulated in response to amino acid starvation as part of the stringent response (11). In a manner analogous to postsegregational killing, this transcriptional attenuation could result in the inability to replace the rapidly degraded antitoxin MazE, leading to MazF-mediated cell death (2, 16). It has been proposed that, under some circumstances, it may be evolutionarily advantageous for a fraction of cells to undergo programmed cell death in order to provide nutrients for the remainder of the population (25). Alternatively, examination of the toxin-antitoxin gene pair *relB*/*relE* (12, 17, 19, 31, 32) supports the model that, unlike plasmid-based toxins, the function of the chromosomal TA pairs is not bacterial "apoptosis" but to modulate the rate of metabolic processes in response to environ-

mental stress (18). Mutant alleles of *relB* confer a defect in the stringent response termed a delayed relaxed phenotype, in which cells are unable to efficiently resume protein synthesis after readdition of amino acids (4, 15, 24). Excess RelE protein has been shown to result in a decrease in the rate of protein synthesis both in vitro and in vivo, and this inhibition is neutralized by the addition of the antitoxin RelB (12, 31). Furthermore, recent evidence suggested that the mechanism of protein synthesis inhibition by RelE is via sequence-specific cleavage of mRNA in the ribosomal A site, with preference for the stop codon UAG (32). The ability of RelB and RelE to reversibly inhibit translation may provide a mechanism to slow the rate of translation in response to nutrient deprivation. Although the precise mechanism of toxicity has not been reported for MazF, overexpression of MazF disrupts both translation and replication in a manner that is reversible by MazE, suggesting this TA pair could also play a similar role in stress adaptation (18, 31).

The cellular target and mechanism of toxicity for the majority of toxin proteins are unknown. However, where a target has been identified, the toxin has been shown to function by interacting with essential proteins known or suspected to be good targets for antimicrobial agents. For example, CcdB inhibits DNA gyrase activity (6, 28), and PemK is thought to poison cells via the replicative helicase DnaB (33). Therefore, identification and study of additional members of this class of proteins may help validate novel targets for antimicrobial therapy or provide insight into novel mechanisms of bacterial cell death.

We used an approach that did not rely upon sequence homology to investigate whether additional TA protein pairs are present in the *E. coli* genome. All pairs of genes in the annotated genome of *E. coli* K-12 (7) that fit the highly conserved size and genetic organization of known TA pairs were identified. Of 32 genes tested, ectopic expression of 6 genes resulted in growth inhibition and/or a reduction in the number of CFU. Paired antitoxin function was demonstrated for several of these, as defined by the ability to restore normal growth upon coexpression. In this report, we describe the initial characterization of a novel family of three homologous TA pairs.

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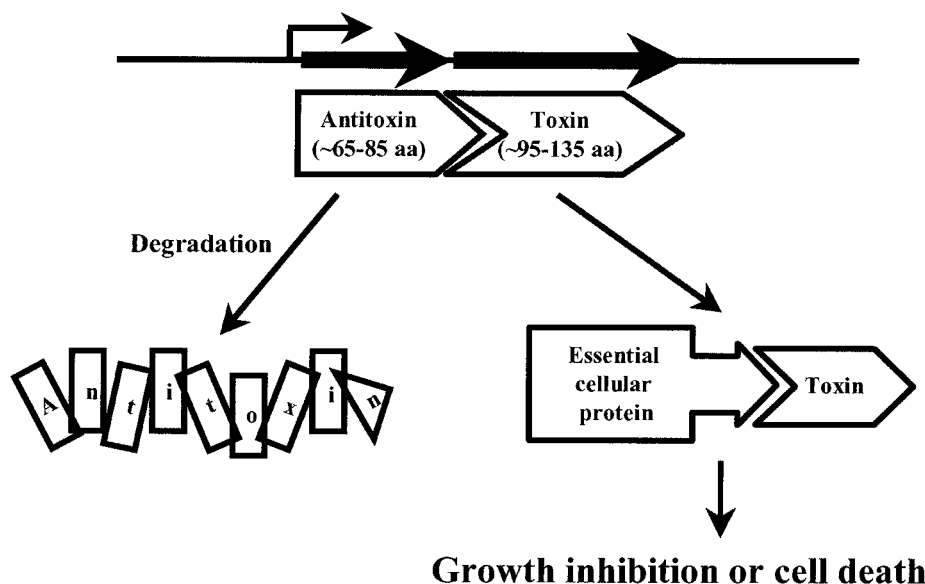
FIG. 1. Characteristics and genetic organization of bacterial TA pairs.

MATERIALS AND METHODS

Identification of candidate toxin genes. A database containing several physical parameters for the proteins encoded by all predicted open reading frames (ORFs) in the *E. coli* genome (G. Schoenhals and K. J. Shaw, unpublished data) was used to identify all proteins 65 to 135 amino acids (aa) in length. The genes encoding these proteins were sorted by chromosomal position and scanned for pairs of ORFs that fell into the desired size range and were predicted to be in the same operon (intergenic space of ≤150 bp). The nomenclature and identification of all of the hypothetical *E. coli* genes identified via genomic sequencing and used in this study are found at the following websites: http://genolist.pasteur.fr/Colibri/ and http://bmb.med.miami.edu/EcoGene/EcoWeb/.

Subcloning of genes of interest. The predicted ORF (start codon to stop codon) for each gene of interest was amplified by PCR from MG1655 genomic DNA. The 5′ end of upstream primers incorporated an *Eco*RI site (italics) as well as a consensus ribosome-binding site (bold), followed by 18 to 22 bp of gene sequence starting with ATG (e.g., *GAATTC* **GGAG**TGAAACG ATG. . .). The 5′ end of each downstream primer contained an *Xba*I site. After amplification, each PCR product was digested with *Eco*RI and *Xba*I (New England Biolabs, Inc., Beverly, Mass.) and ligated into one or both of the arabinose-inducible expression vectors pBAD18 (ampicillin resistance; high copy, ~100 to 300 copies/cell) or pBAD33 (chloramphenicol resistance; low copy, ~15 copies/cell) that had been digested with *Eco*RI and *Xba*I (20). Subcloning into pBAD33 required partial digestion with *Eco*RI due to the presence of a second cut site in the chloramphenicol cassette. Ligation mixtures were transformed into chemically competent *E. coli* TOP10 cells (Invitrogen Corporation, Carlsbad, Calif.), selecting for the appropriate plasmid-borne drug resistance markers. Positive clones were verified by DNA sequencing.

Toxicity assays. Overnight cultures were diluted 100-fold in fresh medium and grown to log phase (optical density at 600 nm [OD_{600}] = 0.4 to 0.5), then rediluted to OD_{600} = 0.01 in fresh medium with or without 0.2% L-(+)-arabinose (Sigma, St. Louis, Mo.). Optical density was monitored using a Spectronic 20D+; when the OD_{600} was ≥1.0, cultures were diluted 10-fold and reread to stay within the accurate range of the instrument. To quantitate CFU, cells were diluted in phosphate-buffered saline (pH 7.1; Invitrogen Corporation), plated on Luria-Bertani (LB) agar plus 100 μg of ampicillin/ml, and incubated overnight at 37°C. All cultures were grown at 37°C in LB plus 100 μg of ampicillin/ml or 30 μg of chloramphenicol/ml, as appropriate, with shaking (~225 rpm).

Coexpression constructs. Genes that are adjacent on the *E. coli* chromosome were amplified directly from MG1655 genomic DNA with the same primers as described above and ligated into pBAD18. Others were constructed using PCR-SOEing (22). As in the single-gene constructs, the upstream primer contained a consensus ribosome-binding site.

Cluster analysis. Multiple sequence alignments were done using ClustalW version 1.8 (36). Formatting was done with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html).

Examination of toxin protein and mRNA levels. Overnight cultures were diluted 100-fold into fresh LB plus ampicillin (100 μg/ml), grown to OD_{600} = 0.5, and then split into new tubes containing LB plus ampicillin (100 μg/ml) with or without 0.2% arabinose. After 30 min, the optical density was monitored and 0.5 ml of each culture was pelleted (5,000 × *g* for 5 min at 4°C). Supernatant was removed, and the pellet was dissolved in 100 μl of B-PER (Pierce Biotechnology Inc., Rockford, Ill.) plus 1× NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen Corporation) per 1.0 OD_{600} unit. A 3.0-μl aliquot of each sample was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a NuPage 12% bis-Tris gel with morpholineethanesulfonic acid running buffer (Invitrogen Corporation) and transferred to polyvinylidene difluoride membrane. Western blotting was performed following the manufacturer's instructions using purified murine monoclonal His$_6$ antibody (Covance Inc., Princeton, N.J.) and anti-FLAG M2 monoclonal antibody (Sigma). Total RNA was isolated from equivalent samples using the Ambion NorthernMax kit according to the manufacturer's instructions (Ambion Inc., Austin, Tex.). Ten micrograms of RNA was used for each sample. Insert DNA isolated after digestion of each expression construct with *Eco*RI and *Xba*I was used as a template for synthesis of ^{32}P-labeled DNA probes using the Radprime DNA labeling system (Invitrogen Corporation).

RESULTS

Identification of toxin genes. We used a method to identify genes encoding putative toxins that did not rely on sequence homology to known proteins. Most previously characterized TA pairs are cotranscribed and consist of a gene encoding a small (~65- to 85-aa) antitoxin protein adjacent to a gene encoding a slightly larger (~95- to 135-aa) toxin protein (Fig. 1). We identified 18 pairs of genes in the annotated genomic DNA sequence of *E. coli* K-12 (MG1655) that fit these size criteria and were linked closely enough to potentially be within the same operon (≤150 bp apart) (*pspB-pspC*, *pspD-pspE*, *yafN-yafO*, *yeeT-yeeU*, *yeeU-yeeV*, *ybdJ-ybdF*, *ykfH-yafW*, *yafW-ykfI*, *ybgE-ybgC*, *ynaK-ydaY*, *yebG-yebF*, *yffM-yffN*, *yfhN-yfhF*, *ypjJ-yfjZ*, *yfjZ-ypjF*, *ygfY-ygfX*, *yheL-yheM*, and *yheM-yheN*).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Insert (description)	Source or reference
E. coli strains		
TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacZ*74 *deoR recAI araD139* Δ(*ara-leu*)7697 *galU galK rpsL endAI rupG*	Invitrogen
MG1655	F⁻ λ⁻ *ilvG rfb-50 rph-1*	7
Plasmids and constructs		
pBAD18	None	20
pRD020	*mazF*	This study
pRD022	*pspC*	This study
pRD023	*ykfI*	This study
pRD024	*yeeV*	This study
pRD025	*ydjM*	This study
pRD026	*yafO*	This study
pRD027	*yfjG*	This study
pRD028	*ydgF*	This study
pRD029	*yafW*	This study
pRD030	*yeeU*	This study
pRD041	*ykfI*-His₆	This study
pRD042	*yeeV*-His₆	This study
pRD049	*yafW-ykfI*	This study
pRD050	*yeeU-yeeV*	This study
pRD052	*ypjF*	This study
pRD061	*yafW-ykfI*-His₆	This study
pRD062	*yeeU-yeeV*-His₆	This study
pRD068	*ykfH*	This study
pRD069	*yeeT*	This study
pRD086	*yfjZ*	This study
pRD095	*yeeU-BamHI-yeeV*	This study
pRD103	*ypjJ*	This study
pRD104	*yeeT-yeeV*	This study
pRD105	*gfp-yeeV*	This study
pRD106	*ykfH-ykfI*	This study
pRD107	*gfp-ykfI*	This study
pRD114	*relE*	This study
pRD124	*gfp-ykfI*-His₆	This study
pRD125	*ypjF*-His₆	This study
pRD134	*UTR-yeeV*	This study
pRD135	*yeeU-UTR-BamHI-yeeV*	This study
pRD136	*Flag-ypjF*	This study
pRD139	*yafW-BamHI-ykfI*	This study
pRD140	*gfp-yeeV*-His₆	This study
pRD141	*yeeU-BamHI-ykfI*	This study
pRD142	*yafW BamHI-yeeV*	This study
pBAD33	None	20
pRD072[a]	*yeeV*	This study

[a] Low-copy-number pBAD33-based construct. All others described are high-copy pBAD18-based constructs.

To determine which, if any, of the candidate genes encode toxin proteins, the predicted ORF for each of the genes listed above was amplified by PCR from *E. coli* MG1655 genomic DNA and subcloned into the arabinose-inducible expression vector pBAD18 (20) (Table 1). To assay for toxicity, log-phase cultures of *E. coli* TOP10 cells transformed with each expression construct were diluted to $OD_{600} = 0.01$ in fresh LB plus ampicillin (100 μg/ml) with or without arabinose, and the optical density was monitored over the course of 6 h. Six genes were identified whose overexpression resulted in significant inhibition of growth compared to the strain carrying the parent

vector (Fig. 2). For five of the overexpressing strains, the growth rate diverged from that of the control strain after 3 h of induction, similar to that seen for strains overproducing RelE or MazF. Growth of the PspC overexpression strain was similar to that of the parent vector control for 4 h before slowing significantly.

yeeV, ykfI, and ypjF define a novel toxin gene family. In this study, we initially focused our investigation on two of the six genes identified, *yeeV* and *ykfI*. These genes encode small proteins (124 and 113 aa, respectively) that share 58% aa sequence identity. Surprisingly, arabinose induction of a third highly similar gene, *ypjF*, did not cause growth inhibition in our assay, even though the YpjF protein is 80% identical to the YkfI protein (Fig. 3A and C). Quantitation of bacterial titers indicated that in addition to inhibiting growth, expression of *yeeV* or *ykfI* also caused a reduction in the number of CFU (Fig. 4A). After increasing at the same rate as the uninduced control culture for the first 2 h after induction, the viability of cells expressing *ykfI* decreased to approximately half of that at time zero, whereas the uninduced cells continued to grow exponentially. CFU of the *yeeV*-expressing strain decreased ~500-fold from time zero.

We constructed C-terminal His₆-tagged forms of each protein to allow analysis of protein expression. Addition of the epitope tag did not affect the phenotype of overexpression (Table 2). Examination of cellular protein concentration showed that in the presence of arabinose, YeeV-His₆ and YkfI-His₆ accumulate in the cell but YpjF-His₆ does not, explaining the lack of toxicity (Fig. 4B). Northern blot analysis showed that the level of *ypjF*-His₆ mRNA was significantly lower as well (Fig. 4B). Because all pBAD18 promoter and regulatory sequences were conserved between the expression constructs, we inferred that the difference in mRNA concentration may be an indirect effect. For example, if the translation rate for YpjF-His₆ were low, mRNA stability could be decreased due to the tight linkage between bacterial transcription and translation. Inefficient translation is known to cause both premature termination of transcription (35) and a decrease in mRNA stability (34). Replacement of the His₆ tag on YpjF with an N-terminal Flag tag resulted in both a large increase in cellular protein accumulation and growth inhibition (Fig. 4B; Table 2). Two slightly different forms of Flag-YpjF were detected by Western blotting.

yeeU and yafW can prevent toxicity. *yeeV*, *ykfI*, and *ypjF* are each preceded on the chromosome by two potential antitoxin genes that encode proteins of approximately the same size, the first of which is slightly smaller (Fig. 3C). In all three cases, none of the upstream genes inhibited growth when overexpressed individually (Table 3). Each of the ORFs was coexpressed with the appropriate toxins to test for the ability to prevent growth inhibition. In all previously characterized TA pairs, the antitoxin is immediately adjacent to the toxin. However, since *yeeT* and *ykfH* encode slightly smaller proteins than *yeeV* and *ykfI*, a common characteristic for antitoxins, these were also assayed for activity. Finally, the gene encoding the unrelated green fluorescent protein (GFP) was coexpressed as a negative control. ORFs that are adjacent in the genome were amplified as a pair from genomic DNA and subcloned into pBAD18 for expression. Others were constructed such that only the sequence of the proximal ORF varied; all regulatory
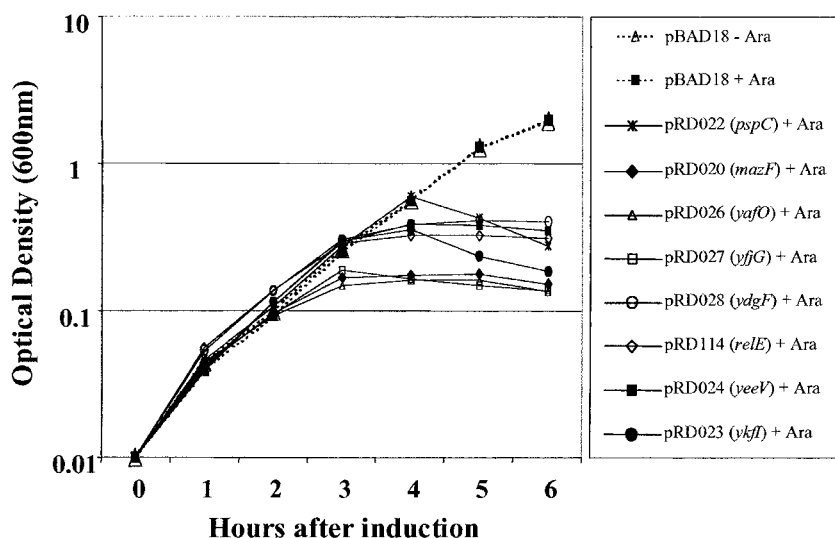
FIG. 2. Ectopic expression of six candidate toxin genes causes growth inhibition. Log-phase cultures (OD_{600} = 0.5) were diluted in LB plus ampicillin (100 μg/ml) liquid medium to OD_{600} = 0.01 and grown in the presence or absence of 0.2% arabinose. The known toxin genes *relE* and *mazF* were included as positive controls.

and untranslated intergenic sequences were conserved. In both cases, coexpression of the adjacent gene (*yeeU-yeeV* and *yafW-ykfI*) restored the growth rate to the level of the uninduced strain (Table 3) and prevented a reduction in the number of CFU (data not shown). Growth of strains coexpressing the other pairs of genes (*yeeT-yeeV*, *gfp-yeeV*, *ykfH-ykfI*, or *gfp-ykfI*) was inhibited to approximately the same degree as growth of those expressing toxin alone (Table 3).

Most known antitoxins prevent toxicity by physically interacting with the toxin partner (16, 18). Therefore, we performed coimmunoprecipitation experiments to detect in vivo protein-protein interactions by using expression constructs in which both the toxin and antitoxin were epitope tagged. Addition of an epitope tag to the N terminus of the antitoxins YeeU and YafW did not affect their ability to prevent growth inhibition. However, although the tagged forms of each protein could be individually immunoprecipitated when expressed, demonstrating that sufficient amounts of proteins were produced for these studies, interactions between YeeU and YeeV or YafW and YkfI were not observed under a wide variety of experimental conditions (data not shown). Instead, Western blotting revealed that antitoxin coexpression resulted in almost a complete absence of His$_6$-tagged toxin proteins in whole-cell extracts, whereas GFP did not (Table 4; Fig. 5). Because all transcriptional regulatory sequences were conserved among all of the expression constructs, it was anticipated that mRNA synthesis should be similar. Therefore, these results suggest that rather than binding to the toxins to prevent toxicity, YeeU and YafW may function as antitoxins by either preventing toxin protein translation or promoting toxin degradation. Both the degree of growth inhibition and the amount of toxin observed upon induction of pRD124 (*gfp-ykfI*-His$_6$) were increased compared to that conferred by expression from pRD041 (*ykfI*-His$_6$). The converse was true for cells transformed with pRD140 (*gfp-yeeV*-His$_6$)—both the cellular toxin level and the degree of growth inhibition were decreased compared to that in a strain carrying pRD042 (*yeeV*-His$_6$). These data, combined

with those shown in Fig. 4, suggest that the degree of growth inhibition is directly related to the amount of toxin protein present in the cell. Therefore, the toxins may be titrating out an essential cellular component that may be present in limiting quantities.

YeeU antitoxin function requires the presence of the intergenic UTR. Each of these novel toxin-antitoxin gene pairs is separated by a conserved 20-bp untranslated region (UTR). However, an additional 68 bp of untranslated mRNA is located immediately downstream of *yeeU*, but not *yafW* or *ypjJ*. To investigate whether the UTR plays a role in YeeU antitoxin function, we compared the coexpression constructs described previously (pRD049 and pRD050 [Table 3]) to pRD095 and pRD139, constructs in which each ORF was amplified separately and ligated together in pBAD18 using a *Bam*HI restriction site (*yeeU*-*Bam*HI-*yeeV*, *yafW*-*Bam*HI-*ykfI*). In the absence of the UTR, YeeU could not prevent growth inhibition mediated by YeeV, whereas if the UTR were amplified along with the *yeeU* ORF before ligation with *yeeV* (*yeeU*-UTR-*Bam*HI-*yeeV*), antitoxin activity was restored (Table 5). The presence of the UTR alone (*UTR-yeeV*) was not sufficient to prevent toxicity (Table 5). Growth of arabinose-induced cells carrying pRD142 (*yafW*-*Bam*HI-*ykfI*) was similar to that of the uninduced control, indicating that the conserved 20-bp portion of the UTR is not required for antitoxin function (Table 5).

We further probed the requirement of *cis* elements for the UTR in YeeU function by expressing *yeeV* and *yeeU* from separate compatible plasmids in the same cell. To do this, a strain containing both pRD072 (*yeeV* subcloned into the low-copy-number arabinose-inducible vector pBAD33) and pRD030 (*yeeU* subcloned into pBAD18) was constructed. We found that expression of *yeeU* was unable to prevent toxicity in *trans*, supporting the hypothesis that the UTR contains critical *cis*-acting regulatory elements (Table 5).

We constructed expression cassettes in which *yeeU* and *yafW* were exchanged to examine the cross-compatibility of antitoxin function. We found that YafW was able to partially block

A

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YpjF     1  MNTLPATISQAAKPCLSPVAVWQMLLTRLLEQHYGLTLNDTPFSDETVIKEH
YkfI     1  MKTLPAITQRAVKPCLSPVAVWQMLLTRLLEQHYGLTINDTPFCNEAVIKEH
YeeV     1  MKTLPVLPGQAASSRPSPVEIWQILLSRLLDQHYGLTLNDTPFADERVIEQH

YpjF    53  IDAGITLADAVNFLVEKYELVRIDHRGFSWQQQSPYISVVDILRARRSTGLL
YkfI    53  IDAGITLADAVNFLVEKYELVRIDRKGFSWQEQSPYLRAADILRARQATGLL
YeeV    53  IEAGISLCDAVNFLVEKYALVRTDQPGFSACTRSQLINSIDILRARRATGLM

YpjF   105  KTNVK----------------
YkfI   105  RQSRNNVVR------------
YeeV   105  TRDNYRTVNNITLGKYPEAK
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B

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YfjZ     1  --------------MSNTTWGLQRDITPRLGARLVQEGNQLHYLADRASITG
YafW     1  --------------MSNPTRGLQREITLRLGARLVQEGNRLHYLADRASITG
YeeU     1  MSDTLPGTTLPDDNHDRPWWGLPCTVTPCFGARLVQEGNRLHYLADRAGIRG

YfjZ    40  KFSDAECPKLDVVFPHFISQIESMLTTGELNPRHAQCVTLYHNGFTCEADTL
YafW    40  KFSDIECRKLDETFPHFILQMESMLTTGELSPHHAHCVTLYHNDLTCEADTL
YeeU    53  LFSDADAYHLDQAFPLLMKQLELMLTSGELNPRHQHTVTLYAKGLTCKADTL

YfjZ    93  GSCGYVYIAVYPTQR---
YafW    93  GSCGYVYIAIYPTQR---
YeeU   105  SSCDYVYLAVYPTPEMKN
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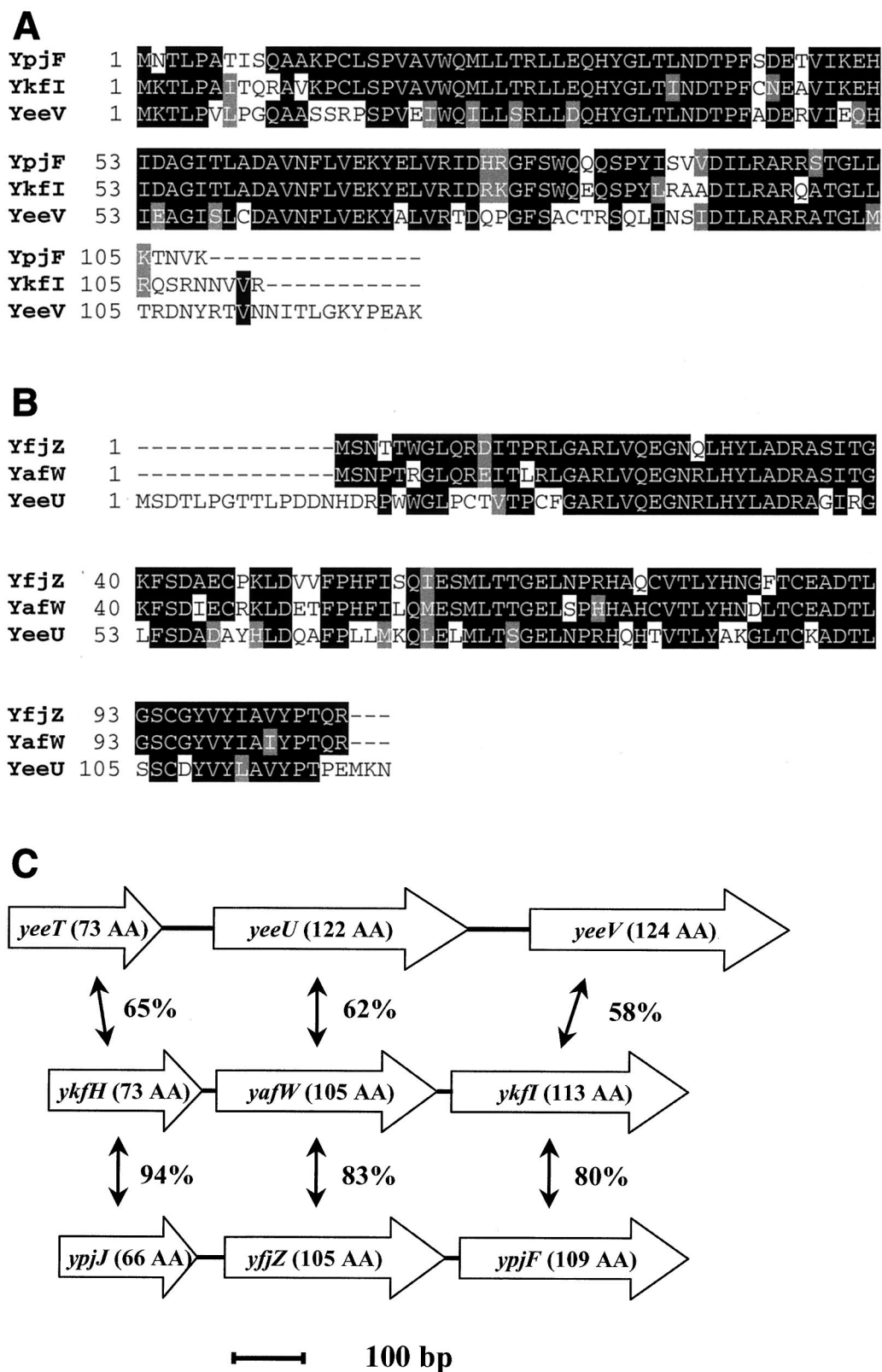
C

FIG. 3. YeeV, YkfI, and YpjF define a novel family of proteins. (A) Amino acid sequence alignment of the toxins YeeV, YkfI, and YpjF. Identical residues are highlighted in black, and chemically conserved residues are highlighted in gray. (B) Amino acid sequence alignment of the gene upstream of each toxin. (C) Chromosomal organization of *yeeV*, *ykfI*, *ypjF*, and the two proximal genes for each. The percent amino acid sequence identity for each homolog is indicated.
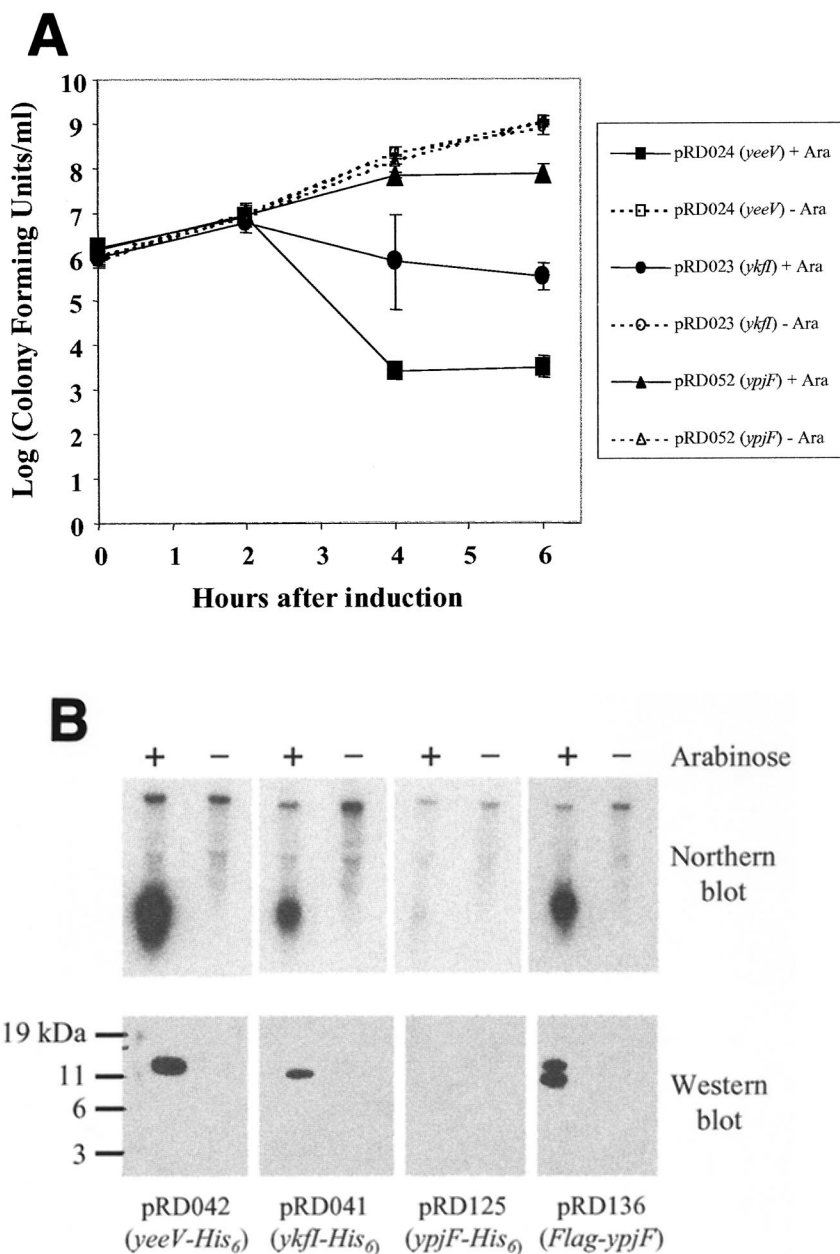
FIG. 4. (A) Viability of strains expressing *yeeV*, *ykfI*, and *ypjF*. Growth in the presence (solid lines) or absence (dashed lines) of 0.2% arabinose was performed as described in the legend for Fig. 2. To quantitate CFU per milliliter, cells were diluted and plated on LB agar plus 100 μg of ampicillin/ml at the times indicated. (B) Analysis of mRNA and protein concentration for strains expressing *yeeV*-His, *ykfI*-His, *ypjF*-His, or FLAG-*ypjF*. Equivalent masses of total RNA and cellular protein isolated from log-phase cultures (OD_{600} = 0.5) shaken at 37°C for 30 min in the presence or absence of 0.2% arabinose were resolved by gel electrophoresis. mRNA was detected by hybridization with ^{32}P-labeled DNA for each gene, and protein was detected using the appropriate antibody to each epitope tag.

YeeV toxicity but was less effective than YeeU (with the UTR present) (Table 5). Finally, YeeU was able to prevent YkfI toxicity to a moderate degree, even in the absence of the 68-bp portion of the UTR, but was less effective than YafW (Table 5).

DISCUSSION

We used a method that relied on protein size, rather than sequence homology, to search for novel bacterial TA pairs.

Out of 32 genes tested, 6 caused growth inhibition when ectopically expressed in *E. coli*. We focused our attention on two of these chromosomal genes, *yeeV* and *ykfI*, that along with a third highly related gene, *ypjF*, define a novel family of toxins. Overproduction of each of these resulted in growth inhibition, although YpjF only accumulated to levels sufficient to inhibit growth when stabilized with an N-terminal FLAG tag. None of the toxin genes contained an unusual number of rare codons,

TABLE 2. Effect of epitope tagging on toxin induced
growth inhibition

Plasmid (gene expressed)	Arabinose	Growth at 6 h[a]
pBAD18	+	+++
pBAD18	−	+++
pRD052 (ypjF)	+	+++
pRD125 (ypjF-His$_6$)	+	+++
pRD136 (Flag-ypjF)	+	−
pRD041 (ykfI-His$_6$)	+	−[b]
pRD042 (yeeV-His$_6$)	+	−

[a] Growth was monitored as described for Fig. 2. Optical density after 6 h of growth is represented as follows: −, $OD_{600} < 0.5$; +++, $OD_{600} > 1.5$.
[b] In this experiment, $OD_{600} = 0.45$.

which might be expected to lead to growth inhibition if the protein were overexpressed. Given that *ypjF* and *ykfI* share 80% sequence homology and the expression constructs are identical outside of the ORF, we suspect that specific sequences near the 5′ end of the *ypjF* gene are responsible for either inefficient translation or increased degradation and that these differences are masked by the presence of the FLAG tag. Coexpression of the adjacent, upstream gene of each toxin (*yeeU-yeeV*, *yafW-ykfI*) restored normal growth and viability. These genes encode proteins of approximately the same size as the toxin, which was somewhat surprising since our initial search was for pairs in which the antitoxin was slightly smaller. Indeed the antitoxin-encoding genes *yeeU* and *yafW* are preceded by the slightly smaller genes *yeeT* and *ykfH*, respectively. However, *yeeU* and *yafW* were not toxic when overexpressed, and *yeeT* and *ykfH* did not prevent toxicity when coexpressed with *yeeV* or *ykfI*. Additionally, we found that *yeeU*-mediated suppression of *yeeV* toxicity required the unique 68-bp intergenic UTR that was not present in the other two pairs. Cluster alignment analysis suggests the UTR may be derived from a duplication of sequence within the *yeeU* coding region (unpub-
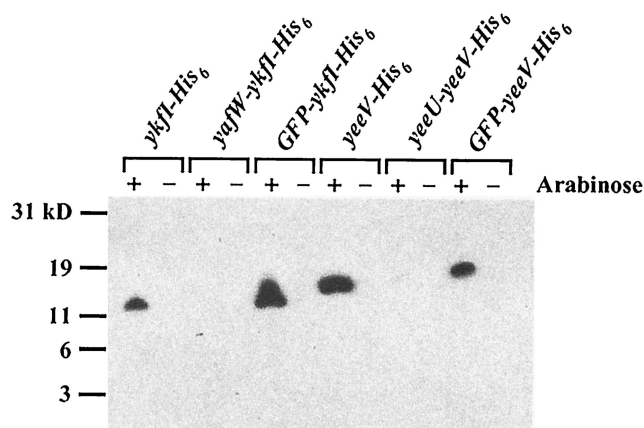

FIG. 5. Quantitation of His-tagged toxin protein. Cellular protein isolated from log-phase cultures ($OD_{600} = 0.5$) shaken at 37°C for 30 min in the presence or absence of 0.2% arabinose was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The mass of total protein loaded was normalized using the optical density of each culture. Detection was performed using an anti-His$_6$ antibody.

lished observation). This may explain why *yafW*, which does not have the extended downstream UTR, does not require it for antitoxin function. The fact that *yeeU* requires the presence of the downstream UTR to prevent growth inhibition caused by *yeeV*, but not *ykfI*, demonstrates that antitoxin function is not modular but relies on specific interactions with the paired toxin gene.

Identifying the mechanism by which *yeeV*, *ykfI*, and *ypjF* induce cell death is of primary importance. A general mode of action for antimicrobial agents can often be determined by assaying for the ability of the compound to inhibit a specific macromolecular synthesis pathway (27). However, in contrast to the immediate effect on cellular metabolism observed upon addition of inhibitor to a culture, the onset of toxicity caused by ectopic expression of YeeV or YkfI was relatively slow (Fig. 2 and 4A), presumably due to a lag in the accumulation of the toxin protein. These kinetics did not allow sufficient resolution to distinguish the primary macromolecular synthetic pathway inhibited by YeeV or YkfI. BLAST searches for related proteins also did not yield insight into the mechanism of action of

TABLE 3. Effect of coexpression of the adjacent gene, the distal gene, or *gfp* on the growth inhibition induced by *yeeV* and *ykfI*

Plasmid (gene[s] expressed)	Arabinose	Growth at 6 h[a]
pRD069 (yeeT)	+	+++
pRD030 (yeeU)	+	+++
pRD024 (yeeV)	+	−
pRD024 (yeeV)	−	+++
pRD104 (yeeT-yeeV)	+	−
pRD050 (yeeU-yeeV)	+	+++
pRD105 (gfp-yeeV)	+	−
pRD068 (ykfH)	+	+++
pRD029 (yafW)	+	+++
pRD023 (ykfI)	+	−
pRD023 (ykfI)	−	+++
pRD106 (ykfH-ykfI)	+	−
pRD049 (yafW-ykfI)	+	+++
pRD107 (gfp-ykfI)	+	−
pRD103 (ypjJ)	+	+++
pRD086 (yfjZ)	+	+++
pRD052 (ypjF)	+	+++

[a] Growth curves were performed as described for Fig. 2. Optical density after 6 h of growth is represented as follows: −, $OD_{600} < 0.5$; +++, $OD_{600} > 1.5$.

TABLE 4. The antitoxin activity of YeeU and YafW is not prevented by the presence of a C-terminal His tag on the YeeV or YkfI toxins

Plasmid (gene[s] expressed)	Arabinose	Growth at 6 h[a]
pRD042 (yeeV-His$_6$)	+	−
pRD042 (yeeV-His$_6$)	−	+++
pRD062 (yeeU-yeeV-His$_6$)	+	+++
pRD140 (gfp-yeeV-His$_6$)	+	+
pRD041 (ykfI-His$_6$)	+	+[b]
pRD041 (ykfI-His$_6$)	−	+++
pRD061 (yafW-ykfI-His$_6$)	+	+++
pRD124 (gfp-ykfI-His$_6$)	+	−

[a] Growth curves were performed as described for Fig. 2. Optical density after 6 h of growth is represented as follows: −, $OD_{600} < 0.5$; +, $0.5 < OD_{600} < 1.0$; +++, $OD_{600} > 1.5$.
[b] In this experiment, $OD_{600} = 0.55$.

TABLE 5. The unique 68-bp UTR between *yeeU* and *yeeV* is required for *yeeU* antitoxin function

Plasmid (gene[s] expressed)	Arabinose	Growth at 6 h[a]
pRD024 (*yeeV*)	+	−
pRD024 (*yeeV*)	−	+++
pRD095 (*yeeU-Bam*HI-*yeeV*)	+	−
pRD134 (*UTR-yeeV*)	+	+
pRD135 (*yeeU-UTR-Bam*HI-*yeeV*)	+	+++
pRD050 (*yeeU-yeeV*)	+	+++
pRD142 (*yafW Bam*HI-*yeeV*)	+	+++
pRD072 (*yeeV*)	+	−
pRD072 (*yeeV*) + pRD030 (*yeeU*)	+	−
pRD023 (*ykfI*)	+	−
pRD023 (*ykfI*)	−	+++
pRD139 (*yafW-Bam*HI-*ykfI*)	+	+++
pRD049 (*yafW-ykfI*)	+	+++
pRD141 (*yeeU-Bam*HI-*ykfI*)	+	++

[a] Growth curves were performed as described for Fig. 2. Optical density after 6 h of growth is represented as follows: −, $OD_{600} < 0.5$; +, $0.5 < OD_{600} < 1.0$; ++, $1.0 < OD_{600} < 1.5$; +++, $OD_{600} > 1.5$.

YeeV-, YkfI-, or YpjF-mediated growth inhibition. These proteins do contain a moderate degree of chemical conservation to SIS domains (sugar isomerase), which are found in a number of proteins known to bind various phosphosugar metabolites (3). The most similar SIS domain to YeeV, YkfI, and YpjF is found in the *E. coli* protein LpcA (9 of 13 amino acids thought to be conserved in SIS domains [3] are chemically related). Deletion of the *lpcA* gene results in the production of a shortened lipopolysaccharide core and supersensitivity to novobiocin (10). However, we did not observe increased novobiocin sensitivity upon induction of *yeeV* or *ykfI*, nor did we observe synergy with several other antimicrobial agents whose mechanism of action is known (unpublished data). Our observation that there is a positive correlation between the cellular concentration of toxin protein and the severity of growth inhibition suggests that the toxins may act by binding to and titrating out an essential protein or metabolite.

These novel TA pairs varied from previously characterized pairs in that we could not detect any physical interaction between the proteins. Instead, we found that expression of the antitoxins resulted in a decrease in the level of toxin protein in the cell. The mechanism by which the antitoxins affect this regulation requires further investigation but could involve preventing the translation of toxin mRNA or increasing the efficiency of degradation of either toxin mRNA or protein. The requirement of the downstream UTR for *yeeU* activity suggests binding of antitoxin protein to the mRNA transcript may be important.

The physiological role of these new TA pairs is currently unclear. If it were true that the toxicity associated with this family of proteins results from the titration of an essential metabolite or enzyme, this would be consistent with the model proposing a role for TA pairs in the regulation of specific metabolic pathways (18). However, a role in programmed cell death cannot be ruled out. For instance, if a pool of untranslated toxin mRNA were present in the cytoplasm, cell death could be triggered by inactivation of the antitoxin. A detailed examination of the in vivo regulation of the endogenous genes and proteins will be required to distinguish between these and other possibilities.

Of the six toxin genes described in this report, only *pspC* has been previously studied (9, 30). This gene is part of an operon that is induced by phage infection, as well as a variety of other stresses (5, 8, 30). Coexpression of *pspB* with *pspC* restored normal growth rates (unpublished data). Although these proteins have not previously been described as a TA pair, they do possess additional characteristics of TA pairs, including physical interaction (1) and autoregulation of transcription (37, 38). PspC positively regulates the transcription of the *psp* operon, for which the only currently known function involves maintaining the proton motive force of the inner membrane under stress conditions (23). Overexpression of PspA, which is thought to be the effector protein in this process, does not inhibit growth (unpublished data). Interestingly, the *Yersinia enterocolitica* homolog of *pspC* was shown to be required for virulence and is likely to regulate additional genes outside the *psp* locus (13, 14).

Another particularly intriguing finding was the toxicity associated with the overexpression of *yafO*. This gene is located just downstream of *yafN*, which has been described as an "orphan" antitoxin gene (19). It has some sequence similarity to *relB* but lacks a transcriptionally linked *relE* homolog. Our results show that *yafN* is indeed directly upstream of a toxin-encoding gene, but one that is unexpectedly divergent from *relE*. YafN antitoxin activity against YafO has not yet been demonstrated.

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