Published in final edited form as:

J Mol Biol. 2007 September 28; 372(4): 894–905. doi:10.1016/j.jmb.2007.07.016.

prlF* and *yhaV* encode a new toxin-antitoxin system in *Escherichia coli

Oliver Schmidt^{1,3}, Verena J. Schuenemann¹, Nicholas J. Hand^{2,4}, Thomas J. Silhavy², Jörg Martin¹, Andrei N. Lupas^{1,*}, and Sergej Djuranovic^{1,5}

¹ Department of Protein Evolution, Max-Planck-Institute for Developmental Biology, 72076 Tübingen, Germany

² Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

Summary

Toxin-antitoxin systems consist of a stable toxin, frequently with endonuclease activity, and a small, labile antitoxin, which sequesters the toxin into an inactive complex. Under unfavorable conditions, the antitoxin is degraded, leading to activation of the toxin and resulting in growth arrest, possibly also in bacterial programmed cell death. Correspondingly, these systems are generally viewed as agents of the stress response in prokaryotes. Here we show that *prlF* and *yhaV* encode a novel toxin-antitoxin system in *Escherichia coli*. YhaV, a ribonuclease of the RelE superfamily, causes reversible bacteriostasis that is counteracted by PrlF, a swapped-hairpin transcription factor homologous to MazE. The two proteins form a tight, hexameric complex, which binds with high specificity to a conserved sequence in the promoter region of the *prlF-yhaV* operon. As homologs of MazE and RelE, respectively, PrlF and YhaV provide an evolutionary connection between the two best-characterized toxin-antitoxin systems in *E. coli*, *mazEF* and *relEB*.

Keywords

mRNA decay; RelE superfamily ribonuclease; stress response; swapped-hairpin barrel; toxin-antitoxin system

Introduction

Toxin-antitoxin (TA) systems consist of pairs of genes located in one operon, which encode two components: a stable toxin and an unstable antitoxin (for recent reviews see refs 1;2;3. The antitoxin sequesters the toxin in a catalytically inactive complex, which acts as a transcriptional repressor of the operon. TA systems were initially discovered as plasmid addiction systems on low-copy number plasmids, where they mediate a phenomenon called post-segregational killing. In this context, cells that lose the plasmid die, because more rapid

*corresponding author: Department of Protein Evolution, Max-Planck-Institute for Developmental Biology, Spemannstr. 35, 72076 Tübingen, Germany, Tel. 49 7071 601 341 / Fax -349, E-mail: andrei.lupas@tuebingen.mpg.de.

³present address: Institute for Biochemistry and Molecular Biology, University of Freiburg, 79104 Freiburg, Germany

⁴present address: Division of Gastroenterology and Nutrition, Joseph Stokes Jr. Research Institute, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

⁵present address: Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

proteolysis of the labile antitoxin unmasks the toxin activity. In recent years, TA systems encoded in the chromosomes of bacteria and archaea were discovered in increasing numbers. These systems would be activated by the up-regulation of cellular proteases in response to environmental challenges. However, in contrast to the systems encoded on extrachromosomal elements, the biological role of the chromosomal systems remains unclear. Furthermore, the general view that chromosomal TA systems function as prokaryotic stress-response elements, causing growth arrest under unfavorable conditions and mediating programmed cell death, has recently been challenged by a study that showed that an *E. coli* strain with mutations in five known TA systems has no clear mutant phenotype 4.

We recently proposed the existence of a new TA system in *Escherichia coli*, consisting of the genes *prlF* and *yhaV*, based on a bioinformatic analysis 5. We identified *prlF* (protein localization locus F) originally in a screen for mutations that affect Sec-dependent protein secretion in *E. coli* 6. The *prlF1* allele, resulting from a duplication of 7 nucleotides close to the 3' end of *prlF*, suppressed the lethality associated with a toxic LamB-LacZ hybrid protein that jams the SecYEG translocator. PrlF1 is unable to repress the transcription of its operon 7, resulting in elevated PrlF levels, which in turn cause hyperactivation of the Lon protease by an as yet unknown mechanism 8, an effect that can be reproduced by PrlF overexpression. Two additional alleles of *prlF* were isolated independently as suppressors of the *htrA* (*degP*) null phenotype 9, *sohA*, both of which were later found to be identical to *prlF1* 10. *htrA* encodes a periplasmic protease required for growth at elevated temperatures and compensation for its absence appears to be fundamentally connected with the activation of Lon 11.

More recently, we identified PrlF as a distant homolog of double-psi barrels 12 and subsequently classified it into a family of prokaryotic transcription factors exemplified by the main transition state regulator of *Bacillus subtilis*, AbrB 5. These transcription factors, whose fold we denoted the swapped-hairpin barrel, share a common ancestor with double-psi barrels, but differ in several aspects of their topology 5. Most notably, they achieve their fold as homodimers of two tightly interleaved monomers, which jointly form a single DNA-binding domain, as seen for example in the structures of AbrB (PDB: 1yfb) and of the antitoxin MazE (PDB: 1ub4). During our analysis of PrlF, we observed that the gene encoding it was followed in most genomes by a gene encoding a homologue of *E. coli* YhaV. YhaV shows distant but significant similarity to ribonucleases of the RelE superfamily 5, which appear as toxins in several bacterial toxin-antitoxin (TA) systems 13. These observations and the genetic organization of the *prlF-yhaV* locus, which is typical for TA systems, prompted us to propose that PrlF and YhaV might form such a system as well 5. Here we report experimental evidence that *prlF-yhaV* is indeed a toxin-antitoxin operon, and thereby assign a new function to the enigmatic PrlF protein.

Results

Characterization of the *prlF-yhaV* operon

E. coli PrlF is a protein of 111 amino acids (12.3 kD), predicted to contain two domains: an N-terminal swapped-hairpin barrel (Fig. 1b) 5 and a C-terminal domain enriched in acidic amino acids, which gives the protein an acidic pI of 4.8. YhaV is a single-domain protein of 154 residues (17.8 kD), with a complementary, basic pI of 9.3. It bears distant similarity to nucleases of the RelE superfamily 5, particularly to strands β 3- β 5, which harbor catalytic residues of RelE (Fig. 1c). This includes two basic residues, Arg85 and Arg94 in YhaV, which are highly conserved in the superfamily 13 and which were shown to be critical for the toxic activity of RelE 14. It should be noted however that RelE requires binding to translating ribosomes to catalyze mRNA cleavage 15, while most other toxins like YoeB, another member of the RelE superfamily 16, or the unrelated MazF are *bona fide* RNases. YhaV can be

distinguished from other RelE homologs by an insertion of ~20 residues between helices 1 and 2.

Sequences closely matching the *E. coli* consensus -35 and -10 promoter elements and ribosome binding sequence are located immediately upstream of *prlF* 7, while no such sequences precede *yhaV*. Furthermore, the stop codon of *prlF* overlaps the ATG of *yhaV* by a single base pair (Fig. 1a, d). Taken together, this suggests transcriptional and translational coupling of the two open reading frames. To investigate whether both genes are expressed, we placed the operon under a lactose-inducible T7 promoter and attached His₆-tags to the N-terminus of PrlF and to the C-terminus of YhaV (plasmid PYnoStop/pET28b). Since the intergenic region remained unchanged, we judged that the tagging should not interfere with translational coupling. Indeed, two gene products of the expected size (14.5 kD for His₆-PrlF and 19.3 kD for YhaV-His₆) were expressed from this plasmid, with an excess of PrlF (Fig. 2).

We constructed a similar plasmid carrying the *prlF1* allele (F1YnoStop/pET28b), which has a duplication of 7 nucleotides at position 271 of *prlF* 7 (Fig. 1d). This mutation causes a frame-shift that alters the C-terminal 21 residues of the protein and introduces a premature stop codon in *prlF*. The resulting mutant protein, PrlF1, has 105 residues (12.0 kD) and lacks the negative charges at its C-terminus (pI = 9.2). After expression from this plasmid, only one protein corresponding to His₆-PrlF1 (14.2 kD) was seen in the western blot (Fig. 2); no YhaV-His₆ could be detected. The polar effect of the *prlF1* mutation on *yhaV* expression shows that the two genes are indeed translationally coupled. The intergenic interval of 24 nucleotides, which arises between the *prlF1* stop codon and the *yhaV* ATG is too large to be bridged by the ribosome in the absence of a ribosomal binding site upstream of *yhaV*.

PrIF and YhaV form a hetero-hexameric protein complex

In TA systems containing a swapped-hairpin barrel antitoxin, the toxin and antitoxin form a hetero-hexameric complex, in which two toxin dimers with basic pI are bound to the acidic C-terminal tails of a core antitoxin homodimer^{2;17}. To test whether the PrlF and YhaV proteins form a complex consistent with a TA pair we performed gel filtration analysis. We expressed PrlF and YhaV from two co-transformed plasmids (PrlF/pET31b, YhaV/pET28b) in *E. coli* C41 (DE3) and purified them in two steps: by Ni-NTA affinity chromatography, using a His₆-tag attached to the N-terminus of YhaV, and subsequently by gel filtration. The proteins eluted as a single peak from both columns, indicating that they form a complex (Fig. 3); in addition, a second, minor peak appeared in gel filtration at approximately twice the molecular weight of the complex, which may reflect a tendency of the complex to dimerize (Fig. 3c). We confirmed the identity of the proteins by peptide mass fingerprinting and their folded nature by CD spectroscopy (data not shown). We determined the molecular weight of the complex by analytical gel filtration to 102.8 ± 9.9 kD, close to the calculated mass of a PrlF₂-His₆-YhaV₄ hexamer (104.36 kD). Mass spectrometric analysis and the intensity of the bands in SDS-PAGE support the assumption of a molar excess of YhaV. The interaction between the proteins appears to be highly stable, as the complex was only partly disrupted in 8 M urea and required 6 M guanidinium chloride for separation.

We also expressed PrlF variants individually. Purified His₆-PrlF, the His₆-PrlF1 mutant, and a construct comprising only the N-terminal β-barrel domain of PrlF, His₆-PrlF-N, differ in their oligomerization state. As judged from gel filtration on a preparative Superdex HiLoad S75 column, the constructs lacking the acidic tail, His₆-PrlF1 and His₆-PrlF-N, form dimers, while His₆-tagged PrlF forms a higher oligomer in the absence of YhaV, most likely a tetramer (Fig. 3a). Untagged PrlF recovered by refolding from the denatured complex is also tetrameric (data not shown), so that an artefactual oligomerization involving the His₆-tag can be excluded.

Refolded His₆-YhaV is a dimer (Fig. 3c), as is the natively purified non-toxic mutant His₆-YhaV R85A R94A (data not shown; mutant described below).

YhaV toxicity is counteracted by PrIF

To test our hypothesis that PrIF and YhaV form a toxin-antitoxin system *in vivo*, we expressed them individually and jointly in the *E. coli* strains NJH134 (MC4100 *prlF::kan* (*StuI*)) and W3110 (wt) under control of the tight arabinose-inducible BAD promoter (Fig. 4a). For coexpression of both proteins in comparable amounts, we constructed pBADtwin vectors containing both ORFs under separate BAD promoters.

Expression of *yhaV* alone led to a pronounced growth arrest within 30 minutes after induction. As for other chromosomally encoded TA toxins 18, the effect was bacteriostatic, not bacteriocidal: we took samples of *E. coli* W3110 expressing YhaV/pBAD or PrIF/YhaV/pBADtwin at different times after induction (10, 120 and 240 min) and compared their colony-forming ability after removal of arabinose. Regardless of the severity of the growth arrest in the *yhaV*-expressing cells, no reduction in colony forming units (cfu) was apparent within 4 hrs after induction (Fig. 4b). However, massive overexpression of *yhaV* from a strong, lactose-inducible T7 promoter (YhaV/pET28b) in the *E. coli* strain C41 (DE3) was toxic and the survival rate of the cells dropped by about three orders of magnitude after 2 hrs of induction (Fig. 4c).

Expression of *prlF* alone did not alter the growth behavior, but, when coexpressed with *yhaV* from pBADtwin, it fully suppressed the growth arrest caused by the toxin (Fig. 4a). Equal results were obtained with the wild-type strain W3110 and with strain NJH134, whose chromosomal *prlF-yhaV* operon is disrupted, excluding an influence of the endogenous genes. Expression of *prlF1* also alleviates the toxicity of YhaV, albeit less efficiently than *prlF*, which may be due to the loss of acidic residues in its tail.

PrIF is highly susceptible to proteolysis

A characteristic feature of TA systems is the inherent instability of the antitoxin relative to the cognate toxin. To test the stability of the PrIF and YhaV subunits in the hetero-hexameric complex we performed proteinase K sensitivity assays. The two proteins showed considerably different susceptibility to proteolysis in the complex, when treated with 10 µg/ml proteinase K (Fig. 5a). His₆-YhaV was rapidly reduced to an 18 kD fragment and then resisted further proteolysis over a period of 60 min. The fragment corresponds to a stable core domain of YhaV which lacks the N-terminal His₆-tag (Fig. 5b). In contrast, PrIF was degraded within 5–10 min to a minimal level that was not degraded further; major proteolytically resistant fragments did not appear. When purified away from the complex prior to proteinase K exposure, His₆-YhaV was also protease resistant; however, the degradation pattern was altered to yield two stable fragments, the larger of which still retained the N-terminal His₆-tag (Fig. 5b). This suggests that YhaV either assumes a different conformation in complex with PrIF, or that proteolytic sites are masked in the complex by fragments of PrIF, which remain associated with YhaV.

YhaV has ribonuclease activity in vitro

Since the RelE superfamily contains several endoribonucleases 13;16;19, we investigated whether YhaV has the same catalytic activity. As we had previously observed that overexpression of YhaV is toxic in the absence of compensatory PrIF overexpression, we isolated YhaV from the co-purified PrIF-His₆-YhaV complex by denaturing Ni-NTA chromatography and subsequent refolding. The purified YhaV protein thus obtained rapidly degraded a cellular RNA preparation from *E. coli* W3110 without obvious specificity, including the 23S and 16S ribosomal RNAs (Fig. 6a), as well as synthetic transcripts of *prlF* and *yhaV* (data not shown). This broad activity may underlie the bacteriocidal effect of YhaV

at high expression levels. Proteinase K treatment did not reduce RNA degradation (Fig. 6d), suggesting that the core RNase domain is highly protease-resistant. Unlike RelE, YhaV does not require ribosome binding for its activity.

To examine the effect of the antitoxin, we tested whether PrIF could block the RNase activity of YhaV. Prior incubation of refolded YhaV with a three-fold molar excess of natively purified His₆-PrIF did not inhibit YhaV endonuclease activity (Fig. 6b). In contrast, when YhaV and PrIF were permitted to fold together, the complex was reformed and was again as inactive (Fig. 6c) as the natively purified complex (Fig. 6d). Complex pretreated with proteinase K remained inactive (Fig. 6d). These observations are compatible with the notion that PrIF only associates with YhaV during folding and thus differ from most TA systems, where co-incubation of the components *in vitro* inhibits the toxin 1;14. By analogy to other TA systems, where the C-terminal tail of the antitoxin mediates the interaction with the toxin, we also tried to inhibit YhaV by coinubation and by refolding with an excess of a synthetic peptide comprising the C-terminal 31 residues of PrIF, but did not observe any reduction of RNase activity in either case (data not shown).

mRNA-interfering toxins of the RelE superfamily contain two highly conserved arginines, which are not found in family members that exert gyrase-poisoning activity 13. Their mutation strongly attenuates the inhibitory effect of RelE on protein synthesis 14. We therefore mutated the two equivalent residues of YhaV, Arg 85 and Arg 94, to alanine. Both single mutants exhibited reduced toxicity and, unlike wild-type YhaV, lacked any growth defect in absence of induction; with induction, they both still caused growth arrest (Fig. 7a). In contrast, the double mutant YhaV R85A R94A was completely non-toxic in growth experiments and could be produced with high yields. The natively purified mutant protein showed significantly lower endonuclease activity than the wild-type protein (Fig. 7b), which was again not inhibited by coinubation with PrIF (Fig. 7c).

The hetero-hexameric PrIF-YhaV complex binds to its own promoter

Our previous work has shown that the *prlF* operon is negatively autoregulated. In a β -galactosidase-based reporter system the *prlF* promoter was upregulated 4 – 5 times in the *prlF1* and *prlF*[−] strains compared to wild-type 7 and we have observed the same effect in two different *yhaV::kan(XcmI)* strains (data not shown).

To investigate the biochemical basis of this autoregulation, we performed electrophoretic mobility shift assays (EMSA) using purified PrIF-YhaV complex with a 250 bp PCR product of the region upstream of the operon, ranging from position -196 to +54 from the *prlF* start codon. The PrIF-His₆-YhaV complex caused a clear and specific band-shift (Fig. 8a) at nearly equimolar DNA:complex ratios. In contrast, His₆-PrIF, His₆-PrIF1 and His₆-PrIF-N caused the DNA to smear and the effect was only observable at considerable excess of protein to DNA (Fig. 8b–d). Binding of all proteins was specific, as none of the proteins bound to control DNA in a competitive EMSA (Fig. 9a). We used a two-fold molar excess of the upstream region of *spoVT* from *Bacillus subtilis* as a control, because SpoVT has a swapped-hairpin barrel domain related to PrIF 5 and is autoregulated as well 20. Specific DNA binding by PrIF-N confirms that the primary binding site of the complex to its recognition sequence lies in the swapped-hairpin barrel domain of PrIF.

The upstream regions of *prlF-yhaV* operons in different species contain a conserved motif of 14 nt with the bipartite palindromic consensus sequence AATGCAtTGCATT (Fig. 8e). *E. coli* contains two copies, one at −71 nt from the start codon of *prlF* and a second, less conserved one at +199 (AATGCAtttTGCgTT). In order to establish whether this is the binding motif for the PrIF-YhaV complex, we divided the initial PCR product into overlapping pieces of 50 bp and tested them in EMSA (Fig. 9b). The DNA fragment with the −71 copy of the motif at its

center was the only one shifted (oligo -96 to -47). Another fragment with the motif at the 5' end (oligo -71 to -22) was not, indicating a need for flanking sequence, but this sequence may not need to be specific, as transfer of the motif into an unrelated DNA created a functional binding site (Fig. 9c). We confirmed the motif as the primary binding site through simultaneous mutation of symmetry-related positions to their complementary bases, which dramatically reduced binding (data not shown). We also analyzed binding to the less conserved motif at +199 and observed a comparable binding affinity and stoichiometry (data not shown).

The bipartite nature of the motif suggests that two PrIF-YhaV hexamers form a ternary complex on the DNA. This is in agreement with the EMSA experiments, which indicate saturation at a 2–3-fold excess of complex. However, we never observed the stepwise shift of DNA bands that might be expected for consecutive binding of two complexes. To investigate this point, we created DNA sequences with half-palindromes, where either the first or the second part of the binding site was mutated (Fig. 9c). These experiments revealed a second, lower band that presumably corresponds to a binary complex of DNA with a single hexamer. This band also appeared when only the first half of the motif (the palindrome AATGCATT) was transferred into the unrelated DNA sequence. Deletion of either half of the motif, however, only reduced and never abolished formation of the ternary complex, indicating that binding of the second complex is driven by cooperativity, and depends more on the presence of an already bound hexamer than on the integrity of the DNA-binding site. This finding is in agreement with the observation that the PrIF-YhaV complex exhibits a tendency to further dimerize in solution (Fig. 3c) and that the DNA-binding motif at +199, which contains a degenerate second half (Fig. 8e), nevertheless binds the complex with comparable affinity to the conserved motif at -71.

Discussion

We have discovered a new toxin-antitoxin system on the *E. coli* chromosome, composed of the proteins PrIF and YhaV. PrIF is a swapped-hairpin barrel and belongs to the AbrB superfamily of bacterial transcription factors, which includes the antitoxins of the TA systems *mazEF*, *pemIK* and *kis/kid* 5. YhaV is a distant member of the RelE superfamily 5, which comprises the toxins of the TA systems *relEB*, *yoeB-yefM* and *parDE* 13. *prIF-yhaV* is thus an intermediate between two of the most extensively studied TA system families, *relEB* and *mazEF*, and strengthens the proposed evolutionary origin of new TA systems by gene shuffling between a few major, analogous classes of toxins and antitoxins 13. Homologs of *prIF-yhaV* are found in various, phylogenetically disparate species of proteobacteria and cyanobacteria. The antitoxins are sometimes misannotated as “AbrB”, but are clearly recognized as PrIF orthologs by cluster analysis 5. The presence of *prIF-yhaV* homologues on the plasmid pSD25 from *Ruegeria* sp. PR1b and plasmid 3 from *Nitrobacter hamburgensis*, as well as in transposons and genetic islands, indicates that the system is also used as a plasmid addiction system.

The *prIF-yhaV* system has the characteristic features of chromosomal TA systems 21. The two gene products form a tight complex (presumably a YhaV₂-PrIF₂-YhaV₂ hexamer like the MazEF complex) and show differential susceptibility to proteolytic degradation *in vitro*, with the antitoxin being much more labile than the toxin, whose nuclease domain forms a protease-resistant core [sentence is too long]. The genes form an overlapping transcriptional unit with the antitoxin at the 5' end, and their translation is coupled such that an excess of antitoxin is produced. The toxin has RNase activity and causes cell growth arrest, presumably by degrading cellular mRNAs, unless it is sequestered into a catalytically inactive complex by the antitoxin. Two copies of the complex bind cooperatively to a conserved palindromic motif in the operator region of the operon, repressing its transcription.

Our data explain the negative transcriptional regulation of the operon by *prlF* and its upregulation in the *prlF*⁻, *yhaV*⁻, and *prlF1* strains by the repressor function of the PrlF-YhaV complex. The mechanism(s) by which the *prlF1* mutation suppresses the jamming of the SecYEG translocator by LamB-LacZ and the temperature sensitivity associated with loss of the periplasmic DegP/HtrA protease remain elusive, and the experiments presented here do not directly address them. Based on the data presented here, it is clear that these effects are neither a consequence of toxin activation, as YhaV is not produced in the *prlF1* mutant, nor of the loss of YhaV activity, since null mutations in *yhaV* do not confer the *prlF1* phenotype 10.

We have previously shown that the *prlF1* mutation leads to post-translational hyperactivation of the Lon protease by an unknown mechanism, and that *lon* null mutations are epistatic to *prlF1* 8. We are left to conclude that the effect on Lon is caused by the antitoxin alone and that whatever activity leads to it is elicited more efficiently by *prlF1* than by *prlF*, since the effects of the *prlF1* mutation can be reproduced by *prlF* overexpression. It should be noted however, that the mechanism by which hyperactivated Lon causes the *prlF1* phenotypes is unclear: turnover of the toxic LamB-LacZ protein is unaffected by *prlF1* and to date we have been unable to identify a proteolytic substrate that would account for the phenotypes.

In conclusion, we have identified a new chromosomal TA system in *E. coli*, which presents parallels to already characterized systems, as well as some novel features, such as the fact that it combines a RelE-like toxin with a swapped-hairpin antitoxin, that the toxin and antitoxin only appear to associate during folding of the latter, and that the antitoxin has an additional function not connected to its antitoxin activity.

Material and Methods

Cloning and expression

prlF, *yhaV* and the *prlF-yhaV* operon were amplified by PCR from genomic DNA of *E. coli* W3110 and cloned into pET28b and pET30b expression vectors (Novagen) using *NdeI* and *HindIII* restriction sites. Point mutations and fusions of DNA fragments were introduced by the megaprimer method as described previously²². Inserts for the pET31b vector (Novagen) were subcloned as *NdeI*/*XhoI* fragments from the respective pET28b constructs. For subsequent purification, proteins were expressed in *E. coli* C41 (DE3) from the respective vectors. Cells were grown in Luria-Bertani broth with appropriate antibiotics at 37°C, and expression was induced in mid-log phase by 1 mM isopropyl thiogalactoside, after which cells continued to grow for 4 hrs at 33°C. To obtain a PrlF-YhaV complex *in vivo*, cells harbouring both PrlF/pET31b and YhaV/pET28b plasmids were used for coexpression.

Growth phenotypes were monitored in pBAD vectors that were constructed by introducing appropriate DNA fragments of *prlF*, *yhaV* or their mutants into the *NcoI* and *HindIII* sites of pBAD/myc-His A (Invitrogen). The pBADtwin vector was constructed by redesigning the pBAD/myc-His A vector for simultaneous expression of two proteins. To this aim, the *prlF* orf and BAD terminator were amplified from PrlF/pBAD, and the BAD promoter and *yhaV* orf from YhaV/pBAD. The *HindIII* site following *prlF* and the *NcoI* site preceding *yhaV* were thereby changed to *XhoI* and *EcoRI* sites, respectively. The resulting PCR fragments were fused, and the full-length fragment was cloned into pBAD/myc-His A using *NcoI* and *HindIII* restriction sites. The resulting plasmid contained the *prlF* and *yhaV* orfs, each preceded by its own BAD promoter. Note that in all pBAD/myc-His-A-based constructs the antitoxin genes contain an additional glycine codon (GGG) after the start codon that was introduced to create the *NcoI* site. A list of all strains and plasmids used in this work is given in table 1.

Protein purification and analysis

All proteins were purified in a two-step purification using Ni-NTA metal affinity chromatography with a linear imidazole gradient from 50 mM to 1M in 30 mM Tris/HCl, 300 mM NaCl, pH 7.9, followed by preparative gel filtration on Superdex HiLoad S75 or S200 columns (GE Healthcare) in 40 mM MOPS/NaOH, 300 mM NaCl, pH 7.2. Foldedness and secondary structure content of the proteins were confirmed by circular dichroism spectra (200–240 nm) on a Jasco J-810 spectropolarimeter at 25°C. To obtain His₆-YhaV alone, natively purified PrIF-His₆-YhaV complex was dissociated by denaturation in 6 M guanidinium chloride, pH 8. The denatured complex was separated by Ni-NTA metal affinity chromatography using a linear gradient from 0–1 M imidazole in 30 mM Tris/HCl, 300 mM NaCl and 6 M guanidinium chloride, pH 8. Purified denatured His₆-YhaV was then refolded by repeated dialysis against 40 mM MOPS/NaOH, 300 mM NaCl, 5% glycerol, pH 7.2. To examine association of PrIF with YhaV during folding, denatured His₆-YhaV and PrIF were mixed in ratios of 1:1 or 1:3 and refolded as described above.

For analytical size exclusion chromatography, a superose 6 column (GE Healthcare) equilibrated with 40 mM MOPS/NaOH, 300 mM NaCl, pH 7.2 was size-calibrated with suitable proteins (1mg/ml each), covering a broad molecular weight range (see figure legends for details). 1–2 mg of purified proteins were loaded onto the column, and the molecular weight was calculated from the elution volume averaged from at least two experiments.

To determine protein expression from the *prIF-yhaV* and the mutated *prIF1-yhaV* operon, PYnoStop/pET28b, F1YnoStop/pET28b and PY/pET30b plasmids were introduced into the *E. coli* strain C41 (DE3). Expression from the lactose-inducible T7 promoter was started at mid-log phase by addition of 1 mM isopropyl thiogalactoside. 3 hrs after induction samples were taken and subjected to SDS-PAGE, followed by semi-dry electro blotting. To stain for His₆-tagged proteins, western blot analysis was done using the Ni-NTA detection kit (Qiagen).

For protease sensitivity assays, proteins (1 mg/ml) were mixed with Proteinase K (10 µg/ml) in 40 mM MOPS/NaOH, 300 mM NaCl, 10% glycerol, pH 7.2 and incubated for 15 min at 4°C. Reactions were stopped with 1 mM phenylmethyl sulfonylchloride. For time course analysis, aliquots were removed at indicated time points and immediately frozen in liquid nitrogen. Subsequently, samples were heated at 95°C for 5 min in SDS sample buffer and subjected to SDS PAGE, followed by Coomassie-Blue staining or western blot analysis, as indicated.

Growth curves and drop dilution test

For growth curves, *E. coli* NJH134 or W3110 cells were transformed with pBAD or pBADtwin plasmids. 5 ml cultures of LB broth with 100 µg/ml ampicillin were inoculated from single clones and grown at 37°C. At OD_{600nm} = 0.5 the BAD promoter was induced with 0.2% L(+)-arabinose, and OD_{600nm} was monitored for 5 hrs. Growth curves were averaged from four independent cultures. At different timepoints (10, 120, 240 min) samples were taken, adjusted to an OD_{600nm} of 0.6, and further diluted logarithmically into LB broth. To compare survival rates, 10 µl of the 1:10², 1:10³, 1:10⁴ and 1:10⁵ dilutions were placed on LB agar with appropriate antibiotics and grown at 37°C until colonies were well visible.

RNase activity assay

RNA preparation from *E. coli* W3110 was performed as described previously 23;24. 10 µg of purified RNA was added to protein adjusted to a final concentration of 0.33 µM (4 µM in Fig. 6d). Calculation of protein concentrations is based on dimeric YhaV in solution and the assumption that the PrIF-YhaV complex contains two dimers of YhaV. Reactions were carried out at 37°C in 13.3 mM MOPS/NaOH, 100 mM NaCl, pH 7.2 (reaction volume 15 µl). At time

points indicated in Fig. 6, the reactions were stopped by addition of 1 µl diethyl pyrocarbonate (DEPC) and 3 µl RNase-free DNA sample buffer (Fermentas) and subsequent shock freezing in liquid nitrogen. All samples were denatured for 5 min at 55°C before being submitted to agarose gel electrophoresis. RNA was visualized in the gel by ethidium bromide staining. For mixing experiments to examine the effect of PrIF, refolded YhaV was incubated at ambient temperature with a 3-fold molar excess of the inhibitor for 30 min prior to RNA addition.

Electrophoretic mobility shift assay (EMSA)

The *prfF* upstream region was amplified by PCR from genomic DNA of *E. coli* W3110. For use in competition experiments, a PCR product of the operator region of *B. subtilis spoVT* was generated. In addition several DNA fragments were used that were generated by annealing of complementary single-stranded oligonucleotides (Sigma-Genosys). The EMSA was performed as described previously^{23;24}. Protein and DNA were mixed in the ratios indicated in Figs. 8 and 9 in 13.3 mM MOPS/NaOH, 100 mM NaCl, 10% glycerol, pH 7.2 and were incubated for 30 min at ambient temperature before electrophoresis.

Sequences of double-stranded DNA oligonucleotides for EMSA:

-71 binding site	
oligo -196>-147	5 -ggactgcataaccagctggcgggtgtttaacccggcaccgggtgacctgatt
oligo -171>-122	5 -ttaacccggcaccgggtgacctgatttcttttggaacgtccctcgcaaaac
oligo -146> -97	5 -tcttttggaacgtccctcgcaaaacatggccttagtgccatgtttttatt
oligo -121> -72	5 -atggccttagtgccatgtttttattgtttaaagtccacacatccattaat
oligo -96> -47	5 -gtttaaagtccacacatccattaataatgcatattgcatcaccttagttca
oligo -71> -22	5 -aatgcatattgcatcaccttagttcaagcttataatttgagcagaaaaacag
oligo -46> +4	5 -agcttataatttgagcagaaaaacaggtgtaaaaggacagtaataatgc
oligo -21> +29	5 -gctgtaaaaggacagtaataatgcccgtaatgctcgctctaacgctgt
oligo +5> +54	5 -ccgctaagtctcgctctaacgctgtactgaccactgaatcaaaggctcact
+199 binding site	
5 -gaacaggaggatcatactatgaatgcatattttgctgtttctggatgcagatatcc	
constructs for Fig. 8c	
wildtype	5 -ccacacatccattaataatgcatattgcatcaccttagttcaagc
chimera	5 -ccaaccaaccaaccaaaatgcatattgcatccaaccaaccaacc
half chimera	5 -ccaaccaaccaaccaaccaatgcatccaaccaaccaaccaacc
wt 1st half	5 -ccacacatccattaataatgcatattggccggaccttagttcaagc
wt 2nd half	5 -ccacacatccattaatggcgggtttgcatcaccttagttcaagc

Acknowledgments

We thank Gerold Schwarz and Florian Altenberendt for mass spectrometry, Heinz Schwarz for electron microscopy, and Natacha Ruiz for critical reading of the manuscript. This work was supported by institutional funds from the Max Planck Society. NJN and TJS were supported by grant GM34821 to TJS from the National Institute of General Medical Sciences.

References

1. Gerdes K, Christensen SK, Lobner-Olesen A. Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol* 2005;3:371–82. [PubMed: 15864262]

2. Buts L, Lah J, Dao-Thi MH, Wyns L, Loris R. Toxin-antitoxin modules as bacterial metabolic stress managers. *Trends Biochem Sci* 2005;30:672–9. [PubMed: 16257530]
3. Condon C. Shutdown decay of mRNA. *Mol Microbiol* 2006;61:573–83. [PubMed: 16803593]
4. Tsilibaris V, Maenhaut-Michel G, Mine N, Van Melderen L. What is the benefit for *E. coli* to have multiple toxin-antitoxin systems in their genomes? *J Bacteriol*. 2007
5. Coles M, Djuranovic S, Soding J, Frickey T, Koretke K, Truffault V, Martin J, Lupas AN. AbrB-like transcription factors assume a swapped hairpin fold that is evolutionarily related to double-psi beta barrels. *Structure* 2005;13:919–28. [PubMed: 15939023]
6. Kiino DR, Silhavy TJ. Mutation prlF1 relieves the lethality associated with export of beta-galactosidase hybrid proteins in *Escherichia coli*. *J Bacteriol* 1984;158:878–83. [PubMed: 6233268]
7. Kiino DR, Phillips GJ, Silhavy TJ. Increased expression of the bifunctional protein PrlF suppresses overproduction lethality associated with exported beta-galactosidase hybrid proteins in *Escherichia coli*. *J Bacteriol* 1990;172:185–92. [PubMed: 2152898]
8. Snyder WB, Silhavy TJ. Enhanced export of beta-galactosidase fusion proteins in prlF mutants is Lon dependent. *J Bacteriol* 1992;174:5661–8. [PubMed: 1512198]
9. Baird L, Georgopoulos C. Identification, cloning, and characterization of the *Escherichia coli* sohA gene, a suppressor of the htrA (degP) null phenotype. *J Bacteriol* 1990;172:1587–94. [PubMed: 2407727]
10. Hand, NJ. Suppression of Lamb-LacZ hybrid jamming in *Escherichia coli*. Princeton University; New Jersey: 2002. PhD Dissertation
11. Hand NJ, Silhavy TJ. Null mutations in a Nudix gene, ygdP, implicate an alarmone response in a novel suppression of hybrid jamming. *J Bacteriol* 2003;185:6530–9. [PubMed: 14594825]
12. Coles M, Diercks T, Liermann J, Groger A, Rockel B, Baumeister W, Koretke KK, Lupas A, Peters J, Kessler H. The solution structure of VAT-N reveals a ‘missing link’ in the evolution of complex enzymes from a simple betaalphabetabeta element. *Curr Biol* 1999;9:1158–68. [PubMed: 10531028]
13. Anantharaman V, Aravind L. New connections in the prokaryotic toxin-antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay system. *Genome Biol* 2003;4:R81. [PubMed: 14659018]
14. Takagi H, Kakuta Y, Okada T, Yao M, Tanaka I, Kimura M. Crystal structure of archaeal toxin-antitoxin RelE-RelB complex with implications for toxin activity and antitoxin effects. *Nat Struct Mol Biol* 2005;12:327–31. [PubMed: 15768033]
15. Christensen SK, Gerdes K. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. *Mol Microbiol* 2003;48:1389–400. [PubMed: 12787364]
16. Kamada K, Hanaoka F. Conformational change in the catalytic site of the ribonuclease YoeB toxin by YefM antitoxin. *Mol Cell* 2005;19:497–509. [PubMed: 16109374]
17. Kamada K, Hanaoka F, Burley SK. Crystal structure of the MazE/MazF complex: molecular bases of antidote-toxin recognition. *Mol Cell* 2003;11:875–84. [PubMed: 12718874]
18. Pedersen K, Christensen SK, Gerdes K. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Mol Microbiol* 2002;45:501–10. [PubMed: 12123459]
19. Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K, Ehrenberg M. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* 2003;112:131–40. [PubMed: 12526800]
20. Bagyan I, Hobot J, Cutting S. A compartmentalized regulator of developmental gene expression in *Bacillus subtilis*. *J Bacteriol* 1996;178:4500–7. [PubMed: 8755877]
21. Engelberg-Kulka H, Glaser G. Addiction modules and programmed cell death and antideath in bacterial cultures. *Annu Rev Microbiol* 1999;53:43–70. [PubMed: 10547685]
22. Sarkar G, Sommer SS. The “megaprimer” method of site-directed mutagenesis. *Biotechniques* 1990;8:404–7. [PubMed: 2340178]
23. Summers WC. A simple method for extraction of RNA from *E. coli* utilizing diethyl pyrocarbonate. *Anal Biochem* 1970;33:459–63. [PubMed: 4910776]
24. Ausubel, FM. Current protocols in molecular biology. Greene Pub. Associates; Wiley-Interscience; New York: 1987.

25. Bachmann, BJ. Derivations and genotypes of some mutant derivatives of *E. coli* K12. In: Neidhart, FC., editor. *Escherichia coli* and *Salmonella typhimurium*. Washington D. C: ASM; 1996. p. 2460-2488.
26. Miroux B, Walker JE. Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 1996;260:289–98. [PubMed: 8757792]
27. Rosenberg AH, Lade BN, Chui DS, Lin SW, Dunn JJ, Studier FW. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 1987;56:125–35. [PubMed: 3315856]
28. Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 1995;177:4121–30. [PubMed: 7608087]

Figure 1.

The *prlF-yhaV* operon in *E. coli*. (a) Schematic depiction of the gene structure; the stop codon of *prlF* (TAA) overlaps by one base with the start codon of *yhaV* (ATG) and both genes are controlled by a shared promoter. (b) Alignment of the N-terminal domain of PrlF with AbrB and three antitoxins of the MazEF family (PemK, MazE, ChpBI). All proteins belong to a superfamily of prokaryotic transcription factors with a swapped-hairpin barrel fold; the secondary structure of the fold (S = β -strand, H = α -helix) and the location of two characteristic sequence motifs 5 are shown above the alignment. For the proteins of known structure, the Protein Data Bank identifiers are given. The organisms are: Ec *Escherichia coli*, Bs *Bacillus subtilis*, Ph *Pyrococcus horikoshii*. (c) Alignment of YhaV with toxins of the RelE superfamily.

Conserved residues of the superfamily are shown bold and the catalytically important residues Arg85 and Arg94 are highlighted in red. Other annotations are as in panel (b). (d) Effect of the *prlF1* mutation on PrlF. The duplication of 7 nt at position 257 – 263 of *prlF* introduces a frame-shift that results in a slightly shorter gene product rich in basic (blue) instead of acidic (red) residues. Moreover, an intergenic gap of 24 nt is formed between the *prlF* and *yhaV* open reading frames.

Figure 2.

Comparison of protein expression from the *prlF-yhaV* operon with *prlF1-yhaV*, both placed under control of a lactose-inducible T7 promotor, indicating that translation of YhaV is abolished by the *prlF1* mutation. For detection in western blot, PrlF and PrlF1 carry a His₆-tag at the N-terminus and YhaV at the C-terminus. A strain expressing the untagged proteins from a similar plasmid (PY/pET30) is used as a control. The cross reaction with an *E. coli* protein of 33 kD serves as a loading control. MW: molecular markers (GE Healthcare LMW-SDS-Marker kit).

Figure 3.

Co-expressed PrIF and His₆-YhaV form a complex and co-elute on both preparative gel size exclusion (a) and Ni-NTA metal affinity chromatography (b). (c) Migration of natively purified PrIF-His₆-YhaV complex and of His₆-YhaV on analytical Superose 6 gel size exclusion columns. A second peak of approximately twice the molecular weight overlaps the main peak of the PrIF-His₆-YhaV hexamer. Lower panel: Coomassie Blue-stained SDS-gel of the peak fractions from the PrIF-His₆-YhaV run. Size calibration of the column: GroEL (800 kD), bovine serum albumin (BSA; 66 kD), carbonic anhydrase (CA; 29 kD), cytochrome c (Cyt c; 12.4 kD), aprotinin (Aprot.; 6.5 kD).

Figure 4.

(a) Growth phenotype of strain NJH134 expressing the plasmids *PrlF*/pBAD, *YhaV*/pBAD, *PrlF*/*YhaV*/pBADtwin, *PrlF1*/*YhaV*/pBADtwin and the pBAD vector as a control. The curves are averaged from four independent cultures and are normalized by setting cell density of each culture at induction time ($t = 0$ min) to 0 % and average density of the vector control at $t = 300$ min to 100%. Error bars show standard deviation. (b) Expression of *YhaV* from *YhaV*/pBAD in the wild type strain *E. coli* W3110 induces growth arrest, from which the cells recover when the inducing agent is removed. No loss of colony forming units is observed in a drop dilution test on LB agar in cells expressing the toxin *YhaV*, compared to cells coexpressing *PrlF* and *YhaV*. Each consecutive drop is a ten-fold dilution of the previous, starting from OD₆₀₀ =

6×10^{-3} . c) High-level expression of His₆-YhaV from the IPTG-induced YhaV/pET28b plasmid in the C41 (DE3) strain reduces the number of viable cells by three orders of magnitude within 2 hrs with respect to cells expressing simultaneously the PrIF/pET31b and YhaV/pET28b plasmids. The YhaV-expressing strain harbored an additional empty pET15b plasmid to provide ampicillin resistance.

Figure 5.

(a) Time course for proteolysis of purified PrIF-His₆-YhaV with proteinase K (PK) (10 µg/ml). A 18 kD protease-resistant fragment of YhaV is formed, while PrIF is almost completely degraded. (b) Differential proteolysis pattern of the PrIF-His₆-YhaV complex and of His₆-YhaV. Left panel: Coomassie Blue-stained SDS-gel; right panel: corresponding western blot probed for His₆-tagged proteins.

Figure 6. RNase activity of YhaV towards a preparation of cellular RNA of *E. coli* W3110 (10 µg/lane), showing (a) His₆-YhaV, (b) His₆-YhaV preincubated for 30 min with a three-fold molar excess of His₆-PrIF, (c) refolded PrIF-His₆-YhaV, (d) PrIF-His₆-YhaV and refolded His₆-YhaV pretreated with proteinase K. RNA alone (control) and RNA with just proteinase K (PK) are shown for comparison; the positions of 23S and 16S ribosomal RNA are indicated.

Figure 7.

(a) Growth curve of *E. coli* NJH134 expressing pBAD plasmids of YhaV and of the mutants YhaV R85A, YhaV R94A and YhaV R85A,R94A. The experimental setup and data processing are as in Fig. 4a. (b) RNase activity of the natively purified double-mutant His₆-YhaV R85A,R94A (0.33 μ M and 16.5 μ M). Control is RNA alone. (c) RNase activity of His₆-YhaV R85A,R94A preincubated with His₆-PrIF.

Figure 8.

Electrophoretic mobility shift assays of a 250 bp PCR product containing the operator region of *prlF* (1.33 pmol/lane), titrated with increasing amounts of (a) PrIF-His₆-YhaV, (b) His₆-PrIF, (c) His₆-PrIF1 and (d) His₆-PrIF-N. The molar ratios of DNA to hexameric complex (PrIF-YhaV), and dimer (PrIF, PrIF1, PrIF-N) are indicated for each lane. The dimer was chosen as the basic unit, since it forms one putative DNA-binding domain. (e) A consensus DNA sequence motif in the operator region of *prlF* in various organisms. The positions are counted from the first nucleotide of the annotated start codon of *prlF* (+1). *E. coli* harbors a second, less conserved motif at position +199, while in *Pseudomonas putida* a second motif overlaps the predicted start codon.

Figure 9.

Electrophoretic mobility shift assays of PrlF constructs.

(a) Protein binding to the operator region of *prlF* in competition with a two-fold molar excess of the operator region of *B. subtilis spoVT*. (b) PrlF-His₆-YhaV binding to overlapping fragments of the *prlF* operator region. (c) Comparison of PrlF-His₆-YhaV binding to the native operator (wt), an unrelated DNA carrying the 14 nt consensus motif (chimera), the same DNA carrying only the first eight palindromic nucleotides of the motif (half chimera), and two additional native operator sequences where either the first (1st half) or last (2nd half) eight nucleotides are retained while the rest of the motif is mutated. Two shifted bands are detected, labelled 'binary complex' and 'ternary complex', which presumably correspond to one and two PrlF-His₆-YhaV complexes bound to DNA, respectively. (d) Schematic model of the PrlF-YhaV complex binding to its recognition site.

Table 1
***Escherichia coli* strains and plasmids used in this work**

Strain/plasmid	Strain/plasmid properties	References
<i>E. coli</i> W3110	Wild-type strain	25
<i>E. coli</i> C41 (DE3)	Expression strain	26
<i>E. coli</i> NJH134	<i>E. coli</i> MC4100 <i>prfF::kan</i> (<i>StuI</i>)	This work
pET28b, pET30b, pET31b, pET15b	Lactose-inducible expression vectors (Novagen)	27
pBAD/myc-His A	Arabinose-inducible expression vector (Invitrogen)	28
pBADtwin	pBAD/myc-HisA derivative for insertion of two orfs under separate arabinose-inducible promoters	This work
PrIF/pET28b	<i>prfF</i> in pET28b	This work
PrIF1/pET28b	<i>prfF1</i> in pET28b	This work
PrIF-N/pET28b	<i>prfF-N</i> (<i>prfF</i> Δ175–333) in pET28b	This work
YhaV/pET28b	<i>yhaV</i> in pET28b	This work
YhaV R85A R94A/pET28b	<i>yhaV</i> CG253GC CG280GC in pET28b	This work
PrIF/pET31b	<i>prfF</i> in pET31b	This work
PY/pET30b	<i>prfF-yhaV</i> in pET30b	This work
PYnoStop/pET28b	<i>prfF-yhaV</i> ΔTAA798 in pET28b	This work
F1YnoStop/pET28b	<i>prfF1-yhaV</i> ΔTAA805 in pET28b	This work
PrIF/pBAD	<i>prfF</i> in pBAD/myc-His A	This work
YhaV/pBAD	<i>yhaV</i> in pBAD/myc-His A	This work
YhaV R85A/pBAD	<i>yhaV</i> CG253GC in pBAD/myc-His A	This work
YhaV R94A/pBAD	<i>yhaV</i> CG280GC in pBAD/myc-His A	This work
YhaV R85A R94A/pBAD	<i>yhaV</i> CG253GC CG280GC in pBAD/myc-His A	This work
PrIF/YhaV/pBADtwin	<i>prfF</i> and <i>yhaV</i> in pBADtwin	This work
PrIF1/YhaV/pBADtwin	<i>prfF1</i> and <i>yhaV</i> in pBADtwin	This work