

Toxin-Antitoxin Systems in Bacteria and Archaea

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Abstract

Almost all bacteria and many archaea contain genes whose expression inhibits cell growth and may lead to cell death when overproduced, reminiscent of apoptotic genes in higher systems. The cellular targets of these toxins are quite diverse and include DNA replication, mRNA stability, protein synthesis, cell-wall biosynthesis, and ATP synthesis. These toxins are co-expressed and neutralized with their cognate antitoxins from a TA (toxin-antitoxin) operon in normally growing cells. Antitoxins are more labile than toxins and are readily degraded under stress conditions, allowing the toxins to exert their toxic effect. Presence of at least 33 TA systems in *Escherichia coli* and more than 60 TA systems in *Mycobacterium tuberculosis* suggests that the TA systems are involved not only in normal bacterial physiology but also in pathogenicity of bacteria. The elucidation of their cellular function and regulation is thus crucial for our understanding of bacterial physiology under various stress conditions.

INTRODUCTION

Regulation of cell death is essential for living organisms. In eukaryotic cells, apoptotic cell death is essential for embryonic development, for maintenance of normal cell homeostasis, and for elimination of cells damaged by stress or pathogen infection (87). In bacteria, regulation of cell growth and cell death is also important under various stress conditions. Almost all bacteria contain a gene that encodes for a small toxin protein approximately 100 amino acid residues in length. This toxin may inhibit cell growth by targeting a key molecule in any one of several essential cellular processes, including DNA replication, mRNA stability, protein synthesis, cell-wall biosynthesis, and ATP synthesis (88, 97). These toxins are co-transcribed and co-translated with their cognate antitoxins from an operon called a toxin-antitoxin (TA) operon.

In normally growing cells, a toxin and its cognate antitoxin form a stable complex that prevents the toxin from exerting its toxicity. Free antitoxins that are not complexed with their cognate toxins are partially unstructured, making them highly unstable in the cells relative to free toxins. As a result, in the normally growing cells, in order to constantly inhibit the toxin function, cognate antitoxins have to be continuously synthesized (110).

The TA systems are not essential for normal cell growth, nevertheless they are widely prevalent in bacteria and archaea (88). Based on the prevalence of TA systems, it may be speculated that they play subtle roles that are advantageous for cell survival in their natural habitats. Toxins may promote cellular adaptation to ever-changing environments by slowing down cell growth, inhibiting cell growth, or causing some cells to die.

The first TA system identified was carried on a plasmid and was shown to play a role in plasmid maintenance (102). When a plasmid encoding the TA system is lost from a cell, the toxin is released from the existing TA complex, given that the antitoxin is more unstable than

toxin. This results in cell growth inhibition and eventual cell death (38). Since this discovery, a number of different TA systems have been identified that are encoded from bacterial genomes. The toxins from these TA systems are proposed to be associated with various cellular processes in regulating gene expression, bacterial population control, and programmed cell death (110).

To date, at least 33 TA systems have been identified in *Escherichia coli* K12, with 12 being well-characterized [MazF-MazE (116, 117), RelE-RelB (90, 100), ChpBK-ChpBI (118), YafQ-DinJ (81, 91), YoeB-YefM (50, 114), HipA-HipB (57, 61), HicA-HicB (49, 75), YhaV-PrfI (95), YafO-YafN (12, 115), MqsR-MqsA (11, 53, 111), HigB-HigA (18), and RnlA-RnlB (59)] (Y. Yamaguchi & M. Inouye, unpublished data). It is interesting to note that *Mycobacterium tuberculosis* contains more than 60 TA systems, whereas its nonpathogenic counterpart, *Mycobacterium smegmatis* has only two TA systems (88). The number of the TA systems may be related to the pathogenicity of a bacterium, and it has been speculated that the TA systems in *M. tuberculosis* may play important roles in the maintenance of the extremely long dormancy of this pathogen inside macrophage (109). On the basis of this assumption, it is quite surprising that *E. coli* also contains a large number of the TA systems. Given that these TA systems have likely been maintained through the evolution of *E. coli*, one can speculate that each of them might have played a positive role in survival of the bacterium under harsh natural environments over those cells that had few or no TA systems. Characterization of these TA systems, including the identification of their cellular targets, the mechanisms of their toxic actions, and the regulatory mechanisms of their expression, therefore, is essential for our understanding of the roles of the TA systems in bacterial physiology, pathogenicity, and evolution. In this review, we describe functions, structures, and possible roles in physiology of all TA systems so far identified in bacteria, archaea, and fungi.

PRESENCE OF TOXIN-ANTITOXIN SYSTEMS IN BACTERIA, ARCHAEA, AND FUNGI

Symbiotic bacteria, such as *Chlamydia muridarum* and *Mycoplasma gallisepticum*, live inside eukaryotic cells. In 2005, an early analysis of a limited number of available bacterial genomic sequences led to the conclusion that symbiotic bacteria do not contain any TA systems (88). However, as more genomic data became available, this conclusion was revisited. At present, at least 20 genomic sequences of symbiotic bacteria have been determined, and computational analysis of these genomes using the PostgreSQL program reveals that most of these symbiotic bacteria, indeed, contain TA systems (97). Many archaea also contain the TA systems. To date, 94 archaea genomes have been determined, and at least 86 of these contain the TA systems homologous to bacterial systems.

The same early analysis of available genomes (88) suggested that TA systems do not exist in eukaryotes. However, BLAST search of the currently available databases of fungal genomes identified fourteen DNA sequences that may encode Doc toxin homologs. These Doc homologs have 26%–33% identity and 42%–51% homology to *E. coli* Doc. At present, it is not known if these loci are expressed to produce Doc-like proteins, whether these proteins, if expressed, are toxic, and whether they have cognate antitoxins. These results indicate that the TA systems are widely prevailed in not only almost all bacteria and archaea but also in some fungi, raising an intriguing question on the general roles of the TA systems in their diverse natural habitats and the advantage these confer to the organisms during evolution.

CLASSIFICATION AND COMMON FEATURES OF THE TOXIN-ANTITOXIN SYSTEMS

The TA operon is classified into three types, Type I, II, and III, according to the nature of the antitoxin and the composition of the TA

systems (Figure 1). In the Type I TA system, the toxin gene expression is regulated by an antisense RNA transcribed from the same toxin region in the reverse orientation (33, 40). In the Type II TA system, both toxins and antitoxins consist of proteins, and the toxin function is neutralized by forming a complex with its cognate antitoxin (110). The Type III TA system, in which RNA antitoxin is proposed to inhibit toxin function by directly interacting with the toxin protein by forming a RNA-protein complex (31), has been recently identified. The RNA antitoxin is not an antisense RNA, and therefore it does not inhibit the translation of the toxin mRNA like the Type I TA system. The ToxN antitoxin RNA is the first such example of the Type III TA system (31). Based on studies on various toxins from these three types of TA systems, it seems that they may have a single cellular target to inhibit cell growth. The toxicity of Type II and III system toxins can be neutralized by their cognate antitoxins irrespective of them being proteins or RNA.

Type I Toxin-Antitoxin System

In the Type I TA system, the toxin gene expression is regulated by an antisense RNA transcribed from the toxin gene but in reverse orientation (32, 40). Examples of this TA system include RdlD-LdrA (55), SymR-SymE (54), and IstR-TisB (103, 106). The antisense RNA antitoxins thus transcribed anneal to the toxin mRNAs to form a double-stranded RNA molecule. This double-stranded RNA is thought to stimulate the degradation of the toxin mRNAs, resulting in the inhibition of toxin production (40).

All the toxins of the Type I TA system consist of a very small hydrophobic polypeptide of 19–38 amino acid residues (32). Overexpression of TisB, one of the Type I TA toxins, has been shown to disturb the cytoplasmic membrane, resulting in inhibition of ATP synthesis (103). All the toxins in the Type I TA system of *E. coli* so far characterized have an identical secondary structure consisting of one α -helical structure and are predicted to be localized in the inner

Doc: toxin in *Phd-Doc* TA system inhibiting translation elongation through its binding to 30S ribosome

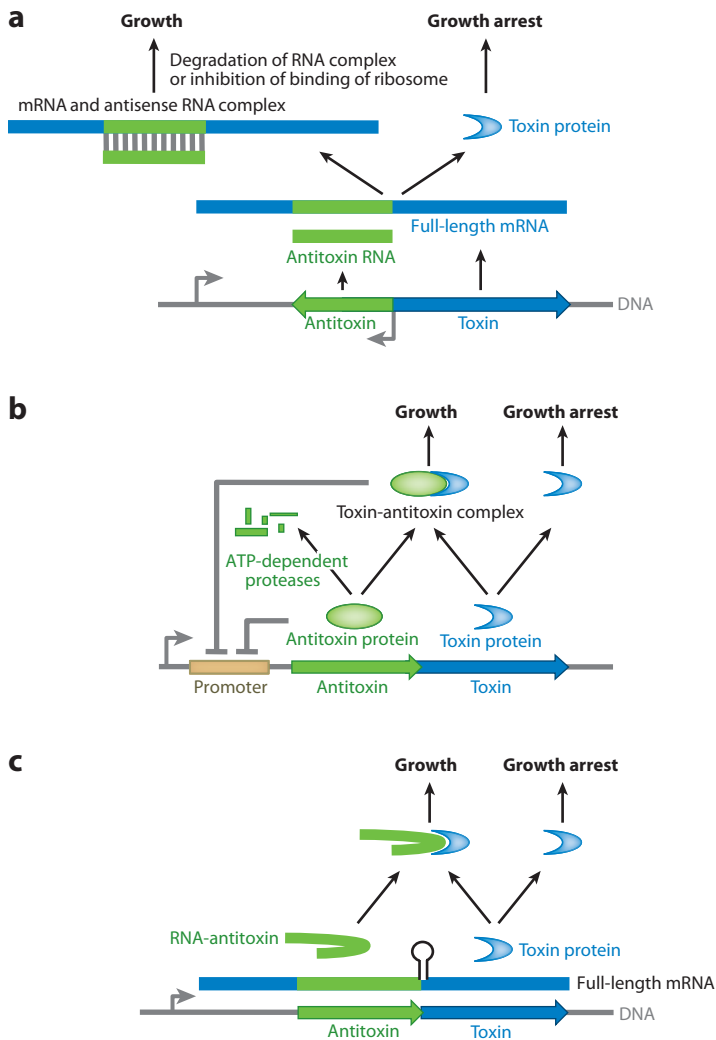


Figure 1

Regulation of TA systems. (a) Regulation of Type I TA systems. Toxin and RNA-antitoxin (antisense RNA) are transcribed separately. RNA-antitoxin binds to mRNAs for toxin to form a duplex inhibiting translation. (b) Regulation of Type II TA systems. Antitoxin and toxin mRNAs are synthesized from the same promoter and both are translated into protein. Antitoxin forms a heterodimeric polypeptide complex with toxin to inhibit toxicity of toxin and to autoregulate the TA module. Antitoxins are subjected to cleavage under stress conditions by an ATP-dependent protease. Antitoxin itself can also autoregulate the TA systems, but more weakly than the TA complex. Toxin, released from the TA complex then attacks its cellular target. This toxin activity leads to bacterial cell growth arrest and eventual cell death. (c) Regulation of Type III TA systems. ToxI RNA binds to ToxN protein and inhibit the toxicity.

membrane, likely forming a pore to destroy the membrane potential and thus to inhibit ATP synthesis (33, 54, 55, 103).

Type II Toxin-Antitoxin System

Among the TA systems, the Type II TA system has been most extensively studied. Examples of this TA system include MazE-MazF (2), RelE-RelB (90), YefM-YoeB (50, 114), and MqsR-MqsA (11, 18, 53, 111). Almost all the Type II TA systems share a number of common features (39, 110): (a) each TA system is encoded by an operon consisting of two small genes usually overlapping by a few bases or apart only by a few bases (110); (b) a toxin and its cognate antitoxin form a stable TA complex to block the function of the toxin; (c) the antitoxin is less stable than the toxin in the cell so that the antitoxin has to be produced continuously to prevent the toxin from exerting its toxic function (2); (d) under stress conditions, antitoxins are degraded by stress-induced proteases to free toxins from the TA complexes in the cell, resulting in cell growth inhibition or cell death (2, 15); (e) the TA complex functions as the repressor for the TA operon transcription by binding a palindromic sequence in the promoter region. Therefore, as the concentration of the TA complex in the cell is reduced as a result of antitoxin degradation, the TA operon expression is derepressed to produce more toxin and antitoxin (56, 76). In this manner, the expression of the TA operon is autoregulated and thus the Type II system is also termed as the addiction module (112); (f) in most of the Type II systems, the antitoxin genes are located upstream of their cognate toxin genes so that the antitoxins appear to have an advantage for their production over their cognate toxins. However, there are many exceptional cases in which the toxin genes are located upstream of their cognate antitoxin genes (18, 111). These differences in the gene order may have subtle effect on in the toxin-to-antitoxin ratio in the cell, which in turn regulates the toxin function, and (g) the pI values of the toxin proteins tend to be basic, whereas those for their cognate

antitoxin proteins are acidic (110). These opposite pI values are considered to stabilize the formation of TA complexes.

Type III Toxin-Antitoxin System

The ToxI-ToxN TA system from a plasmid in the plant pathogen *Erwinia carotovora* subspecies *atrosepticum* (*Pectobacterium atrosepticum*) is the first example of the Type III TA system in which a RNA antitoxin, ToxI-RNA (36 nucleotides), directly inhibits the toxicity of ToxN (Figure 1) (31). The genes for ToxN homologs are found in other plasmids and the chromosomes of diverse bacteria, including human and animal pathogens, and soil and marine bacteria (31). It is not known whether these ToxN genes are also regulated by antitoxin RNAs.

Recently, the X-ray structure of the ToxI-RNA and ToxN complex has been determined (8), and showed that three ToxI-RNA molecules bind to three ToxN monomers to generate a trimeric ToxN-ToxI complex. These authors also showed that ToxN had endoribonuclease activity. However, it is not known at present if this endoribonuclease activity causes the ToxN toxicity.

Regulation of Toxin-Antitoxin Systems

In normally growing cells, all Type I, II, and III TA systems are constitutively transcribed, constantly producing both toxins and antitoxins. In Type I TA systems, RNA antitoxins bind to toxin mRNAs, causing inhibition of ribosome binding to the mRNA or enhancing degradation of toxin mRNAs, resulting in inhibition of toxin production (33, 54, 55, 106). In Type II and III TA systems, every toxin molecule has to form a complex with its cognate antitoxin for the toxin function to be completely inhibited (31, 110). One may wonder why so many TA systems have to be constitutively expressed in the cells rather than being induced when they are needed. Is it not more economical for the cells to produce the toxins only when needed, e.g., under stress conditions? One possible

answer may be that cells must be ready for any sudden environmental changes. It may be advantageous for a cell to be constantly equipped with TA complexes, rather than to initiate toxin production upon sensing an environmental change. Such changes induce stress-responsive proteases, which then preferentially remove antitoxins to free toxins in Type II TA systems.

It is not known at present how the toxins from Type I TA systems are induced. RNA antitoxins may be specifically degraded or the transcription of the genes for the antitoxin RNAs may be repressed.

DIVERSITY OF TOXIN TARGETS

Deciphering the function and cellular target of each toxin is of fundamental importance for our understanding of the roles of the TA systems in bacteria. All Type I toxins cause the inhibition of ATP production, as discussed below, whereas the cellular targets of the *E. coli* toxins of Type II TA systems are highly diverse. Moreover, each TA complex releases toxin by stress-induced proteases under various stress conditions (39, 110).

DNA Replication

Two toxins, CcdB and ParE, block DNA replication by inhibiting DNA gyrase activity, which is essential for cell growth. ParE is a toxin encoded on plasmid RK2 (28, 47, 93), and CcdB is a toxin encoded on the F plasmid (80). Although both CcdB and ParE toxins inhibit DNA gyrase, they share neither sequence nor structural homology between them.

The mechanism of the action of CcdB has been determined in detail (20). CcdB binds to the dimerization domain of GyrA, thereby preventing strand passage and the formation of the functional conformation of GyrA (71). ParE from plasmid RK2 is thought to inhibit GyrA gyrase activity by stabilizing gyrase-DNA complexes similar to CcdB (113). However, *E. coli* harboring a CcdB-resistant GyrA was not resistant to ParE, indicating that ParE inhibits the gyrase activity in a manner different from

that of CcdB (93). Recently, it was shown that ParE2 encoded on the *Vibrio cholerae* genome required ATP in contrast to CcdB (47). This ParE2 inhibited gyrase-dependent DNA supercoiling but not DNA relaxation.

The first such enzyme described was MazF from *E. coli*, which cleaves mRNA at ACA sequences to effectively inhibit protein synthesis (117). A large number of MazF homologs have been identified from bacteria to archaea, and some bacteria have more than one MazF homolog (97). MazF homologs so far identified specifically cleave mRNAs at either three-, five- or seven-base recognition sequences (Y. Yamaguchi & M. Inouye, unpublished data). It is not clear at present why these cleavage sites consist of only odd base numbers. It is interesting to see how the RNA cleavage site expands by two bases at a time from three-base sequences to five-base and seven-base sequences. The RNA recognition and cleavage specificity appears to have been extended mostly at the 3'-end site, which is particularly evident when the *E. coli* MazF cleavage site (A^CA or ^ACA) is compared with the cleavage sequence of the MazF homolog (MazF-hw) from an archaeon (UU^ACUCA) (Figure 2). It seems that more space is available on the MazF-dimer interface for RNA substrate binding, which allows it to extend the recognition specificity at the 3'-end.

mRNA Stability

mRNA is the most frequently used cellular target for TA system toxins. Out of 34 TA systems in *E. coli* so far identified, eight have been identified to regulate mRNA stability (110). Given that all these eight toxins cleave cellular mRNAs, they are termed mRNA interferases (45, 110). Based on how these mRNA interferases cleave mRNAs, they are classified into two different types: those that can freely cleave mRNAs in the absence of ribosomes (ribosome-independent mRNA interferases), and those that cleave mRNA only when they associate with ribosomes (ribosome-dependent mRNA interferases) (110).

Ribosome-independent mRNA interferases are sequence-specific endoribonucleases that cleave mRNA at specific RNA sequences (110).

mRNA interferases: interfering mRNA by cleaving mRNA

Ribosome-independent mRNA interferases: cleaving mRNA freely at specific sites in the absence of ribosome

Ribosome-dependent mRNA interferases: cleaving mRNA only when associated with ribosome

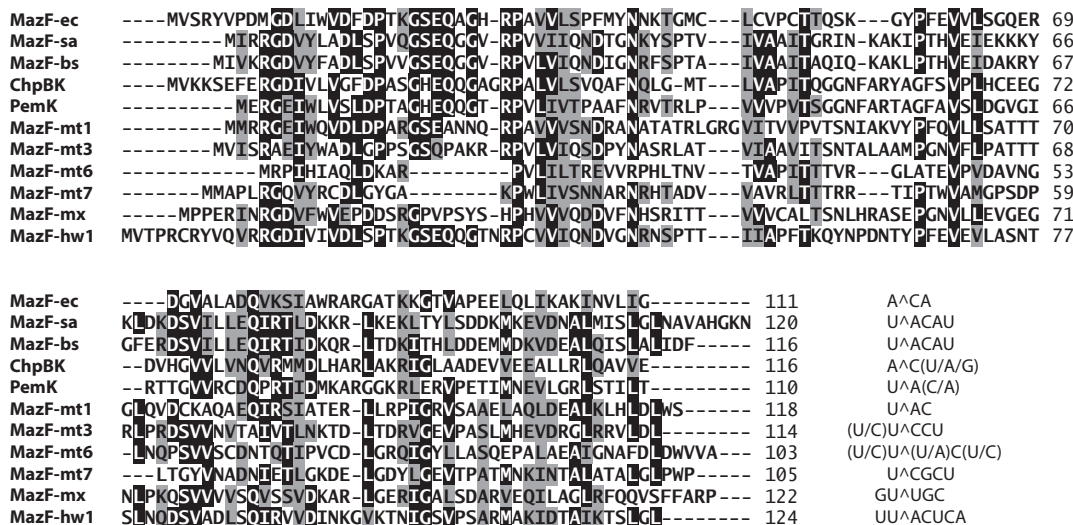


Figure 2

Alignments of MazF homologs from bacteria and archaea. MazF-ec and PemK from *E. coli*, MazF-sa from *Staphylococcus aureus*, MazF-bs (YdcE) from *Bacillus subtilis*, MazF-mt1, -mt3, mt6, and mt7 from *Mycobacterium tuberculosis*, MazF-mx from *Myxococcus xanthus*, and MazF-hw1 from *Haloquadratum walsbyi*. Cleavage sites are shown by ^ . Identical amino acid residues are indicated by black shading and conservative substitutions by gray shading.

It remains to be determined if this odd-number rule for RNA recognition will prevail in all MazF homologs yet to be identified. As the recognition sequences become longer, the target mRNAs become more limited so that MazF homologs cleave mRNA species more and more specifically. Thus, the cellular functions of MazF homologs appear to be highly diverse: Some, such as ACA-specific *E. coli* MazF, eliminate almost all cellular mRNAs, whereas others, such as MazF-hw, are highly sequence-specific and eliminate only a specific subset of cellular mRNAs.

To date, the longest specific recognition sequence consists of seven bases, UU[^]ACUCA (Y. Yamaguchi, H. Nariya, M. Inouye, unpublished data), and was identified in a MazF homolog from the halophilic archaeon *Haloquadra walsbyi*, isolated from a hypersaline pool on the Sinai Peninsula (108). *Haloquadra walsbyi* grows phototrophically using bacteriorhodopsin in the membrane (9) and requires 3–4 M NaCl for growth and is tolerant to 2 M magnesium (10). Theoretically, a specific seven-base sequence exists only once in every 16,384-bases. Interestingly, the seven-base RNA target sequence of MazF-hw (MazF homologs are named by using the initial letter of host species. For example, the MazF homolog from *Haloquadrum walsbyi* is named MazF-hw) is overrepresented in the rhodopsin transcription activator gene and a few other membrane protein genes in this archaeon, suggesting that these mRNAs are more susceptible to degradation than other cellular mRNAs when MazF-hw is expressed in the cells. This suggests that in this archaeon, protein-mediated mRNA interference regulates the cell growth by silencing specific gene expression.

Ribosome-dependent mRNA interferases have no or very weak endoribonuclease activity (110, 114, 115). The RelE in the RelB-RelE TA system is one of the most thoroughly investigated ribosome-dependent mRNA interferases (16). RelE binds to 30S ribosomes, and it is postulated that this binding stimulates an endogenous ribonuclease activity of ribosomes (43, 90). However, at present one cannot exclude a

possibility that this toxin-ribosome interaction may induce a conformational change in RelE protein, which may activate the latent ribonuclease activity of the toxin molecule. RelE associates with the ribosome A-site, leading to cleavage of mRNA preferentially between the second and the third nucleotides of the termination codon (17, 90). In this manner, RelE effectively inhibits protein synthesis. The crystal structure of the RelE-ribosome complex has been determined, showing that RelE tightly interacts with 16S rRNA but not with 50S subunits and ribosomal proteins (85).

Homologs of the RelB-RelE system have been found in other bacteria as well as in archaea, and may be present either on the chromosome or on plasmids (14, 97). YoeB is another ribosome-dependent mRNA interferase. It associates with 50S ribosomes to specifically block translation initiation, resulting in cleavage of mRNAs immediately downstream of the initiation codon (114). Given that YoeB was shown to have a weak ribonuclease activity (50), the mRNA cleavage observed may be directly caused by YoeB protein as a result of conformational change occurring in the YoeB molecule upon its association with 50S ribosomes. Interestingly, YoeB and RelE share a structural similarity, but the former interacts with 50S ribosomes to inhibit translation initiation, whereas the latter associates with 30S ribosomes to inhibit translation elongation.

Ribosomes

The Phd-Doc TA system is derived from bacteriophage P1 (46). Doc toxin inhibits translation elongation through its association with the 30S ribosomal subunit (70). Doc expression inhibits cell growth by inhibiting translation without affecting transcription or replication. Interestingly, Doc does not cleave mRNA, as documented with other toxins like mRNA interferases. Doc appears to function by inhibiting translation elongation in a manner similar to that of an aminoglycoside antibiotic, hygromycin B (HygB) (70). HygB is able to block Doc binding to the 30S ribosomal

subunit, indicating that HygB binds to the same region on the ribosomes as Doc (70). Furthermore, a HygB-resistant strain is not sensitive to the toxicity exerted by the induction of Doc protein (70), suggesting that the Doc-binding site is located on a highly conserved region in 16S rRNAs similar to the HygB binding site at the 30S–50S ribosome interface containing the P and A sites, which are essential for protein translation. Notably, because Doc inhibits translation elongation by binding to the ribosome as well as chloramphenicol, cellular mRNAs become highly stable (four- to tenfold) by arrested ribosome, which physically protects a nuclease-sensitive target site (70).

Cell Division

CbtA (YeeV) toxin inhibits cell division and is the first toxin to date that inhibits cell growth by binding to the cytoskeletal proteins (101). CbtA alters cell morphology, converting normally rod-shaped *E. coli* to lemon-shaped cells (101). Interestingly, CbtA interacts with FtsZ, a tubulin-like protein, and MreB, a prokaryotic actin-like protein. FtsZ is a highly conserved GTPase (23, 92), which forms a ring structure at septa and is essential for cell division (6). MreB is required for maintenance of the typical rod shape of cells, and assembles into a helical structure that coils along the cell length (48, 104). MreB is also involved in cell division (62, 107), chromosome segregation (62, 63) and cell polarity (41). *E. coli* has two CbtA homologs, YpjF and Ykfl (12). CbtA shows 78% and 75% homology to YpjF and Ykfl, respectively, suggesting that YpjF and Ykfl also bind to FtsZ and MreB to inhibit cell division.

ATP Synthesis

All Type I toxins thus far identified appear to inhibit ATP synthesis (33, 38, 55, 103). All the Type I toxins have an identical secondary structure consisting of one α -helix, and they are predicted to form a pore across the inner membrane to inhibit ATP synthesis. Overproduction of Type I toxins TisB or

Hok inhibits cell growth and reduces viability, as measured in colony formation units (38, 103). Both proteins are inserted into the inner membrane, affect membrane integrity, and inhibit ATP synthesis. Consequently, replication, transcription, and translation are severely inhibited. Overproduction of IbsC and ShoB proteins was also shown to destroy the proton gradient across the inner membrane (33). Recently, it was shown that Type I TA system, TisB-IstR-RNA, is involved with persister phenomenon as described below.

PHYSIOLOGICAL FUNCTIONS OF TOXIN-ANTITOXIN SYSTEMS

Up- or downregulation of TA systems has been examined under several stress conditions, including nutrient starvation, addition of antibiotics, DNA damage, and heat-shock in *E. coli* (2, 39, 44, 94). In *E. coli*, it has been proposed that stress-induced toxins cause cell growth arrest and eventually programmed cell death (29, 110). In *Mycococcus xanthus*, a gram-negative bacterium that forms fruiting bodies upon development, MazF plays an essential role. It is induced upon the initiation of fruiting body formation to kill about 80% of the cell population, causing cell lysis so that the remaining 20% of cells are able to form fruiting bodies (84). When the gene for MazF is deleted, this obligatory cell death is severely hampered and prevents normal fruiting body formation, demonstrating that MazF is essential for programmed cell death in this bacterium. In contrast, cell lysis is not observed when *E. coli* cells are starved of nutrients or treated with antibiotics, which induces MazF production in these cells (2, 39, 44, 94). Notably, MazF-induced *E. coli* cells are metabolically active and capable of synthesizing proteins if their mRNAs do not contain any ACA sequences (99). These states are called quasis dormancy, as cells may recover and proliferate if enough MazE is produced to neutralize MazF toxicity. HipA, another of the *E. coli* TA system toxins, also causes complete cell growth arrest leading to multidrug-resistance or the persistence state (82).

PERSISTENCE STATE AFFECTS ANTIBIOTIC SENSITIVITY

Persister cells are those that are able to survive in the presence of antibiotics even if they are genetically sensitive. It is believed that persister cells are in a dormant state so that antibiotics are unable to function. Induction of the persistence state by some toxins may render cells functionally resistant to antibiotics, even in the absence of genetic resistance (66, 105). Antibiotic resistance due to this persistence phenomenon presents a major problem when treating patients with antibiotics.

Bactericidal antibiotics such as kanamycin are reported to repress the TA systems, *mazE-mazF*, *relB-relE*, *mqsR-mqsA*, and *bigB-bigA* in *E. coli* (60). When the expression level of all the TA systems was examined after the addition of the same antibiotics (kanamycin and ampicillin) in *E. coli*, not only the four TA systems described above but also other TA systems were also repressed (Y. Yamaguchi & M. Inouye, unpublished results), suggesting that cell death mediated by bactericidal antibiotics may be associated with many TA systems in *E. coli*.

TisB was shown to be involved with the persister phenomenon after treatment with an antibiotic, ciprofloxacin (27). The persister mechanism caused by TisB expression is not well understood; however, the inhibition of ATP synthesis caused by TisB seems to be an important factor. Interestingly, several toxins in the Type I TA system (SymE, HokE, and TisB) are induced under SOS response (19, 30, 54, 89, 106). It is predicted that the induction of these toxins from the genome causes growth arrest by inhibition of ATP synthesis but does not cause cell death (27, 103).

STRUCTURAL HOMOLOGY IN TYPE II TOXIN-ANTITOXIN SYSTEM

Three-dimensional structures of a number of TA complexes, toxins, and antitoxins have been determined mainly by X-ray crystallography

and in some cases by nuclear magnetic resonance (NMR) spectroscopy. Comparing these structures, one can find that many TA systems share three-dimensional structural homology (Figures 3 and 4) (8). The analysis of these structural homologies observed in TA systems is highly informative and useful for our understanding of their functions and of evolutionary relationships between the TA systems. The structurally and functionally distinct domains in TA systems are described below.

Neutralization of Toxin with Antitoxin

In general, the antitoxin consists of two distinct domains: a DNA-binding domain and a toxin-binding domain. The major role of the antitoxin is to neutralize the toxic function of its cognate toxin mediated by the toxin-binding domain. The toxin-binding domain in antitoxins is folded distinctly to specifically neutralize the toxicity of their cognate toxins. Structural models of a number of the TA complexes reveal that the interaction between toxin and its cognate antitoxin is mediated through a combination of hydrophobic and electrostatic interactions; however, the electrostatic interaction mainly contributes to the specificity of the antitoxin binding to the cognate toxin (21, 51).

In the MazE-MazF TA system, the C-terminal (residues 55–77) domain of MazE contains a total of six negatively charged residues that directly interact with the MazF homodimer (51). Interestingly, the C-terminal domain of RelB, which is required for neutralization of the RelE toxicity, has a structure similar to that of the MazE C-terminal domain; however, their cognate toxins, RelE and MazF, do not show high structural homology (Figure 3a–c) (51, 69). Although *E. coli* RelE toxins have structural homology with *M. tuberculosis* RelE, their cognate RelB proteins show different folding patterns in the C-terminal neutralization domains (4). Thus, even though two different bacteria have homologous toxins, their toxicity may be neutralized by different mechanisms, as their cognate antitoxins have different structures.

NMR: nuclear magnetic resonance

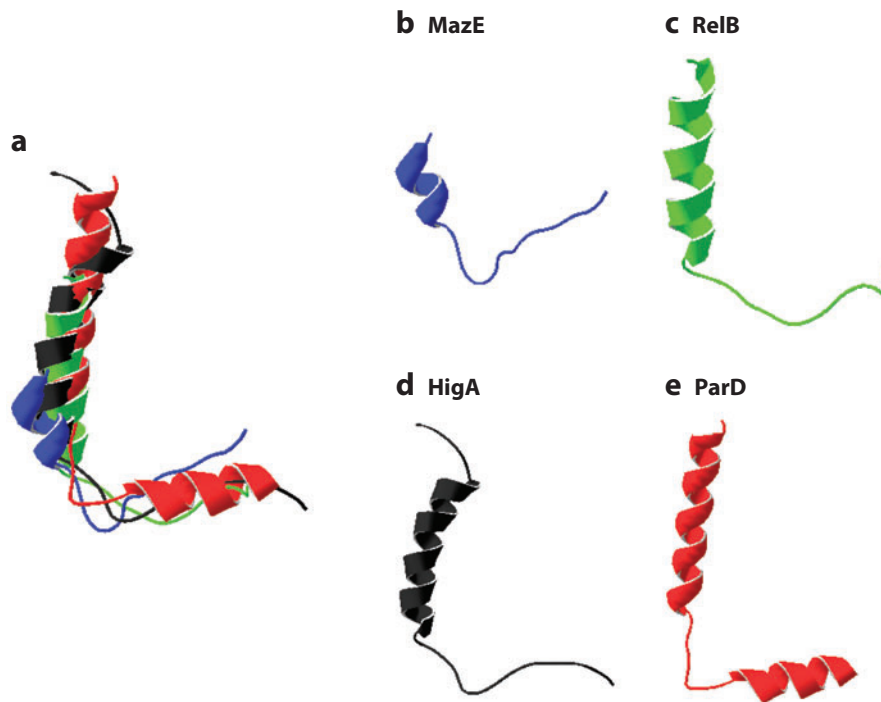


Figure 3

C-terminal toxin-binding regions of four antitoxins having structural homology. (a) Structural aligned C-terminal domains of four antitoxins, MazE (PDB ID: 1UB4, blue, residues 46–76), RelB (2KC8, green, residues 47–79), HigA (2ICT, black, residues 60–94), and ParD (3KXE, red, residues 43–79). Magic Fit and Fit molecules (from selection) functions were used for structural alignment in Swiss-Pdb Viewer 4.0.1. (b) The structure of C-terminal of MazE. (c) RelB. (d) HigA. (e) ParD. All structures were imaged in Swiss-Pdb Viewer 4.0.1.

Many antitoxins neutralize the toxicity of their cognate toxins by direct binding to their active sites of toxins. Less frequently, antitoxin binding to toxins neutralizes activity by causing substantial conformational changes. Toxins in the latter class include the MqsA-MqsR, HipB-HipA, Phd-Doc, and ϵ - ζ TA systems (11, 36, 78, 96). MqsR belongs to the RelE family, based on structural homology. It functions as a GCX (X; U, C, and A)-specific mRNA interferase (18, 111). However, unlike the RelB-RelE TA system, MqsR is a ribosome-independent mRNA interferase and the MqsA antitoxin does not wrap the region on the MqsR molecule that interacts with the RNA substrate. Instead, upon interaction with MqsR, MqsA may induce a conformational change that inhibits mRNA binding (11).

Disorder-Order Binding Model

Disorder-order binding occurs in the many TA systems in which an unstructured region of the free antitoxin is reorganized into a well-defined secondary structure upon binding to its cognate toxin (68, 86), including CcdA, YoeB from *E. coli*, and Phd from Phage P1 (73, 86, 35). The disordered domain in the antitoxin is highly susceptible to stress-induced proteases (3, 13, 26, 65).

The CcdA antitoxin has an intrinsically disordered structure in its C-terminal domain in the absence of the interaction with the CcdB toxin (24, 25). This disordered CcdA domain is able to reactivate the gyrase activity of CcdB-bound gyrase by removing CcdB from the CcdB-gyrase complex using a zipper-like

mechanism. After forming the complex with CcdB, CcdA attains ordered folding in the C-terminal domain, leading to a conformational change in the CcdB molecule, resulting in inactivation of the CcdB toxin function (24). From the crystal structure of the YefM-YoeB complex in *E. coli*, one can see that the disordered YefM C-terminal domain becomes an ordered-structure upon its binding to YoeB (13, 50).

The disorder-order binding mechanism is not common to all TA systems, however (11, 64). Some TA antitoxins lack a disordered domain, including YefM from *M. tuberculosis* and MqsA from *E. coli*. YefM from *M. tuberculosis* appears not to follow the disorder-order binding mechanism for its binding to YoeB (64). Free *M. tuberculosis* YefM shows a well-ordered structure containing significant secondary and tertiary structures (64). MqsA, an antitoxin for MqsR, is also well-structured even in the absence of the interaction with MqsR (11).

Binding of Toxin to Substrate

Toxins of the TA systems have been classified into six groups according to their structural homology (8). The structures of many toxins and toxin-target complexes solved to date are from the Kid family. The Kid toxin belongs to the Kid family together with ToxN, MazF, YdcE, and CcdB (8). The Kid protein is encoded from the *parD* in *E. coli* plasmid R1 and forms a compact, closely interwoven dimer in which each monomer contains a twisted antiparallel β -sheet at the core (42). Kid is an mRNA interferase that cleaves RNA at UAC sequences (83). Notably, the sequence identity of Kid with CcdB is very low, but their structures are very similar (42). The residues involved in the RNA cleavage and the RNA recognition have been identified by comparison of NMR structures of the Kid protein with and without an uncleavable RNA substrate (**Figure 4a,b**) (52, 116) (replacement of the uridine at the cleavage site with a deoxyuridine blocks RNA cleavage). The residues at the RNA recognition site in Kid interact with RNA by hydrogen bonds as well as

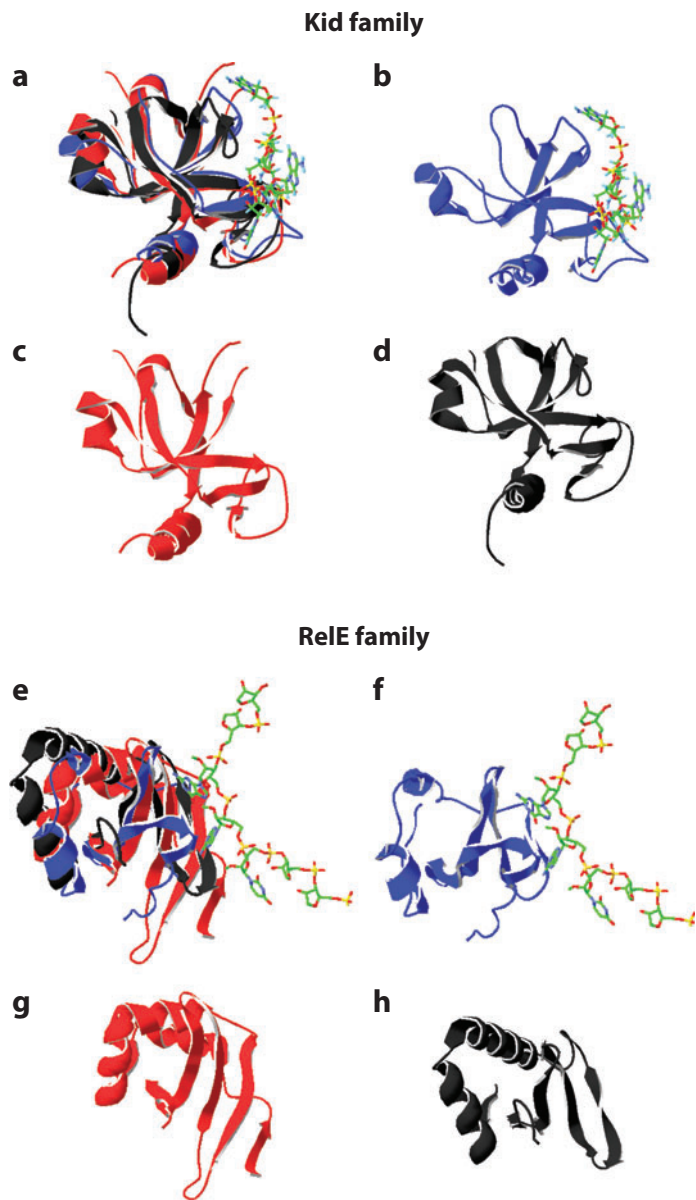


Figure 4

Toxins in two structural families (a)–(d) Kid family. (a) Structural aligned Kid family, Kid monomer with 5 dU-RNA (rAdUrArCrA) (PDB ID: 2C06), MazF monomer (1UB4), and YdcE monomer (1NE8). (b) Kid monomer with 5 dU-RNA. (c) MazF monomer. (d) YdcE monomer. (e)–(h) RelE family. (e) Structural aligned RelE family, MqsR monomer (PDB ID: 3HI2), RelE monomer (3KIQ) with 2'-O-methylated RNA, and YoeB monomer (2A6Q). (f) RelE monomer. (g) MqsR monomer. (h) YoeB monomer. (a) and (e) Magic Fit and Fit molecules (from selection) functions were used for structural alignment in Swiss-Pdb Viewer 4.0.1. All structures were imaged in Swiss-Pdb Viewer 4.0.1.

RHH:
ribbon-helix-helix

HTH:
helix-turn-helix

hydrophobic contacts, and three residues (D75, R73, and H17) were proposed to be part of the active site of the Kid (52).

To elucidate the RNA-binding mechanism of *E. coli* MazF dimer, a 13-base single-stranded DNA (which cannot be cleaved) was incubated with MazF dimer for NMR spectroscopic study (68). The substrate-binding sites were shown to be located on the concave interface of the MazF homodimer (67), consisting of a highly basic region underneath the two loop regions, between β -sheet 1 and 2 and β -sheet 3 and 4 (**Figure 4a,c**). The binding of a substrate molecule to one of the two identical interfaces created in a symmetrical MazF dimer prevented binding of a second RNA molecule to another interface through negative cooperativity. The crystal structure of the complex of CcdB with its target, the dimerization domain of GyrA protein (residue 363–494), was determined (22). Structure–function analysis showed that Arg462 in this region is required for binding to CcdB (5). When the CcdB-GyrA complex is formed, small conformational changes in GyrA protein are induced as a result of hydrogen bond formation (22). From mutagenesis studies of a MazF homolog from *Bacillus anthracis*, His59 and Glu78 were proposed to play a role in an acid-base couple mediating the enzymatic activity (1). On the basis of the crystal structure of the ToxI-ToxN complex of the Type III TA system, ToxN toxin can be classified to the same group as toxins in the Kid family (7). They share structural homology within the β -sheet core region (S1-S2 and S3-S4). However, ToxN has an additional helical region in helix-3, which gives a broader RNA-binding region that is not present in other toxins in the Kid family. The RNA-binding regions are different between Kid and ToxN, and their RNA recognition specificities are different.

DNA-Binding Domain of Toxin-Antitoxin Complex or Antitoxin

In the TA systems, the expression of both toxin and antitoxin are negatively autoregulated by

the antitoxin or the TA complex by binding to the palindromic sequence located in the promoter region of the TA operon. In other words, the TA complex or the antitoxin by itself functions as the repressor for its own operon. This binding is mediated by the DNA-binding domain of the antitoxin, which has a specific structure, ribbon-helix-helix (RHH) or helix-turn-helix (HTH), for binding to double-stranded DNA (11, 58, 68, 73, 77, 79, 86). It is important to note that the binding of the TA complex to the promoter region is much stronger than the binding of the antitoxin, so that the production of the TA complex is more favorable than the production of only the antitoxin (35).

In the MazE crystal structure, the N-terminal hairpin contains many positively charged residues and this region is predicted to bind to two palindromic sequences in the *mazE-mazF* promoter region (51, 72). The N-terminal DNA-binding segment of CcdA has an RHH fold. The cocrystal structure of CcdA with the 6-bp palindromic DNA sequence in the promoter region of the *ccd* operon reveals that association with DNA does not induce a major conformational change. The N-terminal domain of CcdA is composed of a positively charged β -sheet, which binds to the major groove of DNA (73). RelB from *E. coli* was also shown to bind to DNA by the same mechanism as that of CcdA (68).

The RHH domain in MqsA is distinctly different from the RHH domain of other known antitoxins, as it requires a zinc ion for structural stability. MqsA can bind not only to its own promoter region but also to other promoters such as the promoters for *mcbR* and *spy* (11). HipB antitoxin employs an HTH domain for binding to the HipA-HipB promoter region (8). Notably, some antitoxins, such as Phd, contain neither RHH nor HTH motifs in their DNA-binding domain, however the domain essential for dimerization is used for DNA binding (37, 56, 74, 98). In these antitoxins, the structure of the DNA-binding domain is highly conserved.

Binding of Ribosome-Dependent mRNA Interferases to mRNAs and Ribosomes

As described above, there are two types of mRNA interferases, ribosome-independent mRNA interferases (**Figure 4a–d**) and ribosome-dependent (**Figure 4e–h**) based on their mechanisms for RNA cleavage (110). It is speculated that ribosome-dependent mRNA interferases must interact with ribosomal subunits to induce a conformational change essential to activate enzymatic activity (43). Thus, ribosome-dependent mRNA interferases always cleave only mRNAs associated with ribosomes.

Recently, a cocrystal structure of *E. coli* RelE associating with 70S ribosomes from *Thermus thermophilus* was determined (85). Structural analysis showed that RelE occupies the ribosomal A site, pulls the mRNA into its active site

to cause its reorientation, and then cleaves it after the second nucleotide of the codon in the A site, resulting in inhibition of translation (85). This mRNA reorientation is stabilized by RelE residue Y87 and 16S rRNA residue C1054, although these residues have not been directly demonstrated to participate in the cleavage reaction (8, 85). These data indicate that not only RelE but also 16S rRNA are essential for mRNA cleavage by RelE. Like RelE, YoeB is also a ribosome-dependent mRNA interferase, which interacts with 50S ribosomes (114). As seen above, each ribosome-dependent mRNA interferase may function in a manner different from that of others, because RelE and YoeB bind to different subunits of rRNA (**Figure 4**). More structural studies on the complex of ribosome-dependent mRNA interferases with ribosomes will be important for our understanding of the function of these toxins.

SUMMARY POINTS

1. Almost all bacteria and some fungi contain the TA systems in their genomes, and constitutively express toxins and their cognate antitoxins. *E. coli* contains at least 34 TA systems.
2. In normally growing cells, the toxins form stable complexes with their cognate antitoxins. However, under stress conditions, stress-induced proteases digest less stable antitoxins to free the toxins in the cells, which interact with various cellular targets to inhibit DNA replication, protein synthesis, cell-wall biosynthesis, and ATP production.
3. *Mycobacterium tuberculosis* contains more than 60 TA systems, whereas a related non-pathogenic strain contains only two, suggesting that the TA systems may play important roles in bacterial pathogenicity.
4. The TA systems are not essential for cell growth but are considered to play important roles in survival under stress conditions. They also participate in fruiting body formation in developmental bacteria such as *M. xanthus*.
5. There are three types of TA systems, Type I, II, and III, distinguished by the mechanisms of TA gene regulation and the nature of the antitoxins.
6. Toxins can be released by stress-induced proteases induced by some antibiotics, causing cells to enter a persistence or quasi-dormant state in which they are completely resistant to antibiotics. Toxins thus released may also play important roles in eliminating damaged cells from their populations.

7. In general, the antitoxin consists of two distinct domains, a DNA-binding domain and a toxin-binding domain. The toxin-binding domain in antitoxins is folded distinctly to specifically neutralize the toxicity of their cognate toxins.
8. The toxins of the TA systems are classified into six distinct families on the basis of three-dimensional structural homology in the target binding domains.

FUTURE ISSUES

1. To elucidate the physiological roles of the TA systems in *E. coli* and other bacteria under various stress conditions.
2. To elucidate the roles of the TA systems in pathogenicity of bacteria such as *M. tuberculosis* and *Staphylococcus aureus*.
3. To identify the cellular target of each toxin and to elucidate the molecular mechanism for its toxic function.
4. To elucidate the three-dimensional structure of the TA complexes.
5. To elucidate evolutionary relationships of the TA systems in bacteria.
6. To study the physiological roles and evolutionary origin of TA systems found in some fungi.

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Contents

Comparative Genetics and Genomics of Nematodes: Genome Structure, Development, and Lifestyle <i>Ralf J. Sommer and Adrian Streit</i>	1
Uncovering the Mystery of Gliding Motility in the Myxobacteria <i>Beiyang Nan and David R. Zusman</i>	21
Genetics and Control of Tomato Fruit Ripening and Quality Attributes <i>Harry J. Klee and James J. Giovannoni</i>	41
Toxin-Antitoxin Systems in Bacteria and Archaea <i>Yoshihiro Yamaguchi, Jung-Ho Park, and Masayori Inouye</i>	61
Genetic and Epigenetic Networks in Intellectual Disabilities <i>Hans van Bokhoven</i>	81
Axis Formation in Hydra <i>Hans Bode</i>	105
The Rules of Engagement in the Legume-Rhizobial Symbiosis <i>Giles E.D. Oldroyd, Jeremy D. Murray, Philip S. Poole, and J. Allan Downie</i>	119
A Genetic Approach to the Transcriptional Regulation of <i>Hox</i> Gene Clusters <i>Patrick Tschopp and Denis Duboule</i>	145
V(D)J Recombination: Mechanisms of Initiation <i>David G. Schatz and Patrick C. Swanson</i>	167
Human Copy Number Variation and Complex Genetic Disease <i>Santhosh Girirajan, Catarina D. Campbell, and Evan E. Eichler</i>	203
DNA Elimination in Ciliates: Transposon Domestication and Genome Surveillance <i>Douglas L. Chalker and Meng-Chao Yao</i>	227
Double-Strand Break End Resection and Repair Pathway Choice <i>Lorraine S. Symington and Jean Gautier</i>	247

CRISPR-Cas Systems in Bacteria and Archaea: Versatile Small RNAs for Adaptive Defense and Regulation <i>Devaki Bhaya, Michelle Davison, and Rodolphe Barrangou</i>	273
Human Mitochondrial tRNAs: Biogenesis, Function, Structural Aspects, and Diseases <i>Tsutomu Suzuki, Asuteka Nagao, and Takeo Suzuki</i>	299
The Genetics of Hybrid Incompatibilities <i>Shamoni Maheshwari and Daniel A. Barbash</i>	331
Maternal and Zygotic Control of Zebrafish Dorsoventral Axial Patterning <i>Yvette G. Langdon and Mary C. Mullins</i>	357
Genomic Imprinting: A Mammalian Epigenetic Discovery Model <i>Denise P. Barlow</i>	379
Sex in Fungi <i>Min Ni, Marianna Feretzaki, Sheng Sun, Xuying Wang, and Joseph Heitman</i>	405
Genomic Analysis at the Single-Cell Level <i>Tomer Kalisky, Paul Blainey, and Stephen R. Quake</i>	431
Uniting Germline and Stem Cells: The Function of Piwi Proteins and the piRNA Pathway in Diverse Organisms <i>Celina Juliano, Jianquan Wang, and Haifan Lin</i>	447

Errata

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