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REVIEW

Toxin–Antitoxin Systems in Bacteria: Apoptotic Tools or Metabolic Regulators?

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Abstract—The results of recent (10–12 years) research in the functions of two-gene chromosomal modules are considered and generalized. One of the genes encodes a toxin protein; the product of the other gene is an antitoxin protein. In the course of balanced bacterial growth, the toxin is constantly neutralized by the antitoxin; however, certain metabolic changes (amino acid starvation, etc.) disturb the balance and then the toxin "poisons" the cell (in most cases, by destroying mRNA). As a result, bacterial growth ceases. In accordance with one group of the data, long-term inhibition of growth of most cells results in their programmed death and destruction, corresponding to apoptosis; this allows a minor part of the population to survive due to an additional nutrient source. The results of other works show that growth inhibition is mostly reversible and the functions of the relevant gene modules are restricted to the regulation of cell metabolism, i.e., transition of bacteria to the hypometabolic state. There is also a compromise point of view. The possibilities of biotechnological applications for "toxin–antitoxin" systems are discussed.

Key words: "toxin–antitoxin" modules, proteases, endoribonucleases, ectopic expression, metabolism, apoptosis.

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Apoptosis is the term for genetically programmed cell death; in multicellular organisms, whole tissues are sometimes eliminated. Apoptosis of the tail tissue cells of a tadpole during its metamorphosis into a frog is a well-known example. Apoptosis occurs at various stages of embryo development and is considered a vitally important process. Although bacteria are unicellular organisms equipped with a system of adaptations for autonomous existence, bacterial communities (cultures in liquid medium, colonies on solid medium, biofilms, etc.) have long been considered as more than just cell assemblies (see, e.g., reviews [1–3]). One of the reviews of this subject [4] bears the typical title "Thinking about Bacterial Populations as Multi-Cellular Organisms." In such communities, interrelations between the individual bacteria may be similar to those between eukaryotic cells in various tissues; thus, death of some bacterial cells is a possible (or even necessary) event in the course of their development or under changing conditions of their existence. These processes are interpreted as manifestations of apoptosis in bacteria; sometimes they are termed a model of altruistic behavior [5, 6]. Of course, from a "human point of view" it is difficult to say whether such death is "voluntary" and/or "compulsory." Whole systems of genes are involved in the apoptotic processes in bacteria. This is the case for cyanobacteria, root nodule nitrogen-fixing bacteria, myxobacteria, and actinomycetes (see articles [7–10], respectively). Mother cell death during sporulation in bacilli may also be considered apoptosis [11].

In many bacteria, the chromosome contains modules, usually consisting of two genes; one of them encodes formation of a product that stops cell growth, which may lead to cell death; the other gene is responsible for the synthesis of an "antidote" neutralizing the effect of the toxic product. These modules are called "toxin–antitoxin" systems (abbreviated as TA systems). The action of these systems is qualified in some works as a kind of apoptosis, i.e., genetically programmed "suicide" of some part of the population during starvation or other stresses, allowing the rest of the bacteria to survive (although other points of view exist). The presented review considers the structure and functions of "toxin–antitoxin" systems in bacterial cells.

"Toxin–Antitoxin" Systems in Plasmids

"Toxin–antitoxin" systems were first studied (beginning from the mid-1980s) in low-copy, usually big, plasmids. These systems perform quite a specific function: to provide stable coexistence of bacteria and their plasmids over many generations by eliminating the cells that have lost plasmids or stopping their growth. Plasmid TA systems are usually termed "addiction modules." The word "addiction" means attraction to some harmful habit, which passes into

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dependence (e.g., narcotic dependence resulting in withdrawal pains when giving it up is attempted). Thus, TA systems have the same purpose as the mechanisms of active plasmid segregation (i.e., distribution of the plasmids between daughter cells).

As we will see below, practically all plasmid TA modules occur in the chromosomes of different bacteria; they circulate between plasmids and chromosomes. The roles they play in the chromosome and in the plasmid are quite different. Brief consideration of the main characteristics of the plasmid TA modules is worthwhile, not involving detailed analysis of the peculiarities of each module (see reviews [12–14]).

TA system components are usually two genes located one after the other (sometimes overlapping), possessing at least one promoter and forming an operon. The toxin gene determines formation of a long-lived product "poisoning" the bacterium, while the antitoxin gene codes for a labile product that can neutralize this toxin by binding (or preventing its formation). The first gene in the operon with the preceding promoter is usually an antitoxin gene, with a toxin gene following it. The toxin–antitoxin protein complex binds to the promoter, suppressing transcription of the operon [13, 14]. Thereby, the equilibrium of toxin and antitoxin concentrations is maintained; the system is self-regulated. The cell that has lost the plasmid still contains a certain quantity of the products of the TA module genes. Antitoxin is quickly degraded by proteases (as a rule, Lon or Clp [13]). Long-lived toxin "poisons" the cell, affecting different targets. As a result, the plasmidless cell dies or at least stops to grow.

"Toxin–Antitoxin" Systems Located on the Bacterial Chromosome

Several years after the discovery of the plasmid systems, the modules similar to TA systems were found in the chromosome of *Escherichia coli* and other bacteria.

At first, we will discuss the functions of the modules studied by the conventional methods of genetics, biochemistry, and molecular biology and then will dwell on the data obtained *in silico*, i.e., by using computer simulations.

The *mazEF* system. This system is the most widely discussed. The antitoxin and toxin genes (*mazE* and *mazF*, respectively) are components of the *E. coli relA* operon. This locus was initially designated as *chpA* [15]. Both genes were shown to have a certain homology with the "addiction module" *pemI–pemK* of the plasmid R100. Three years later, evidence of involvement of this system in the phenomenon of "programmed bacterial death" (a form of apoptosis) was reported [16]. In that article, the genes were designated as *mazE* and *mazF* and this renaming took roots. In Hebrew, *ma ze* means "what is it?" [17]; most of the works with this module were carried out in Israel.

The *relA* operon begins with the *relA* gene under the control of the *p1* promoter; the following two promoters (*p2* and *p3*) control the expression of the *mazE* and *mazF* genes. The operon is closed up with the *mazG* gene described later. The *mazG* product, nucleotide pyrophosphohydrolase, contributes to the effect of the antitoxin [18, 19]. The results of more than ten-year study of this system may be generalized as follows.

The product of the *relA* gene is ppGpp synthase I synthesizing 3',5'-bispyrophosphate (ppGpp); synthesis of the latter is induced by unfavorable conditions of existence (especially amino acid starvation). PpGpp negatively controls the expression of three "underlying" *maz* genes; i.e., it inhibits the transcription of polycistronic mRNA from these genes. "The point of application" of ppGpp is the *p2* promoter located upstream the *mazE* gene; it is active in the logarithmic growth phase [16]. Moreover, the reading of *maz* genes is self-regulated; i.e., it is suppressed under the excess of their products through the binding of the MazE–MazF protein complex (or, to a lesser extent, of MazE alone) to the two operator regions located near the promoter of these genes [20]. As was already mentioned, the *mazF* gene encodes the protein toxic for the cell, while the *mazE* gene encodes the antitoxin (proteins MazE and MazF of 12.1 and 34 kDa, respectively). The MazF toxin is an endoribonuclease, which cuts the single-stranded mRNA at ACA sequences [18, 21]. The action of this enzyme is so specific that it can be used in biotechnology as a restriction endonuclease "working" on mRNA. MazF is a "long-lived" protein with the "lifetime" in a cell of more than 4 h. MazE, on the contrary, is very labile: its half-lifetime is 30 min; it is degraded by the ClpPA serine ATP-dependent protease [16]. The antitoxin interacts with the toxin with formation of a complex, a linear heterohexamer consisting of alternating toxin–antitoxin homodimers [22]. The binding of antitoxin and toxin "neutralizes" the latter (see below).

If the action of the antitoxin ceases for some reason, the resulting imbalance has fatal consequences for a cell. This was originally demonstrated in [16]. Artificially induced "oversynthesis" of ppGpp (due to superexpression of the *relA* gene) stopped the synthesis of products of both the *mazE* and *mazF* genes. However, the "short-lived" antitoxin MazE was destroyed by the ClpPA protease, so that only the toxin remained in the cell and interacted with any mRNA, cutting it by the ACA sites (if there were any). As a result, translation of mRNA molecules corresponding to most of the genes ceased, the growth stopped, and the cells died. Superexpression from the *mazF* gene cloned in a plasmid in the cells carrying chromosomal *ts* genes *mazEF* decreased the viability at 42°C by three to four orders of magnitude. The phenomenon of growth inhibition followed by the death of bacterial cells was classified as "programmed cell death" (PCD), i.e., a manifestation of apoptosis. It should be noted that PCD occurred only during the logarithmic, but not the stationary, phase of culture growth.

The situation regarding PCD reproduction under ppGpp oversynthesis was certainly artificial. The experiments where PCD was induced by amino acid starvation (achieved by tenfold or higher dilution of the exponential-phase culture with the minimal medium), were closer to natural conditions, although, in the long run, starvation also induced ppGpp synthesis. In one of the test works of Danish researchers [23] it was mentioned that starvation induced not the death of cells, but rather suspension of protein synthesis and inhibition of culture growth. The process was reversible and a modulation of metabolism under starvation conditions rather than an apoptotic manifestation. However, the work of supporters of the PCD hypothesis introduced the concept of "point of no return" [24]. Experiments were carried out with the strains in which the relevant chromosomal genes were knocked out. Plasmids with the cloned toxin or/and antitoxin genes under the control of IPTG-induced *lac* promoter were introduced into the cells of such strains. Thereby, it was possible to induce ectopic expression of the genes located on the plasmid at any growth phase and to discriminate between the separate stages of the process. A "window" of several hours was revealed, when cessation of protein synthesis was reversible. This "window" was followed by the "point of no return," whereupon cell death became irreversible and cells could not be "revived" by cessation of the toxin action. It was suggested that impaired translation caused by mRNA damage was only the first step of a "fatal cascade" involving proteins with modified conformation; residual synthesis of these proteins could proceed, in particular, due to residual translation from mRNA damaged by the toxin [24–27].

It was shown that PCD could be induced not only by starvation, but also by a number of other impacts resulting in the cessation of transcription and translation, DNA damage, and various stresses. All these diverse impacts had in common the fact that they were much more effective for the cells with complete *mazEF* systems than for the cells with deletions of respective genes or the *clpP* gene [24].

First of all, PCD induction by low concentrations of the antibiotics inhibiting transcription (rifampicin interacting with the β-subunit of RNA polymerase) and translation (chloramphenicol, the target of which is one of the ribosome subunits, and spectinomycin affecting translation) will be discussed. Application of these antibiotics resulted in a more pronounced decrease in the CFU number in the growing cultures of strains with initial genotype than in strains with deletions of the *mazEF* genes [26, 27]. The mutants resistant to the above antibiotics exhibited no such effect. The cells that had survived PCD induction (about 10% of the total population) were not antibiotic-resistant, and the PCD effect could be reproduced in them after transfers. It seems that "the alternative of life or death" was dictated by the level of MazE in a particular cell, because this level abruptly decreased in the culture treated with antibiotics (according to assessment of MazE concentration by Western blot). The effect of PCD was much stronger for the cells growing on the minimal medium compared to rich medium. Antibiotics with another cell target (e.g., ampicillin) did not stimulate PCD [28].

The difference between the cells with impaired *mazEF* system and the cells of the wild genotype was also observed under some stresses: increasing the temperature of the medium (to 48–50°C) and treatment with hydrogen peroxide (oxidative stress) or mitomycin C (DNA damage). Interestingly, about 100% of wild type cells and only 10% of the cells with *mazEF* deletions died after 10-min incubation at 50°C. However, both cell types died equally already at 52°C [29].

The *mazEF* module was concerned also with so-called thymineless death: the phenomenon described over 50 years ago, in 1954 [30]. It was shown [31] that thymine starvation (caused by addition of sulfonamides or trimethoprim to the medium) decreased the level of expression of the *p2* promoter; as a result, antitoxin MazE disappeared from the cells and they died.

The works should be mentioned that report on the interaction between the *mazEF* system and the "addiction module," i.e., prophage P1 functioning in the genome (prophage P1 exists in a cell as a plasmid). The products of this module are the short-lived antitoxin Phd and long-lived toxin Doc. In a cell with the cleaved prophage P1, an excess of the Doc toxin develops; it inhibits protein synthesis, i.e. acts like the antibiotic chloramphenicol. Work [32] compared the results of induction of the prophage "addiction module" in the cells with the functioning *mazEF* system and with deletion of this chromosomal module. It was shown that the postsegregational death of bacteria was much more effective in the cells with the *mazEF* system. It was suggested that Doc, apart from its independent action, induced the action of the toxin from TA module (similarly to its induction by translation-inhibiting antibiotics, see above, [24–27]). Interesting results were obtained in the experiments with thermal induction of prophage P1 present in the cells of this genotype and in the cells with deleted *mazEF* module. The cells died after induction in both cases, but the death of the wild genotype cells was almost unaccompanied by lysis and the release of mature phage particles was low. On the contrary, induced cells with deletion were lysed with subsequent release of numerous phage particles [33].

One more essential component of the *mazEF* system, the so-called extracellular death factor, was found recently [34, 35]. This is a small pentapeptide Asn-Asn-Trp-Asn-Asn sensitive to heating at 80–100°C, proteinase K, and "extreme" pH values. It is present in the culture supernatant in the logarithmic but not in the stationary growth phase. Its employment in the *mazEF* system was revealed as follows. PCD after stress impacts occurs only if cell concentration in the growing culture is rather high: 3×10^7–

10^8 cells/ml, but not in the same culture at the beginning of growth. However, PCD induction became possible if the supernatant from the mid- or late-exponential phase culture was added to a culture with low cell concentration. The purified peptide was active at the concentrations of 2.5 ng/ml and higher. It did not activate PCD of a strain with "knocked out" *mazEF* genes, but its action was exhibited if a plasmid with the corresponding cloned undamaged genes was introduced into such strain.

The origin of this peptide in a cell is of interest. It is a small fragment of the enzyme glucose-6-phosphate dehydrogenase (the *zwf* gene product): the fragment was cut out of the region between the catalytic and structural domains of this enzyme with further modification. EDF and similar tri- and hexapeptides were also synthesized artificially. Only the synthetic product, which was absolutely identical to the pentapeptide from the culture supernatant, possessed the activity corresponding to the activity of "natural" EDF, although other synthetic oligopeptides with partial amino acid substitutions also showed minor activity [35].

Surprisingly, only five ORFs with regions that could encode this (or similar) peptide were revealed among innumerable combinations of genomic nucleotides. At the same time, deletions of only two genes prevented the formation of active EDF: the *zwf* gene proper and the *yeo* gene with unclear functions [34].

The "fatal peptide" seems to participate in the initial stages of the PCD pathway. Its role resembles the functions of other signal peptides in bacteria (e.g., competence pheromones of streptococci and bacilli; see [36]). However, data on any particular target of this substance are still absent.

The *relBE* system. One more well-studied chromosomal TA system is the *relBE* system. Work with the latter, also on *E. coli*, was carried out mainly in Denmark at K. Gerdes' laboratory.

The products of the *relB* and *relE* genes are two proteins: an antitoxin of 9.1 kDa and a toxin of 11.2 kDa, respectively [37]. The purified toxin preparation forms in vitro a stable complex with the toxin [38]. This complex and, to a lesser extent, the antitoxin alone are bound to the promoter, inhibiting transcription of the operon (i.e., the system is self-regulated). In contrast to the *mazEF* system, ppGpp is not involved in the module regulation. The functions of the operon are influenced by the Lon protease, which destroys the labile antitoxin. This stimulates transcription of the operon. However, out of the *relBE* gene products, only the stable toxin RelE "survives." Similar to the *mazEF* system, an excess of this long-lived toxin results in impaired culture growth [39]. The Lon protease is activated in stress situations (under laboratory conditions, this is amino acid starvation as a result of manifold culture dilution with the minimal medium).

What happens to the cell after toxin accumulation? In the early work of Gerdes' laboratory [37], the mutants with deletions of the *relBE* genes were used for toxin "overproduction": plasmids with the cloned *relE* gene under the control of lactose promoter were introduced into respective cells. The promoter was induced by IPTG. Cell growth was stopped, and the quantity of individuals that could form colonies at inoculation on a solid medium decreased by hundreds of times. This could be evidence of either irreversible death of the cells or their conversion into a state similar to uncultured anabiosis, when the cells are alive but incapable of reproduction under the given conditions. The latter assumption was verified in subsequent work [40] using the same strains with the *relBE* deletion but bearing two plasmids: one with the cloned *relE* gene under the control of arabinose promoter and another one with the *relB* gene under lactose promoter. If *relE* was induced due to the arabinose promoter, the CFU number dropped already after 30 min, decreasing by six orders of magnitude 100 min after the induction. However, upon induction of the lactose promoter, nearly all cells recovered the ability to form colonies, i.e., the "poisoning" with the toxin was reversible and the cells recovered from the state of nonculturability due to production of antitoxin RelB. Similar results were obtained by Danish researchers when testing the reversibility of the action of toxin MazF [23] (see above). Thus, at least in the case of the *relBE* system, the reversibility of cell death casts doubt on the PCD hypothesis [13]. However, the cells in the nonculturable state were more sensitive to heat shock, osmotic stress, and some other impacts than regular cells [40].

What is the target of the RelE toxin? It was shown that, similarly to the toxin MazF, RelE cleaves mRNA, i.e., RelE is an endoribonuclease that inhibits translation and, eventually, protein synthesis. However, it cleaves not the free transcript but mRNA present in the ribosome. The action of the toxin was codon-specific: mainly the stop codon suffered, although the sense codons could be damaged as well. The damaged mRNA "occluded" the ribosome, and polypeptide synthesis was suspended. The ribosome was "rescued" owing to the joint action of antitoxin RelB and the so-called tmRNA (transfer-messenger RNA combining the properties of transport and information RNA and known also as SsrA; see [41]). Antitoxin RelB was bound to the toxin and "neutralized" the latter; the suspended polypeptide synthesis was resumed on the tmRNA template; as a result, a "composite" polypeptide was obtained with an "alien" region of ten amino acids (this additional region was translated from tmRNA). This process is called transtranslation. The "composite" polypeptide is highly sensitive to various proteases. Eventually, the ribosome was released from the "incorrect" polypeptide, the translation cycle was resumed, and the cells became capable of normal growth [42]. Mutations in the *ssrA*

gene (the product of which is tmRNA) made the cell supersensitive to the action of the toxin RelE [43].

The *yefM–yocB* and *dinJ–yafQ* systems. The role of the genes of these systems as TA modules was initially suggested in [44] due to the revealed homology with the "addiction module" *phd–doc* genes of phage P1. In *E. coli*, the peculiar features of the *yefM–yocB* module (antitoxin and toxin genes, respectively) were studied in detail in [45, 46]. The mass of both proteins was about 10 kDa. In the experiments with ectopic expression, the induction of toxin "oversynthesis" stopped the growth immediately; after 4 h, the number of colony-forming cells decreased 100-fold. The colonies formed by surviving cells were very small in size (though this property was not retained in further transfers). It seems that although the cells remained viable, their metabolic processes were seriously disturbed. The simultaneous ectopic expression of both toxin and antitoxin production had no significant effect on culture growth. Thus, this module in *E. coli* behaved as a typical TA system. This system proved to be homologous to the Axe–Txe system of plasmid pR4B from the cells of the multiresistant clinical isolate of *Enterococcus faecium* [45].

The toxin and antitoxin proteins had different types of molecular conformation. The antitoxin belonged to a less common type with an unfolded molecule and was very labile. The conformation of the antitoxin molecule made it extremely sensitive to various impacts, in particular, to the action of Lon protease. The toxin molecule, on the contrary, had a compact conformation. The "toxin–antitoxin" complex was characterized by high stability. This complex contained two toxin molecules and one antitoxin molecule [46, 47].

In this system, like in other such systems, transcription was self-regulated [48]. Transcription was inhibited under the excess of the module products. The toxin was an endoribonuclease, the "point of application" of which was mRNA [49]. The cuts made by the toxin were codon-specific (similar to the *relBE* system). In *ssrA* mutants, the toxin oversynthesis greatly decreased the ability of the cells to form colonies; apparently, in this case tmRNA was also involved in the "rescue" of occluded ribosomes (see above). The recovery of antitoxin synthesis (in the experiments with ectopic expression) also recovered cell viability [50]. The Lon protease destroys the antitoxin and releases the toxin from under its control, which eventually disrupts translation [50].

The *dinJ–yafQ* module was found during computer searching for the homologues of the *relBE* system. This module was located in the beginning of *E. coli* genetic map [37]. Its properties were investigated in only few works, together with other AT systems. The YafQ toxin is an endoribonuclease [49]. It inhibits translation [50]. An unexpected property of the *dinJ* gene (encoding the antitoxin) is the similarity of one of its regions to the regions of the *lexA* gene, repressor of the genes of SOS response to DNA damage.

Because of the similarity of both modules *(yefM–yocB* and *dinJ–yafQ)* with each other and with the *relBE* system, it was later suggested to name them *relBE2* and *relBE3*, respectively [51].

The *ccd* 0157 system. Plasmid F has a *ccd* module (with the toxin damaging DNA gyrase and the antitoxin inactivated by the Lon protease). Recently, the genes homologous to the plasmid genes were found during the analysis of complete genome sequence in 5 out of 17 clinical *E. coli* isolates. The respective modules were located between the chromosomal genes *folA* and *apaH*. All five strains also bore the F plasmid with its own module of *ccd* genes. The functions of the chromosomal genes of the *ccd* module of one of such strains (*E. coli* 0157:H7) and their interactions with plasmid genes of the same system were studied in the cited work [52]. All of the above genes were cloned for the experiments with ectopic expression. Superexpression of the chromosomal gene of the toxin was shown to kill *E. coli* K-12 cells, while induction of the expression of the antitoxin gene prevented the lethal effect. Thus, the functions of the chromosomal genes of the *ccd* system corresponded to the functions of these genes in plasmid F. However, the experiments with cross expression of TA gene pairs of chromosomal and plasmid origin showed that, while the "chromosomal" toxin was neutralized by the "plasmid" antitoxin, the "chromosomal" antitoxin had no effect on the "plasmid" toxin. Moreover, the loss of a plasmid with the *ccd* genes resulted in "postsegregational death" of the cells, although they contained the chromosomal *ccd* genes which, it would seem, could neutralize the consequences of plasmid loss. Probably, the "capture" of respective genes by the chromosome occurred very long ago (the ratio of GC pairs in the chromosomal genes of *ccd* system and in other chromosomal genes was almost the same) and the functions of the "captured" genes somewhat changed in comparison with their plasmid "twins."

The *yeeV–yeeU, yufI–yafW*, and *ypjF–ypjZ* systems. In [53], a new principle was used for searching for TA modules: these modules were sought for among all the nucleotide sequences of the *E. coli* genome not by homology with the known modules but by another criterion. In earlier works, it was established that the toxin and antitoxin molecules are small (about 8–11 kDa) and that the toxin is slightly bigger than the antitoxin. Based on these data, the researchers sought the "paired" genes corresponding to the proteins consisting of 65–85 (antitoxin) and 95–135 (toxin) amino acid residues and separated by an interval of about 150 bp. Thirty-two "candidates" for TA modules were found. All genes corresponding to the toxins were cloned under the control of arabinose promoter. Six of the found modules inhibited growth of the cells into which they had been introduced. However, growth was recovered after the expression of cloned antitoxin

genes. Three of these modules (mentioned in the subheading) were examined in the work. The properties of TA systems manifested themselves most markedly in the *yeeV–yeeU* module (expression of the toxin gene inhibited growth, assayed as CFU values, 500 times). However, it remained unclear how the antitoxin interfered with the toxin: no complexes of these proteins were found. Probably, the antitoxin somehow inhibited the synthesis of the toxin. Moreover, it is unknown what cell systems were targeted by the toxin (the rate of synthesis of all macromolecules decreased rather slowly, though the culture growth was abruptly inhibited immediately after induction with arabinose). These TA modules should probably be isolated into a separate group.

The *ecnAB* system. *E. coli* contains a so-called entericidic locus including two structural genes, *ecnA* and *ecnB*, and their regulatory elements [54]. This module, which probably also belongs to TA systems, is not like the modules described above. The *ecnA* and *ecnB* genes correspond to the antitoxin and toxin, respectively; the products of these genes are lipoproteins similar to the cell membrane lipoproteins. EcnB is involved in the lysis of bacterial cells during the stationary growth phase under changing NaCl concentrations. The mechanism of lysis is not quite clear. The antitoxin probably does not interact with the toxin directly, but rather inhibits its formation through the regulation systems. The *ecnA* and *ecnB* genes were cloned, and the experiments with ectopic expression showed that the toxin overproduction induced the lysis even of the cells with the initial genotype, i.e., those capable of antitoxin production. The survived cells are nourished with the lysis products. In this respect, this system corresponds to "classical" altruism more than other bacterial TA systems: the process of active lysis is certainly irreversible, and there is no need to resort to the "point of no return."

The *higBA* system. The functional analysis of this system was performed in *Vibrio cholerae*. This bacterium, like other vibrios, belongs to the few bacteria possessing not one but two (big and small) chromosomes: 2400 and 1600 bp, respectively. The small chromosome probably originated from a megaplasmid (see[55]).

Investigation of the TA systems in *V. cholerae* in two independent works [56, 57] yielded similar results. The small chromosome (only this one!) carries seven gene pairs of the *relBE* system already studied in *E. coli*. Further, the small chromosome of the vibrio has two loci of the *higBA* system, which was previously described only for the Rts1 plasmid of *Proteus vulgaris*. In this system, the toxin and antitoxin are products of the *higB* and *higA* genes, respectively; in contrast to other TA systems, the toxin gene on the chromosome of *V. cholerae* is upstream of the antitoxin gene. Both loci of *higBA* exhibited a certain similarity, but not identity.

Deletions of the *higA* gene resulted in cell death, as opposed to deletions of the *higB* gene and the whole *higBA* tandem. The genes were cloned jointly and separately; respective plasmids were introduced into the cells of *E. coli* and *V. cholerae*. Superexpression of the toxin gene inhibited the growth of both *E. coli* and the vibrio and strongly decreased their viability. The antitoxin HigA1 neutralized the action of its "own" toxin HigB1 but not HigB2, and vice versa [56]. Both antitoxins had only 16% similarity, while both toxins had 26% similarity. Thus, the products of the genes belonging to the same TA family could noticeably differ from each other, which probably affected their interactions in the cell.

The Loci of "Toxin–Antitoxin" Systems in Various Bacterial Genomes According to Data of Computer Analysis

Several attempts have been made to determine the presence of TA systems in various bacterial genomes. It concerned the modules *mazEF* [58] and *relBE* [59]. In the later work [60], the maximum number of genomes (126!) was analyzed by BLASTP and TBLASTN computer systems and comprehensive data on TA systems of these genomes were collected. In addition, a search for TA systems was recently carried out at our laboratory in 27 species and subspecies of lactobacilli and bifidobacteria [61].

In the work [60], 16 of the tested genomes belonged to archaea, while the rest of them belonged to gram-positive and gram-negative eubacteria in equal proportions. Sometimes, the data of genome sequences of different strains of the same species were analyzed (e.g., *E. coli* K-12 and three more isolates). In the few cases when bacterial species had two chromosomes, TA was searched separately in each of them.

The chromosomal genes of the following TA systems were sought: *relBE, mazEF, higBA, phd/doc, ccdAB, vapBC*, and *parDE*. The first five of them were already mentioned in this review in connection with the functional analysis of the chromosomal TA systems. The functions of the systems *vapBC* and *parDE* have been as yet studied only in plasmids. Ninety-five genomes (including all archaeal genomes) contained at least one of these systems; no TA was found in the genomes of 31 bacterial species (19 gram-positive and 12 gram-negative bacteria). Moreover, 37 genes of putative toxins had no "pair" in the corresponding chromosomes. Finally, a number of "paired" genes were found, similar to TA in size and other characteristics but as yet not annotated.

Analysis of the data bulk confirmed that the genes of antitoxins are located upstream the genes of toxins (except for the *higBA* system with reversed order). Both genes are components of an operon. The antitoxin gene in all bacteria was always slightly less than the toxin gene. A total of 671 loci corresponding to TA genes of the above systems were identified in

95 genomes. The maximum distribution was shown for the *vapBC* and *relBE* systems (42 and 23% of the number of all found TA loci) and the minimum distribution was shown for *ccdAB* (only five loci and only in gram-negative bacteria). Both the number of TA loci and their affiliation with a specific TA system strongly varied from one genome to another. Most of the genomes (63) contained one to five TA loci. However, in some genomes the number of TA loci was very large and they belonged to different TA systems. For example, *Myc. tuberculosis* H37Rv had 3 *relBE*, 24 *vapBC*, 8 *mazEF*, 1 *higBA*, and 2 *parDE* loci (38 TA loci in all). *Nitrosomonas europaea* had 45 TA loci, and the archaeon *Archeoglobus fuldigus* had 28 loci (4 *relBE* and 24 *vapBC*).

Although one could expect large genomes to have proportionally more TA loci, such correspondence was not observed. There was no distinct correlation between the presence of different TA systems and Gram reaction either. However, one pattern was quite clear: many intracellular parasites or symbionts had no TA systems, in contrast to the related bacteria leading "free lifestyle" or, at least, being facultative parasites. TA loci were absent in mycoplasmas, chlamydia, some rickettsia, and some spirochaetes, namely, obligate parasites (*Treponema pallida, Borrelia burgdorferi*), in contrast to the leptospirosis pathogen *Leptospira interrogans*, which is able to live and reproduce in aquatic environments. The same tendency was observed in mycobacteria: the leprosy pathogen *Myc. leprae*, a strictly obligate parasite, had no TA systems at all, unlike *Myc. tuberculosis* and *Myc. smegmatis*. Such absence of TA loci was interpreted as a result of "secondary simplification," i.e. reductive evolution accompanying transition to the parasitic lifestyle.

A certain tendency was revealed in the location of TA modules (if they were numerous) as islands of the loci from the same family. Sometimes, TA loci were alternated with ATTC sites, targets for integrases; such constructions could probably be mobile elements. For example, all TA loci of the small chromosome of *V. cholerae* were components of a superintegron.

As mentioned above, we checked the presence of TA system genes in gram-positive lactic acid bacteria of the genera *Lactobacillus* and *Bifidobacterium* [61]. The sizes of their genomes vary within 1.8–3.3 Mb. The genomes of many probiotic bacteria useful for the vital activity of their hosts have been recently completely sequenced, including the genomes of the lactic acid bacteria [62]. We analyzed the relevant database using the same computer systems as in [60]. Being of substantial interest for the food industry, numerous species and subspecies of lactic acid bacteria (inhabitants of human and animal intestines) are extensively used in functional nutrition.

The gene analysis of 17 species and subspecies of lactobacilli demonstrated that 14 of them bear the genes of the "classical" *mazEF* system; the same number of genomes bear the genes of the *hipBA* system (its involvement in apoptosis is disputable; it is concerned with development of the persistent state of bacterial cells [63]; see below). The modules of this system were repeated in some genomes one to four times, which may be considered a strain-specific characteristic. The genes of the *relBE* system and the *yefM* and *dinJ* genes (sometimes referred to the *relBE* system) were present in 11 genomes. Other genes of apoptosis occurred only in few genomes and often were absent at all.

In ten genomes of bifidobacteria, TA systems were generally less numerous than in lactobacilli (three to four times less pergenome). The genes of the *relBE* system and related genes *yefM* and *dinJ* (nine genomes) occurred most frequently; two genomes were found to bear the genes of the *maxEF* system. The repeats of the modules of the same system in one genome were less numerous (no more than two identical modules). Bifidobacteria also showed strain-specific distribution of TA system genes (e.g., *B. adolescentis* ATCC 15703 contained the *mazEF* genes, while in *B. adolescentis* L2-32 they were absent; at the same time, strain L2-32 had the *relBE* genes, in contrast to ATCC 15703).

The relative scarcity of the TA system genes in bifidobacterial genomes, compared to lactobacilli, could result from different tempos of evolution in the same ecological niche (intestines) and different adaptation to the conditions of this niche. Of course, not only bioinformational, but also functional analysis is needed for comprehensive consideration of the differences between genomes of these bacterial genera.

Possible Functions of the Chromosomal "Toxin–Antitoxin" Systems

In contrast to the TA systems localized in plasmids, there is no general consensus on the functions of the chromosomal TA systems. Originally, a hypothesis was suggested that at least some of these systems (first of all, *mazEF*) induced PCD acted as apoptotic tools; this viewpoint was typical mainly of Israeli researchers [16, 26, 27]. General reasoning comes to the following propositions. Under unfavorable conditions eventually disturbing protein synthesis, most of the cells in bacterial populations stop growing and then die under the influence of toxin. Thereby, nutrient resources are preserved for the few surviving bacteria; moreover, dead cells themselves become a nutrient source for their live "mates." This pattern resembles "classical" apoptosis in eukaryotes, and the analogy seems to be still more complete as the bacterial population may be to some extent likened to a multicellular organism [1–3]. Danish researchers Gerdes and his coworkers expressed doubts in the validity of this hypothesis in their articles. These doubts were based first of all on the fact that the action of a toxin in their experiments resulted not in cell death but in growth cessation and this process was reversible. In other words, the effect of

the toxin was bacteriostatic but not bactericidal [42, 43].

Further, researchers from one Belgian laboratory recently published work [64]; they have constructed an *E. coli* strain simultaneously bearing deletions of the chromosomal loci corresponding to five TA systems: *mazEF, relBE, yefM–yocB, chpB* (a *mazEF* homologue located in another region of the chromosome), and *dinJ–yafQ*. The cells were exposed to a number of stress factors, including treatment with rifampicin and amino acid starvation. In all cases, the drop in CFU number was the same as in the control (in the strain with the initial genotype). Moreover, in some experiments the bacteria with deletions were grown in the same flask with the strain of the initial genotype (in equal proportions) for "purity of the experiment." After stress impacts, CFU was counted for both cell types; CFU counting revealed no difference between the strains in their response to the stresses.

The counterarguments supporting the "apoptotic" hypothesis have been mentioned above: existence of the "point of no return," after which growth inhibition irreversibly changed into cell death. The discrepancy between the conclusions drawn from the same experiments in different laboratories was explained in different ways (the media of different "nutritional value," the cultures from different growth phases, etc.). However, the authors of some articles continue expressing doubts in the rightfulness of opinions that confine the functions of chromosomal TA systems only to apoptosis [66, 67]. Instead, alternative hypotheses are suggested, where the functions of the chromosomal TA systems are confined to control of the growth rate up to its complete stop. Supposed molecular mechanisms of such control are described in the review [51]. Eventually, it is postulated that growth inhibition and cessation allow the bacteria to survive the "difficult times" and, as a last resort, to fall into a state resembling the state of rest, i.e., to form so-called persistent cells [68].

The peculiar features of persistent cells and the mechanisms of their formation are a special issue. It is worth mentioning here that nearly all the vital functions in such cells are switched off and, as a result, the targets for antibiotics are switched off as well. For example, in normal cells aminoglycosides interrupt translation, thereby killing the bacterium; however, since the translation process in persistent cells is suspended, their tolerance to these antibiotics is much higher. The state of persistency and tolerance in bacteria results not from mutations but from enhanced of expression of certain genes, mainly those inhibiting the translation processes (and consequently other important functions). These are the genes of the toxins comprising TA systems. First of all, the *hipBA* module, which was not mentioned before, should be named here, because it is traditionally considered associated mostly with formation of persistent cells, rather than with apoptotic systems [63]. Its presence was originally shown in the *hipAF* mutant of *E. coli* with increased frequency of formation of persistent cells; this module possesses all the features of TA modules: its products, HipB and HipA (antitoxin and toxin, respectively), form a complex. The antitoxin gene is almost five times smaller than the toxin gene. Superexpression of the *hipA* gene results in cessation of cell growth. The HipB protein is a repressor of the operon, which is typical of antitoxins (see [51, 63, 68]). Moreover, persistent cells exhibit superexpression of the chromosomal modules *mazEF* and *relBE* described above [68]. Thus, the state of persistency is actively maintained by the expression of TA systems.

Some authors suggest a compromise solution [69]. In [70], the authors assume the possibility that some of the cells die in the course of transition from the dormant state to active growth. The authors say, "we believe, however, that different assumptions are not completely incompatible. Stress may activate chromosomal TA systems, thereby bringing bacterial population into stasis. The escape from that state may be different in various cells... A few 'lucky beggars' may obtain nutrition at the expense of their less lucky neighbors" (p. 677).

We also believe that such a point of view, which assumes versatility of the functions of TA systems in bacteria, is acceptable. It is more difficult to explain the reason for the presence of numerous loci (sometimes belonging to the same system) in the genomes of some bacteria. The mechanisms of "accumulation" of these loci may be different: horizontal gene transfer, successive incorporation of plasmids with TA loci into the chromosome, or amplification of genomic regions. However, this is not an answer to the direct question of how, e.g., the archaeon *Archeoglobus fuldigus* may benefit from the presence of as many as 24 *vapBC* loci in its rather small genome (2.8 Mb). It is suggested that at least some part of the genes of TA systems are selfish, something like harmless symbionts "at the chromosomal level" [71]. However, it is hardly possible to consider all TA loci only as selfish genes, because it would be difficult to explain their reduction (as well as, for example, reduction of the systems of synthesis of many amino acids) in intracellular obligate parasites. Obviously, such questions can be answered only by the functional analysis of specific TA systems. Such attempts have already been made. In [72], the *mazF* genes of *Myc. tuberculosis* were cloned and respective plasmids were incorporated into *E. coli* cells. At least four such toxin genes "worked" in *E. coli* cells, causing growth inhibition due to ectopic expression. Although all these toxin genes were from the same family and their products cleaved the mRNA molecule, each of them cleaved it at different codons. It was suggested that the *mazEF* system in the tuberculosis pathogen also transfers the bacteria into a latent state, facilitating their survival in host tissues.

Possible Practical Aspects of the Study of Chromosomal "Toxin–Antitoxin" Systems in Bacteria

Whatever the functions of TA systems in bacterial cells, these systems seem to be necessary for the latter. Therefore, the question of their application in pharmacology, medicine, and biotechnology is debated [47, 51, 73, 74]. We can sum up the respective viewpoints as follows.

A. Artificial induction of the mechanism of "programmed cell death."

In accordance with the conclusions of a number of works, apoptosis is the main function of TA systems. The reviews referring to the genetic control of PCD often abound with metaphors: "time bomb" [12], "lethal insert" [69], "the way to destruction" [75], etc. Indeed, it would be very tempting to find the methods of inducing TA systems, this "Achilles heel" of the bacterial genome, to intentionally provoke the death of the genome owner. In particular, [51] contains considerations on the application of TA systems for construction of genetically modified microorganisms, which lysed or stopped growing under certain cultivation conditions.

B. The search for substances the targets of which would be products of the chromosomal TA systems.

The biotarget-directed screening of inhibitor substances is the main line of development of medicines of the new generation [76–78]. The key moment of such studies is the correct choice of a biotarget [79–81]. Some of the components of TA systems presented in this review are potentially interesting as a basis for biotarget-directed screening [47, 74]. For example, in [47] it is proposed to screen the substances that prevent formation of a complex of the toxin YocB and the antitoxin YefM (see above). The appropriateness of this search does not depend on whether TA systems function asapoptotic tools or as modulators of cell metabolism; disturbance of any of the useful functions will damage the bacterium.

C. Application of toxin preparations as a novel class of antibiotics.

The problem of multiple drug resistance of pathogenic microorganisms has become so threatening in the last decade [82] that it has forced researchers to look for the new strategies of development of antibacterial medicines [79, 80, 83]. Hence, the question about application of some purified natural or modified "toxins" as antibacterial preparations is discussed as well [51, 73]. This approach is attractive, because the toxins of TA systems usually have no "targets" in eukaryotic cells and act only at the level of bacterial cells. In addition, the resistance of microorganisms to conventional antibiotics does not extend to the toxins of TA systems.

D. The toxins of TA systems may supplement the arsenal of substances used in laboratory practice (e.g., ribonucleases recognizing specific sites on mRNA molecules [51]).

Do Bacteria Possess Programmed Death Systems of Eukaryotic Type?

This review pursues the analysis of only one of the apoptotic tools in bacteria, the "toxin–antitoxin" systems. In the very beginning of the review, we also mentioned the existence of other apoptotic systems that function in microorganisms during their differentiation [7–11]. They include the systems of apoptosis that work during multistage processes of formation of the hyphae and aerial mycelium in streptomycetes [84–86] and in the "societies" of biofilm-forming bacteria [87–89]. However, the regulatory systems recently found in bacteria (especially in actinobacteria) involve, in particular, serine–threonine protein kinases, apoptotic ATPases, Toll interleukine receptors (TIR), BCL2-like protein families, etc., usually participating in the apoptotic processes in eukaryotes [27, 69, 90–94]. There is evidence of the presence of classical mechanisms of "eukaryotic" programmed death in bacteria: cell lysis performed by caspaselike proteases, DNA fragmentation by specific nucleases, etc. [10, 74, 84, 95]. It may be supposed that coexistence and functioning in microorganisms of the "two-level" mechanisms of programmed death, i.e.,TA systems (probably originating from plasmids) and various systems analogous to those in eukaryotes, is quite real. A number of systems previously considered as a prerogative of eukaryotes, e.g., serine–threonine and tyrosine protein kinases, have been studied in bacteria [96, 97]. It should be expected that such analogies will be continued.

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