**Regulation of toxin-antitoxin systems by proteolysis.**

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Keywords: proteolysis, toxin-antitoxin, regulation

**Abstract**

Toxin-antitoxin systems are widely distributed among many bacterial species, including human pathogens. Regulation of type II TA system functions, where both components are proteins, relies on proteolysis. In this paper, we discuss the significant features of the antitoxin proteins important for proteolysis. Examples of the best described processes of antitoxin degradation are presented as well as the particular case of the *ω*-*ε*-*ζ* TA system.

**Introduction**

The wide spread of toxin-antitoxin (TA) systems in bacterial genomes is a consequence of their capacity to move by horizontal gene transfer (Koonin and Wolf, 2008; Pandey and Gerdes, 2005). Originally, TA systems were discovered on plasmids; yet, they are also abundant in chromosomes, especially in genomic islands, often found in multiple copies. The omnipresence of these systems among clinical strains and their absence in eukaryotic organisms creates opportunities to eliminate such bacteria by selecting TA systems as molecular targets for new therapeutics. Therefore, it is important to better understand how these systems function in different bacteria species, what factors induce their activity and how they are regulated by cellular components, especially proteases.

In general, TA systems are constituted by two components, toxin and antitoxin. In type II systems, which seem to be the largest group gathering the most extensively studied TA cassettes, both components are proteins. At least ten families of typical type II TA loci have been identified: *ccdAB*, *mazEF*, *vapBC*, *phd/doc*, *parDE*, *ε*-*ζ*, *higBA*, *relBE*, *hipBA* and *hicAB*, all of which can be plasmid- or chromosomally-encoded (Fivian-Hughes and Davis, 2010; Gerdes et al., 2005; Leplae et al., 2011). Except the *higBA* operon (Tian et al., 1996), genes of TA loci are organized in one operon in which antitoxins are located upstream of the toxin gene. Usually, transcription of TA cassettes is regulated by the antitoxin alone or by a complex formed by both proteins; however, three component systems are also known, where the third protein plays specifically a regulatory role (de la Hoz et al., 2000; Smith and Rawlings, 1997). The presence of the antitoxin in excess to the toxin ensures formation of a tight complex between these two proteins and assures toxin inactivity. In a situation when expression from the TA cassette does not occur, in effect of plasmid loss or stress conditions, the toxin protein cannot be neutralized any longer due to a faster reduction of the antitoxin protein. This leads to growth arrest or cell death caused by the toxin freely acting on a specific cellular target. To avoid this, it is necessary for the bacterial host to stably maintain the plasmid which possesses the TA system. The phenomenon by which plasmid-encoded TA systems ensure plasmid stability is called plasmid addiction or post-segregational killing. The role of the chromosomal TA systems is still under discussion. Their putative functions include involvement in persister cell formation, bacterial apoptosis, genome shuffling and cell cycle arrest; yet, the most popular hypothesis is that they are involved in general stress response, as suggested by extensive studies on *mazEF* and *relBE* (Christensen et al., 2001, 2003; Engelberg-Kulka et al., 2006; Gerdes, 2000). They have also been linked to biofilm formation (Kim et al., 2009).

Regulation of the TA system functions, in case of type II systems, relies on proteolysis of antitoxin proteins. In contrast to the stable toxin, the antitoxin protein has a shorter half-life due to its vulnerability to degradation by cellular proteases (Jensen and Gerdes, 1995). Therefore, the antitoxin instability constitutes the molecular basis of the TA system and plays a key regulatory role.

**Features of addiction antidotes important for proteolysis**

In the cell, abnormally folded or heat-damaged proteins are recognized and eliminated by the proteolytic machinery systems. Unfolded proteins may be identified by exposed regions of the polypeptide chain, which are otherwise hidden within the protein core.

In many cases, in the absence of the toxin, the C-terminal domain, or sometimes the whole antidote protein, remains unstructured, which makes it highly susceptible to cellular proteases. After binding to the toxin, conformation of the C-terminal domain of the antitoxin changes by adopting an extended conformation. This disorder-order binding occurs in many TA systems.

It is believed that low thermodynamic stability and intrinsically unfolded domains are common features of class II antitoxins (Yamaguchi et al., 2011). Moreover, antitoxin proteins are often strongly acidic, which, together with their unorganized structure, enable conformational changes necessary for tight binding with positively charged toxins. Protein unfolding is the first step indispensable during the degradation process; therefore, the unfolded nature of antitoxins enables their degradation. As was shown for the Phd antitoxin protein of the P1 phage addiction system, its unfolded state has a physiological significance (Gazit and Sauer, 1999). In P1 lysogens, Phd binds the Doc toxin, protecting the cell from Doc-mediated killing (Lehnherr and Yarmolinsky, 1995). In a tight complex, the Phd protein acquires ordered folding induced by binding with the Doc toxin. This suggests that such interaction can also protect Phd from degradation. Similarly to the Phd protein, the MazE antitoxin is in large part unstructured, which potentially explains its high vulnerability to proteases (Loris et al., 2003). The unstructured C-terminal part of MazE interacts with the MazF toxin, which most likely increases its stability (Kamada et al., 2003). The C-terminal region of the Kis antitoxin, which is encoded together with the Kid toxin by the *parD* operon on the *Escherichia coli* R1 plasmid, is also mostly unstructured (Kamphuis et al., 2007). Similarly, the presence of an unstructured and flexible C-terminal domain is also characteristic for the HipB antitoxin (Schumacher et al., 2009). This region of the HipB protein is critical for its proteolytic degradation by the Lon protease (Hansen et al., 2012); yet, in contrast to other antitoxins, the C-terminal domain does not adopt an ordered structure after binding with the cognate toxin (Schumacher et al., 2009). Alternatively, when the toxin and antitoxin form a complex, HipA blocks the access of Lon to HipB, due to partial shielding of the C-terminal part of the antitoxin from the solvent. Therefore, degradation of HipB occurs rapidly only when the antitoxin is free. The disordered C-terminal region is also present in CcdA (Madl et al., 2006) and ParD (Oberer et al., 2007) antitoxins of plasmid F and RK2/RP4, respectively. The thermodynamic stability of CcdA is low enough to keep the protein close to the unfolded state under *in vivo* conditions, which enables its easy degradation by the cellular protease (Dao-Thi et al., 2000).

It was also confirmed that degradation of the *E. coli* RelB antitoxin by the Lon protease is initiated at the proteolyticallyunstable C-terminus of the antitoxin (Overgaard et al., 2009). The rate of antitoxin degradation is reduced when the RelB and RelE complex is formed. This study supports the observation that RelE interacts with the C-terminus of RelB and, thus, stabilizes the antitoxin.

It should be mentioned that the C-terminal domain of certain antitoxins appears to be structured, e.g. ParD of *Caulobacter crescentus* (Dalton and Crosson, 2010), MsqA of *E. coli* (Brown et al., 2009) or YefM of *Mycobacterium tuberculosis* (Kumar et al., 2008). Free *M. tuberculosis* YefM shows significant secondary and tertiary structures; therefore, the disorder-order binding mechanism does not occur. Interestingly, YefM of *E. coli* was found to be a natively unstructured protein, lacking secondary structure even at low temperature or in the presence of a stabilizing agent (Cherny and Gazit, 2004).

To summarize, it is commonly accepted that the unfolded state of antitoxin proteins is the reason of their physiological instability and is regarded as a critical element in the functioning of the TA module.

**Proteases involved in antitoxin degradation**

Proteolytic regulation of TA systems has been studied in great detail in *Escherichia coli*. Among four proteolytic systems of this bacterium: Lon, ClpP, FtsH and HsIVU (ClpQY), the first two have been shown to be engaged in antitoxin degradation. Lon protease, which is a homo-oligomer with ATPase domain and proteolytic domain within one polypeptide chain, represents a major class of ATP-dependent proteases. It was described to be involved in degradation of RelB (Christensen et al., 2001; Overgaard et al., 2009), MazE (Christensen et al., 2003), ParD (Roberts et al., 1994), CcdA (Van Melderen et al., 1994, 1996), PemI/Kis (Tsuchimoto et al., 1992), PasA (Smith and Rawlings, 1998), HipB (Hansen et al., 2012), YefM (Christensen et al., 2004), MqsA (Wang et al., 2011), DinJ (Prysak et al., 2009) and HicB (Jørgensen et al., 2009) antitoxin proteins. Moreover, the Lons protease from *Synechocystis* sp., sharing 20.7% sequence similarity with Lon from *E. coli*, was shown to breakdown the RnlB antitoxin (Koga et al., 2011).

ClpP is the second well characterized class of proteases. This two-component protease needs two functional elements for proper activity: a cylinder-like proteolytic core and ATPase-active chaperon rings positioned at one or both ends of the proteolytic component. The chaperon is responsible for substrate recognition, unfolding in an ATP-dependent manner and translocation into the proteolytic chamber. All chaperons are members of a large AAA (ATPase associated with various cellular activity) ATPase family, defined by the presence of a P-loop domain with Walker A and B motifs. Different chaperones, namely ClpA, ClpC, ClpE and ClpX, can interact with the ClpP protease core, forming an active chaperon-protease complex. The most ubiquitous of the Clp proteases pair is ClpXP, which is found in almost all bacteria. ClpA is found in Gram-negative proteobacteria, while ClpC in Gram-positive bacteria and cyanobacteria. ClpA and ClpX are chaperons that are essential to ClpP-mediated antitoxin degradation in *E. coli*: ClpAP protease degrades MazE(Aizenman et al., 1996), while ClpXP protease degrades Phd (Lehnherr and Yarmolinsky, 1995) and DinJ (Prysak et al., 2009). Homologous ClpP2s/Xs protease from *Synechocystis* sp. degrades the RnlB antitoxin (Koga et al., 2011). As was shown for the three known TA systems from *Staphylococcu*s *aureus* (*mazEF, axe1-txe1* and *axe2-txe2*), the ClpC chaperon is the only ATPase chaperon necessary for antitoxin breakdown in this strain (Donegan et al., 2010).

**Antitoxin degradation**

Generally, determination of protease(s) involved in antitoxin protein degradation was performed by *in vivo* analysis. In large part, the level of antitoxin in protease-deficient strains was compared to those in the wild-type strain using Western blot analysis (Christensen et al., 2001; Ning et al., 2011; Hansen et al., 2012). Another method used for describing the stability of antitoxin in mutant strains is the pulse-chase labeling technique. The amount of [35S]-methionine-labeled antitoxin protein was estimated by autoradiography (Van Melderen et al., 1994). As TA systems are autoregulated by antitoxins, the transcription activation can depend on a cellular protease degrading these proteins. Chromosomally-encoded TA systems are activated by stress conditions; therefore, to verify which protease is responsible for antitoxin degradation, the transcription response to amino acid starvation was tested in protease mutant strains (Christensen et al., 2003; Jørgensen et al., 2009; Winther and Gerdes, 2012). Additionally, in a few cases, this process was further elucidated by *in* *vitro* degradation assays.

Below presented are examples of the best described antitoxin degradation processes.

CcdA is an antitoxin protein encoded together with its cognate CcdB toxin by the *ccd* operon of plasmid F. Pulse-chase experiments performed in strains overproducing CcdA or CcdB alone showed that the antitoxin protein exhibits lower stability in comparison to the toxin and also that presence of CcdB extends by two-fold the half-life of CcdA. Probably, CcdB protects CcdA by masking regions recognized by Lon. The stability of CcdA was also verified by the same method in protease mutant strains HflA-, ClpP- and Lon-. The CcdA protein was stable only in the *lon* mutant strain (Van Melderen et al., 1994). Results obtained in *in vivo* experiments were confirmed by *in vitro* analysis using the purified CcdA and Lon (Van Melderen et al., 1996). Lon protease cleaves the CcdA protein primarily between aliphatic and hydrophilic residues in an energy-dependent manner. As was shown, degradation of CcdA by Lon does not require involvement of any additional proteins. Moreover, the fluorescence and hydrodynamic measurements suggest that due to the interactions of CcdA and CcdB, which form a tight bimolecular complex, CcdA is converted by CcdB to a more compact conformation and regions recognized by Lon in the antitoxin protein are hidden. Hydrolysis of ATP is necessary for initiation of CcdA degradation by Lon. Possibly, energy is needed for disruption of secondary structure of CcdA, which in consequence increases susceptibility of the protein to proteolysis.

RelB is an antitoxin*,* which counteracts the toxic effect of RelE. Both proteins are encoded by the *relBE* operon, which is autoregulated by RelB dimers as well as by the heterohexamer RelB4/RelE2. Initial *in vivo* experiments indicated that RelB is degraded by the Lon protease (Christensen et al., 2001). These results were confirmed by surface plasmon resonance analysis of purified proteins, which showed that RelB is degraded *in vitro* by the Lon protease in magnesium- and ATP-dependent manner (Overgaard et al., 2009). This degradation is likely to be initiated on the flexible and proteolytically unstable C-terminal domain of RelB, which plays the main role in the interaction with RelE. Thus, under normal cellular conditions (e.g. exponential growth), in the excess of antitoxin, the RelB2-RelE complex is formed, which neutralizes the toxin and protects the antitoxin from degradation. In a steady state or upon nutrient stress, the Lon protease degrades RelB at a high rate, both the free form and in complex with RelE. Moreover, during stress conditions the general rate of translation is reduced, which shifts the ratio between toxin and antitoxin as a consequence of lower antitoxin stability. This results in derepression of the *relEB* promoter, due to the low level of the RelB4/RelE2 complex. Although the transcription level of this operon increases, the enhanced activity of the Lon protease liberates the toxin, which then cleaves mRNA and, in consequence, leads to reduction of energy consumption in the cell. Hence, Lon is required both for activation of *relBE* transcription and for activation of the mRNA cleavage activity of RelE.

The degradation process was also examined for a homologous *relBE* system found on the P307 plasmid. As was verified by *in vivo* experiments, the RelBP307 antitoxin is more labile than the RelEP307 toxin and its instability is abolished in the *lon* mutant strain (Grønlund and Gerdes, 1999). The Lon protease is required for both - activation of the RelE toxin, due to degradation of RelB, and plasmid stabilization.

HipB is an antitoxin that specifically abolishes the activity of the HipA toxin. Both proteins are encoded by the *hipAB* operon of *E. coli*. Similarly to other known TA systems, expression of the *hipAB* operon is regulated by antitoxin protein dimers, which bind to four operator sequences. This repression is enhanced by two molecules of the HipA toxin, which sandwich the HipB-DNA complex by contacting the sides of the HipB dimer (Schumacher et al., 2009). Regulation of the *hipAB* TA system by proteolysis was examined both, *in vivo* and *in vitro*. Comparison of the antitoxin level showed a meaningful increase of the half-life of the HipB protein in *lon* mutant strain, compared to the wild type or *clp* and *hslVU* mutant strains. These results strongly indicate that the Lon protease is the main protease degrading HipB. *In vitro* analysis confirmed involvement of the Lon protease in proteolysis of HipB with ATP and magnesium ions as required factors (Hansen et al., 2012). The unstructured C-terminal domain of HipB, encompassing the last 16 amino acid residues, serves as the degradation signal for the Lon protease. This region of HipB does not play any role in operator or HipA binding. It should be mentioned that degradation does not occur when HipB forms a tight complex with DNA or HipA. As the activity of the *hipBA* promoter under different growth conditions is still not well characterized, the regulator of the Lon activity that directs it to act on HipB has not yet been identified.

Proteins MqsA and MqsR (motility quorum sensing regulator), constituting another antitoxin–toxin system from *E. coli*, have been linked to motility and biofilm formation (Gonzalez Barrios et al., 2006). This unique TA pair is characterized by the larger size antitoxin than the toxin as well as the basic nature of both proteins (Brown et al., 2009). Moreover, the antitoxin is structured throughout its entire sequence and, unlike other TA systems, regulates not only transcription of its own operon, but also binds to the *cspD*, *mcbR*, and *spy* promoters (Brown et al., 2009; Kim et al., 2010). Whole transcriptome studies showed that genes encoding proteases Lon, ClpP and ClpX were all induced upon oxidative stress as well as the *mqsR* gene (Kim et al., 2010). Further analysis showed that simultaneous production of Lon and MqsA significantly reduced the ability of MqsA to increase motility. Hence, this confirmed that the Lon protease degrades MqsA under oxidative stress conditions, when *lon* is induced (Wang et al., 2011). Degradation of MqsA by Lon has further consequences for the cell due to the regulatory functions played by the MqsA antitoxin, and involves mainly enhanced biofilm formation and reduced motility.

A few cases are known when degradation of the antidote can be carried out by more than one protease. For the DinJ protein, which is an antitoxin that counteracts the activity of the YafQ toxin, participation of both Lon and ClpXP proteases in its degradation was described (Prysak et al., 2009). It was shown that DinJ is substantially stabilized in protease mutants *lon,* *clpP* and *clpX*, but not in the wild type or the mutant *clpA* strains. Indirect experiments performed with MazE, the antitoxin encoded by the *mazEF* operon from *E. coli*, showed that two proteases are engaged in its degradation. During steady-state growth, ClpAP is responsible for MazF degradation (Ainzenman et al., 1996), while upon amino acid starvation this role is ascribed to the Lon protease (Christensen et al., 2003). The switch between the two protease activities under different conditions needs to be further investigated. Also, in chromosomally-encoded *relNEs* from the *Synechocystis sp*. PCC 6803 TA system, both proteases, Lons and ClpP2s/Xs, are responsible for RelN antitoxin degradation (Ning et al., 2011). Based on the genetic organization and sequence similarity the *relNEs* system is classified as member of the *rel* family. This TA system is the first from cyanobacteria for which proteolytic regulation was studied and the RelN antitoxin is the first substrate identified for cyanobacterial ATP-dependent proteases. Knowledge regarding protease systems in cyanobacteria is mainly gathered from studies on the model *Synechococcus elegans* PCC 7942 strain (Andersson et al., 2009; Schelin et al., 2002). Among eight *clp* genes encoding proteolytic and ATPase regulatory subunits, two of them have significant (~60%) similarity to *E. coli* proteases. Therefore, besides the Lons protease, which shares 20.7% similarity with *E. coli* Lon, involvement of ClpP2s and ClpXs in RelN degradation was verified. Ectopically overproduced ClpP2s and ClpXs proteases formed a functional protease complex, which degraded the free RelN antitoxin or in complex with RelE, as well as Lons.

**Regulation of chromosomally-encoded TA systems by proteolysis**

In consequence of autorepression during normal bacterial growth, expression from the TA operon occurs at very low level. Concurrently, genetic organization of the TA systems ensures excess of antitoxin protein over the toxin in the cell; therefore, the antitoxin can effectively neutralize the toxin by complex formation. Appropriate cellular levels and turnover rates of the toxin and antitoxin are maintained by combination of autoregulation and proteolysis. As was shown, nutritional stresses, such as amino acid and glucose starvation, activate MazF, RelE and HicA toxins (Aizenman et al., 1996; Christensen et al., 2001; Jørgensen et al., 2009). This is connected with an increased activity of the Lon protease upregulated during stress conditions (Tsilibaris et al., 2006; Van Melderen and Aertsen, 2009). Therefore, the susceptible antitoxin is degraded at a high rate. Breakdown of the antitoxin has not only an effect on the release of the toxin from the complex, but also has an influence on the very rapid increase of the TA operon transcription rates. In turn, increased transcription ensures high toxin level during stress periods, due to insensitivity of the toxin protein for cellular proteases. The balance between the toxin and antitoxin is also shifted by reduction of the global rate of translation during stress conditions, which results in derepression of the promoter due to loss of cooperativity in the repression complex. Activation of TA systems through the ATP-dependent antitoxin degradation could result in a variety of phenotypes, but the most frequently observed are those connected with growth inhibition, persistence, programmed cell death or biofilm formation (Engelberg-Kulka et al., 2005; Hansen et al., 2012; Wang and Wood, 2011). MqsR/MqsA is the first TA system related to biofilm formation (Gonzalez Barrios et al., 2006; Ren et al., 2004). Degradation of the MqsA antitoxin by the Lon protease leads to induction of *rpoS*, which in turn increases c-di-GMP (3,5-cyclic diguanylic acid, which controls the switch from motility to sessility), inhibits motility and increases cell adhesion/biofilm formation (Wang et al., 2011). Additionally, the toxin-encoding gene, *mqsR,* is the most highly induced gene in persister cells as compared to non-persisters (Shah et al., 2006). Different effects of TA systems activation have recently been reviewed elsewhere (Hayes and Van Melderen, 2011; Yamaguchi et al., 2011).

**Particular case of the *ω*-*ε*-*ζ* TA system**

The *ω*-*ε*-*ζ* cassette from the pSM19035 streptococcal plasmid is one of the best described Gram-positive bacteria TA systems (Lioy et al., 2010; Zielenkiewicz and Cegłowski, 2005). The unusual feature for this system, which is widely found in many human pathogens (Mutschler and Meinhart, 2011) is lack of transcription regulation by the free antitoxin or in complex with the toxin. Existence of a third regulatory component - the Omega protein ensures autorepression of the TA operon (de la Hoz et al., 2000; Dmowski et al., 2006). The unfolded structure, a common feature for many antitoxins, is not characteristic for Epsilon, which is folded into a three-helix bundle (Meinhart et al., 2003). As expected for antitoxin proteins, the Epsilon protein has a shorter half-life *in vivo* than the Zeta toxin protein (Camacho et al., 2002). However, *in vitro* analysis showed its higher stability against urea-induced unfolding and higher resistance against unspecific proteolytic degradation in comparison with the Zeta toxin. Therefore, it is quite possible that *in vivo* liability of the Epsilon protein is associated with a specific protease, which degrades this antitoxin under certain cellular conditions. Also it is noteworthy that femtomolar affinity of PezA and PezT, chromosomally encoded homologous PezAT system of *Streptococcus pneumoniae*, is the strongest reported among TA systems (Mutschler et al., 2010). Ambiguous and divergent results obtained by independent groups on *in vivo* degradation of Epsilon are not conclusive. Genetic results (Camacho et al., 2002; Lioy et al., 2006) suggested that in an *E. coli* null *lonA* mutant strain and, to a minor extent, in the null *clpP* cells, the Epsilon antitoxin was not specifically degraded. Our unpublished results show, in turn, that *in vivo* degradation of Epsilon in *B. subtilis* depends mostly on the ClpXP protease. The pleiotropic effect of mutations in the *lonA* and *clpP* proteases-coding genes in *B. subtilis* causes many difficulties both in carrying out and interpreting *in vivo* experiments. Therefore, the stability of the purified Epsilon protein was tested in the presence of crude lysates from several protease-deficient *B. subtilis* strains. The lack of Epsilon degradation was observed only in the *clpP* mutant (unpublished data).

The activity of the *ω*-*ε*-*ζ* cassette was examined in various Gram-positive bacteria with a low DNA G/C content (Brzozowska et al., 2012). As was shown, the efficiency of the cassette functioning differs in the studied strains, but does not depend on the plasmid copy number. Observed differences are probably connected with host-dependent cellular factors. We suppose that one of these factors could be the presence or absence of specific proteases responsible for Epsilon degradation, but this needs to be further explored.

**Concluding remarks**

While proteolysis is a well-studied process for *E. coli* antitoxins, it is generally still weakly described for other bacterial species. Up to now, only one work concerning the involvement of a cellular protease in antitoxin breakdown in Gram positive bacteria has been described. *Staphylococcus aureus*, a major human pathogen, possess three known TA systems: *mazEF, axe1-txe1* and *axe2-txe2*, which are regulated by the ClpPC protease (Donegan et al., 2010). This is the first example of cooperativity between the ClpP protease and ClpC chaperon in antitoxin degradation. It is worth noting that in *S. aureus* the Lon protease is absent. Lack of this protease, which is essential for *E. coli*, could be compensated by two other ATP-dependent proteases, FtsH and HslVU. While little is known about cellular functions of HslVU in *S.* *aureus*, the role of FtsH is connected with osmotic and heat shock tolerance and general cell growth (Lithgow et al., 2004). Some bacterial species, like *Nitrosomonas europeae*, *Sinorhizobium meliloti* and *Mycobacterium bovis,* contain more than 50 putative TA systems in their genome. Proteolytic regulation in species containing numerous TA family members has not been studied yet, but could provide valuable information as to what conditions lead to the activation of these TA systems and what phenotype will occur after their activation. Such studies are relevant, especially in case of human pathogens possessing multiple TA systems. *Mycobacterium* *sp.* possesses several proteases, including ClpXP, but similarly to *S.* *aureus*, its genome does not contain genes encoding an apparent Lon protease. Additionally, the ClpA ATP-dependent subunit is also absent (Cole et al., 1998). As mentioned above, there are cases when two proteases are responsible for antitoxin degradation. One of the explanations of this phenomenon is that under different conditions, e.g. steady state or nutrient stress, different proteases are responsible for activation of a given TA system (Ainzenman et al., 1996; Christensen et al., 2003). It cannot be excluded that a similar correlation could take place more often, especially in bacteria with multiple TA systems.

Various sets of proteases in different bacterial species sharing low homology to *E. coli* proteases, as well as the possible existence of a cofactor modulating the specificity of the ATP-dependent protease are the reason for further investigation of proteolytic activation of TA systems.

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**Fig.1. Regulation of type II TA systems; general scheme.** Toxin and antitoxin are produced from the same operon, which is autoregulated by the TA complex or the antidotum alone. The antitoxin, present in excess, forms a dynamic complex with the toxin. Cellular proteases, Lon or ClpP, degrade the susceptible antitoxin protein, which in consequence (decreased antitoxin level) leads to the appearance of a free toxin form. The uncomplexed toxin can act on a specific cellular target which results in arrest of bacterial cell growth or cell death.

Toxin gene and protein are shown in red, antitoxin gene and protein - in green.

Dashed lines symbolize the possible protease action on the antitoxin complexed with the toxin as well as the reversible toxin-antitoxin complex formation process.

Specific features of different TA systems are not shown.

**Fig. 2. Pictorial view of antitoxin structural changes upon toxin binding.**

A. unstructured C-terminal domain adopts order structure,

B. unstructured C-terminal domain remains disordered but buried inside the toxin,

C. unstructured antitoxin adopts order structure.

Antitoxins are shown as cartoon representation (green), toxins as molecular surface (gray). Fragments of antitoxins critical for presented structural changes are marked in magenta.

**Tab.1. Antitoxins and proteases involved in their degradation.**

Only references relevant for antitoxin proteolysis are indicated.