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Escherichia coli dinJ-yafQ operon shows characteristic features of bacterial toxin–antitoxin modules

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Bacterial toxin–antitoxin (TA) systems are pairs of genes which constitute an operon and code for a stable toxic protein and a labile antitoxin. The antitoxin neutralizes the toxin's inhibitory effect on cell growth and viability via direct protein–protein interactions. TA modules are widespread on the chromosomes of free-living bacteria and archaea, including many pathogens, where they presumably act as stress response elements. The chromosome of *Escherichia coli* K-12 encodes four known TA pairs, as well as the *dinJ-yafQ* operon, which is hypothesized to be a TA module based on similar operon organization to known TA genes. We have cloned the genes from the *dinJ-yafQ* operon of *E. coli* separately and together. Expression of YafQ protein from a low-copy number tightly regulated expression plasmid inhibited cell growth, but did not cause a reduction in viable cell counts. Concomitant expression of DinJ counteracted the toxic effect of YafQ. Thus, *dinJ-yafQ* exhibits the characteristics of an active TA module. Homologues of *E. coli dinJ* and *yafQ* genes were found in a variety of bacterial species. Sequence alignment of DinJ and YafQ homologues and other most similar TA protein pairs from bacteria and archaea has revealed that *E. coli* DinJ and YafQ possess conservative amino acids shown to be important for protein–protein interactions and toxin catalytic activity.

Key words: chromosomal toxin-antitoxin systems, *dinJ-yafQ* operon, *E. coli* stress response

INTRODUCTION

Microorganisms respond and adapt to changing environments in numerous highly sophisticated ways sometimes employing intriguing molecular mechanisms. As it recently has emerged from several studies, bacterial toxin–antitoxin systems (TA) could be involved in stress management by modulating the global level of biological processes such as translation or DNA synthesis [1, 2].

Bacterial TA systems typically consist of two genes organised in an operon which codes for a stable toxin and a proteolytically labile antitoxin. The toxin protein can inhibit essential cellular processes causing cell death or bacteriostasis while the antitoxin binds to the toxin and blocks its activity by forming an inhibitory complex [3]. TA systems initially have been found located on low-copy plasmids of *E. coli* and other bacteria [4]. Extra-chromosomally encoded TA components are responsible for the post-segregational killing effect resulting in death of cells that have lost the plasmid. In the absence of plasmid-directed *de novo* protein synthesis in a daughter cell, antitoxin is degraded faster by cellular proteases than the more stable toxin, allowing the toxin to kill the cell. The plasmid-borne TA systems have been named "addiction modules" as they cause cells to be "addicted" to the short-lived antitoxin product, because its synthesis is essential for cell survival [5].

Over the recent years, pairs of genes homologous to some of extra chromosomally borne addiction modules have been found in the chromosome of *E. coli* and in the genomes of other bacteria and archaea [6]. Chromosomal TA systems have a characteristic organisation where the antitoxin gene precedes the toxin and both genes are co-expressed [1]. Toxins are basic proteins (11–12 kDa), whereas antitoxins are acidic and highly unstable (9 kDa).

There are at least five structurally similar *E. coli* chromosome-encoded TA loci: *relBE, mazEF, chpBIK, dinJ-yafQ and yefM-yoeB* [1]. The *dinJ-yafQ* and *yefM-yoeB* systems are most homologous to *relBE*, whereas *chpBIK* is homologous to *mazEF* [1].

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Table. **Bacterial strains and plasmids**

Strain / plasmid	Genotype / description	Reference / source
BW25113	Δ(*araD-araB*)567 Δ*lacZ*4787(::*rrnB*-4) *lacI*p-400(*lacI*^{Q}) λ^{-} *rpoS*396(Am) *rph*-1 Δ(*rhaD-rhaB*)568 *rrnB*-4 *hsdR*514	[21]
pBAD30	Expression plasmid	[12]
pBAD30-*dinJ*	620 bp DNA fragment, containing *dinJ* gene, restricted with *Eco*88I and cloned into pBAD30 *Hinc*II from plasmid pUC-*dinJ* [13]	This work
pBAD30-*yafQ*	362 bp PCR fragment with *yafQ* gene cloned into pBAD30	This work
pBAD30-*dinJ yafQ*	930 bp PCR fragment with *dinJ yafQ* operon cloned into pBAD30	This work
SC37	MG1655 Δ(*dinJ-yafQ*)	[22]
MG1655	F^{-} λ^{-} *ilvG*$^{-}$ *rfb*-50 *rph*-1	*E. coli* Genetic Stock Center, CGSC 6300

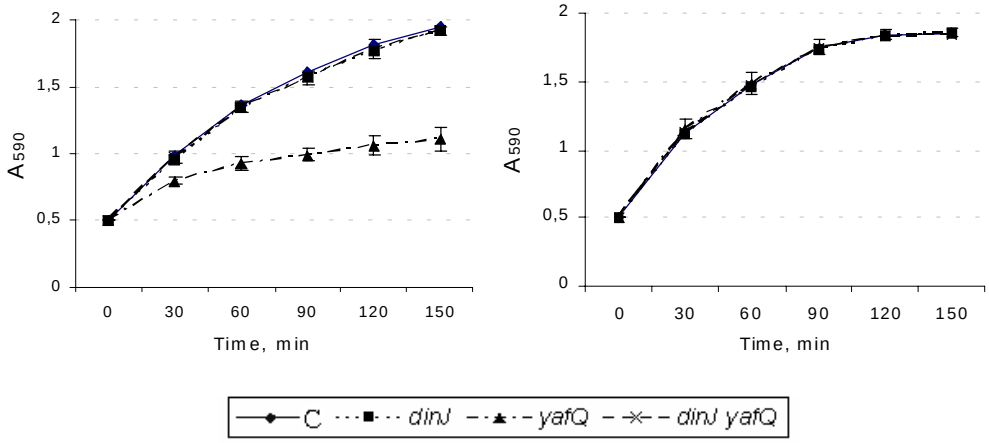

Fig. 1. Effect of extrachromosomal expression of *dinJ yafQ* operon genes on bacterial growth in liquid medium. Strains BW25113 pBAD30 (control, ♦), BW25113 pBAD-*dinJ* (■), BW25113 pBAD-*yafQ* (▲) and BW25113-*dinJ yafQ* (×) were grown in LB medium until mid-exponential phase and shifted to medium with (A) 0.2% L-arabinose or (B) 0.2% D-glucose. Growth was determined by measuring A_{590} every 30 minutes after addition of sugar. The values are means of three independent experiments. Bars indicate SD

The best studied TA systems are *mazEF* and *relBE*. It has been recently demonstrated that RelE, MazF, ChpBK, YoeB toxins inhibit the translation process by cleavage of translated mRNAs at specific codons [7–10]. Translation inhibition by mRNA cleavage by RelE and MazF is induced during nutritional stresses such as amino acid and glucose starvation [9]. Inhibition of translation and colony formation caused by overexpression of MazE or RelE toxins is reversed by the subsequent expression of their cognate toxins [11]. These observations propose a model in which chromosome-borne TA systems such as *mazEF* and *relBE* might function as modulators of metabolic processes in response to environmental stress [5].

E. coli dinJ-yafQ operon codes for a protein pair with the highest homology to the bacterial *relBE* TA system [1]. Despite its similar genomic organisation and some sequence homology to chromosomal TA systems, neither molecular characterization nor functional similarity to bacterial TA systems have been reported for *the dinJ-yafQ* locus so far.

Here we show that the *E. coli dinJ-yafQ* system exhibits characteristic features common to other known bacterial TA systems. Ectopic expression of YafQ protein inhibited cell growth, whereas concomitant expression of putative DinJ antitoxin counteracted the inhibitory effect of YafQ. Protein homologues of *E. coli dinJ-yafQ* system are present on the genomes of enterobacteria and less related bacterial species. DinJ and YafQ proteins preserve conservative amino acids shown to be important for TA complex formation and ribonuclease catalytic site of toxin proteins of related TA systems.

MATERIALS AND METHODS

All the materials listed below were purchased from Sigma, Merck and Roth. Kits for molecular biology were from AB Fermentas. All enzymes were used as recommended by the supplier.

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1.

DNA manipulation and plasmid construction. 362 and 930 bp DNA fragments containing *yafQ* and both *dinJ* and *yafQ* genes, respectively, were PCR-amplified from *E. coli* genomic DNA with the primer pairs YafQ3 (5'-ctggatttggaaagcttacat-3') / YafQ4 (5'-catgattcaaagggatattga-3') for *yafQ* and YafQ1 (5'-tcacattatcaccaaaagag-3')/ DinJ2 (5'-gtagctgaaagagatatgtg-3') for *dinJ yafQ*. The *dinJ yafQ* fragment was ligated into the pBAD30 vector [12] through the *Sma*I site, producing the plasmid pBAD-*dinJ yafQ*. The *yafQ* fragment was firstly ligated into the pUH25-2 vector through the *Pae*I and *Hind*III sites. A *Hind*III site was introduced into the DNA fragment with the primer YafQ3 (underlined in the primer sequence). The resulting plasmid was cut with *Eco*RI and *Hind*III to give a fragment in which *yafQ* was preceded by the SD sequence from pUH25-2. The 376 bp fragment was then ligated into pBAD30, producing the plasmid pBAD-*yafQ*.

The 620 bp DNA fragment containing *dinJ* gene was restricted with *Eco*88I and *Hinc*II from the plasmid pUC-*dinJ* described elsewhere [13] and ligated into pBAD30, resulting in the plasmid pBAD-*dinJ*.

Growth rate analysis. ***Liquid medium***. *E. coli* cells harboring either of the plasmids pBAD30, pBAD-*dinJ*, pBAD-*yafQ* or pBAD-*dinJ yafQ* were grown at 37 °C in LB medium with 0.2% D-glucose to an A_{590} = 0.5. At this point, the culture was divided into two equal parts. The growth medium of one part was changed into fresh LB medium containing 0.2% D-glucose and that of the other into fresh LB medium containing 0.2% L-arabinose. Incubation was continued at 37 °C. Bacterial growth was assessed by measuring A_{590} every 30 minutes.

Solid medium. *E. coli* cells, harboring the same recombinant plasmids as above, were grown at 37 °C in LB medium with 0.2% D-glucose to an A_{590} = 0.5. At this point, cells were washed in 0.9% NaCl solution, diluted 10^6 times and plated on solid LB medium plates with increasing concentrations of L-arabinose (0%; 0.0002%; 0.002%; 0.02%; 0.2%). The plates were incubated at 37 °C overnight. Bacterial growth was assessed by visually inspecting the size of the colonies.

Assays for *E. coli* growth and survival at different stresses. The *E. coli* strains MG1655 and MG1655 Δ(*dinJ-yafQ*) (Table 1) were tested for growth in carbon and nitrogen starvation conditions and for survival of heat and oxidative stress. To assess growth under carbon and nitrogen limitation, cells were grown in minimal medium [14] supplemented with 0.2% or 0.002% of glucose (carbon starvation) and 0.1% or 0.02% of casamino acids (nitrogen starvation). Cell growth was monitored at A_{600} for 24 hours. For studies involving heat stress, cells were grown at 37 °C in LB medium to an A_{590} = 0.5 and then subjected to heat shock at 42 °C for different time periods. Ten-fold PBS dilutions of cell cultures were plated on solid LB medium plates. After 12 hours of incubation at 37 °C, viable colonies were counted. For oxidative stress assay, cells were grown at 37 °C in LB medium to an A_{590} = 0.5, then aliquots of H_2O_2 were added to the final concentrations 5 mM and 10 mM. Cells were incubated for different time periods, plated on solid medium and grown as described above.

RESULTS AND DISCUSSION

Cloning and expression of *E. coli dinJ yafQ* operon genes. The organization of *E. coli dinJ-yafQ* operon is similar to that of other known bacterial TA operons: *relBE*, *mazEF*, *chpBIK* and *yefM-yoeB*. The first gene in the operon, *dinJ*, codes for a small acidic protein (pI = 5.2) with a deduced molecular mass of 9.4 kDa. The second gene, *yafQ*, codes for a basic protein (pI = 9.2) with a deduced molecular mass of 10.8 kDa. Co-expression of both genes was confirmed by detection of ~620 nt length mRNA by Northern blot analysis using a *dinJ* DNA fragment as a probe (data not shown). The small size and basic / acidic features of the YafQ and DinJ proteins resemble those of known *E. coli* chromosome toxin / antitoxin protein pairs. The DinJ and YafQ proteins exibit a low but significant similarity to the *E. coli* RelB-RelE system. Based on the similarity among the toxin proteins, the RelBE, DinJ-YafQ and YefM-YoeB systems were grouped into a separate TA gene family [1]. To characterize the DinJ and YafQ proteins, we cloned the *dinJ* and *yafQ* genes either individually or together. *E. coli* DNA corresponding to the whole *E. coli* K-12 *dinJ yafQ* operon and the separate genes *dinJ* and *yafQ* were cloned into the low-copy plasmid pBAD30 under a regulatable arabinose promoter as described in Materials and Methods and transformed into the *E. coli ara*⁻ strain BW25113 (Table 1). The *dinJ-yafQ* operon genes and *dinJ* gene in plasmids pBAD-*dinJ yafQ* and pBAD-*dinJ*, respectively, are preceded by the native SD sequence, whereas the *yafQ* gene cloned into plasmid pBAD is preceded by a SD of an expression plasmid. DNA inserts in the plasmids pBAD-*dinJ yafQ*, pBAD-*dinJ*, pBAD-*yafQ* were verified by DNA sequencing.

Expression of *E. coli yafQ* inhibits cell growth. Overexpression of RelE, MazF, ChpBK and YoeB toxins of *E. coli* chromosomal TA loci inhibited cell growth and caused reduction in viable cells counts. When these toxin proteins were overexpressed together with their cognate antitoxins, no inhibitory effect was observed due to formation of stable toxin / antitoxin complex [8, 10, 11, 15]. To determine whether *E. coli* YafQ is toxic when produced indepen-

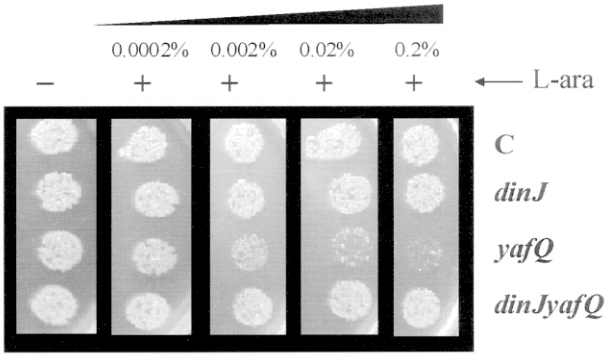

Fig. 2. Effect of extrachromosomal expression of *dinJ yafQ* operon genes on bacterial growth on solid medium. Strains BW25113 pBAD30 (control, C), BW25113 pBAD-*dinJ* (*dinJ*), BW25113 pBAD-*yafQ* (*yafQ*) and BW25113-*dinJ yafQ* (*dinJ yafQ*) were grown in liquid LB medium until mid-exponential phase and plated on solid LB medium plates with increasing concentrations of L-arabinose

dently of DinJ, we grew cultures of BW25113 strain transformed with pBAD-*dinJ*, pBAD-*yafQ*, pBAD-*dinJ yafQ* and pBAD30 vector alone in LB with ampicillin (100 μg/ml) until mid-exponential phase. Then cells were shifted to fresh medium containing either arabinose or glucose (0.2%) and optical density was monitored over the course of 2.5 hours. As can be seen in Fig. 1, A, the presence of arabinose caused a marked inhibition of pBAD-*yafQ* strain growth, but did not affect the pBAD-*dinJ yafQ*, pBAD-*dinJ* and control (pBAD30) strains. The toxic effect was due to overproduction of pBAD-*yafQ* derived protein, since the inhibition of expression from P_{ara} by glucose did not affect bacterial growth in either of the strains tested (Fig. 1, B). The co-expression of the *E. coli* DinJ protein in pBAD-*dinJ yafQ* strain counteracted the toxicity of YafQ, indicating that similarly to the known TA pairs, DinJ might prevent the cellular activity of YafQ.

Next we examined the effect of the expression of YafQ on colony formation ability. Strain BW25113 with one of the plasmids pBAD-*yafQ*, pBAD-*dinJ*, pBAD-*dinJ yafQ* and pBAD30 was grown in LB until mid-exponential phase and plated on solid medium containing different concentrations of arabinose as described in Materials and Methods. Again, a strong inhibitory effect on cell growth on solid medium was observed in the pBAD-*yafQ* plasmid strain. (Fig. 2.). Growth inhibition was observed starting from the 0.002% concentration of arabinose and increased with increasing the concentrations of the inductor. It must be noted that overproduction of *E. coli* YafQ at the presently used conditions caused growth inhibition rather than loss of cell viability. Overproduction of *E. coli* RelE, MazF and ChpBK toxins resulted in inhibition of colony formation [9, 16]. Thus YafQ could be less detrimental to the cell than toxins of other bacterial TA loci. Nevertheless, the results presented above argue that YafQ could act as an active toxin. YafQ activity could by pre-

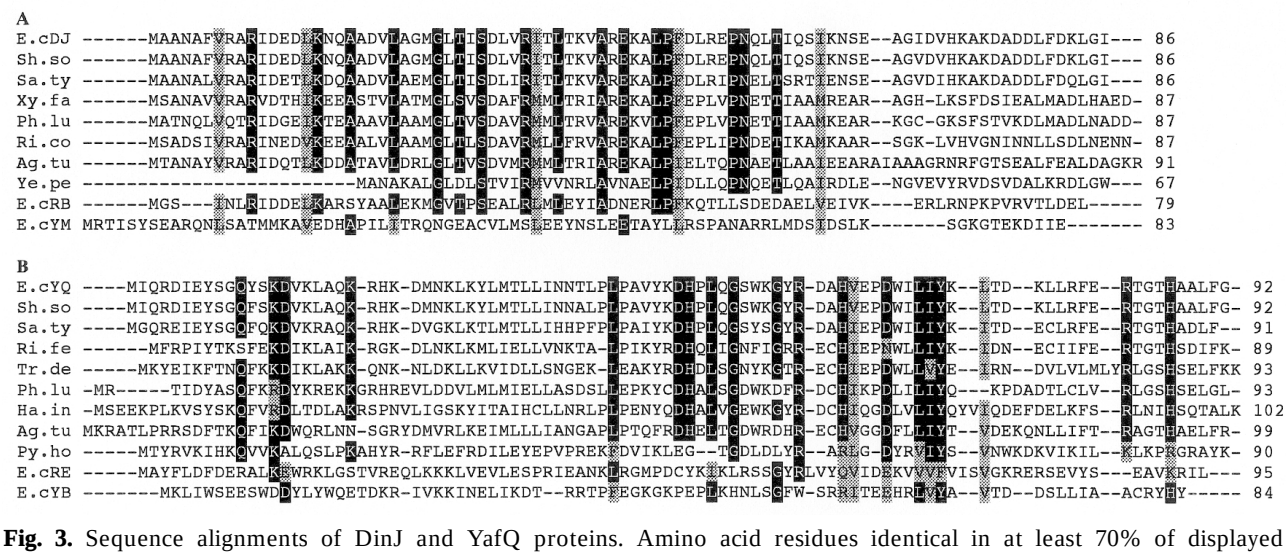

Fig. 3. Sequence alignments of DinJ and YafQ proteins. Amino acid residues identical in at least 70% of displayed sequences are shaded in black, conserved amino acid substitutions are shaded in gray. (A) Sequence alignments of DinJ antitoxin and its homologues: E.cDJ – DinJ of *E. coli*, Sh.so – DinJ of *Shigella sonnei*, Sa.ty – STM3517 gene product of *Salmonella typhimurium*, Xy.fa – RelB of *Xylella fastidiosa*, Ph.lu – DinJ of *Photorhabdus luminescens*, Ri.co – DinJ of *Rickettsia conorii*, Ag.tu – Atu0935 gene product of *Agrobacterium tumefaciens*, Ye.pe – RelB of *Yersinia pestis*, E.cRB – RelB of *E. coli*, E.cYM – YefM of *E. coli*. (B) Sequence alignments of YafQ toxin and its homologues: E.cYQ – YafQ of *E. coli*, Sh.so – YafQ of *Shigella son*nei, Sa.ty – STM3516 gene product of *Salmonella typhimurium*, Ri.fe – RF_0334 gene product of *Rickettsia felis*, Tr.de – TDE0505 gene product of *Treponema denticola*, Ph.lu – YafQ of *Photorhabdus luminescens*, Ha.in – protein HI0711 of *Haemophilus influenzae*, Ag.tu – Atu0934 gene product of *Agrobacterium tumefaciens*, Py.ho – PHS013 gene product (aRelE) of *Pyrococcus horikoshii*, E.cRE – RelE of *E. coli*, E.cYB – YoeB of *E. coli*. Sequences were aligned using Clustal W version 1.82 [23]

vented by interaction with DinJ. This hypothesis is supported by our observation that both YafQ and DinJ proteins could be trapped on Ni^{2+} resin from the extracts of IPTG-induced pET-*dinJ yafQ* His cells (Suþiedëlienë et al., unpublished).

Amino acid sequence aligment of DinJ, YafQ and RelBE family proteins. Chromosomal TA loci are surprisingly abundant in free-living microorganisms, including archaea. According to their similarity, TA systems have been grouped into seven TA gene families [6]. The *relBE* family, which includes *dinJ-yafQ* gene loci, is one of the most abundant. The family members are present in bacteria and archaea [6]. We performed a search for *E. coli* DinJ and YafQ protein homologues in the databases (Fig. 3). Members of RelBE family proteins encoded by bacterial *relBE*, *yefM-yoeB* locus and archaeal *relBE* locus were included into the alignment. As one can see in Fig. 3, A, conservative amino acids of DinJ homologues are clustered in the N-terminal part of the proteins, whereas the C-terminal part exhibits a weak degree of conservation. The N-terminal residues 5–25 of *E. coli* DinJ protein are predicted to form an α-helix structure which contains several conserved hydrophobic amino acids (leucine (L) and isoleucine (I)). A recent structural analysis of *E. coli* RelB-RelE homologs from the hyperthermophilic archaeon *Pyroccocus horikoshii* revealed a heterotetrameric structure of the complex formed by two RelB and two RelE monomers [17]. Surprisingly, the *P. horikoshii* antitoxin RelB appears to have a definite tertiary structure only when bound to the toxin. However, the presence of the hydrophobic residues 8–28 in the N-terminal part of RelB suggests that they might participate in hydrophobic contacts during RelB dimer formation [17]. Other members of the *relBE* gene family, *E. coli* YefM protein dimer and YeoB protein monomer, interact by forming a heterotrimeric complex in a manner different from that observed for *P. horikoshii* RelB-RelE [18].

Of the chromosomal relBE family toxins, *E. coli* RelE, YoeB, ChpBK and *P. horikoshii* RelE have been shown to be sequence-specific endoribonucleases [1, 10, 17]. MazF toxin, encoded by *mazEF* family TA loci, exhibits a similar activity [8]. Toxins act by cleaving mRNA at specific codons and inhibit translation in response to nutritional starvation and presumably to other stress conditions [1]. Site-directed mutagenesis of *P. horikoshii* RelE and *E. coli* YoeB toxins revealed amino acids essential for their catalytic activity [17, 18]. Mutation of Arg85 of *P. horikoshii* RelE (shown in Fig. 3, B) resulted in a complete loss of inhibition of protein synthesis. Mutations at positions Arg40, Leu48, Arg58 and Arg65 significantly reduced the inhibitory activity of RelE [17]. The residues critical to RNase activity of YoeB were found to be Glu46, Arg65, His83 and Tyr84 [18]. In the microbial RNase reaction mechanism, histidine and glutamic acid residues participate as general acid and base groups in the catalytic reaction. Arginine is important for binding the reactive phosphate [19]. Residues important for *E. coli* YoeB RNase activity, except for Arg65, are not conserved in RelE toxin (Fig. 3). However, on the basis of the results of YoeB and RelE structural analysis, it has been proposed that RelE has a microbial RNase fold similar to YoeB [18]. Notably, His83 important for YoeB activity is also highly conserved in *E. coli* YafQ protein (His87) and its homologues (Fig. 3, B). The conserved residues that could potentially serve for YafQ RNase activity are located at different positions as compared to *E. coli* YoeB (*E. coli* YafQ D68 and R83, Fig. 3, B). It was proposed that YafQ could posses a YoeB-like RNase fold, although it could have a different type of catalytic site [18].

Response of *E. coli dinJ-yafQ* TA locus to stress. It has been shown that nutritional stress, such as amino acid and glucose starvation, activates *E. coli* RelE and MazF toxins [1]. Upon entering a stress condition, cellular proteases such as Lon or Clp degrade antitoxins [9, 16]. Whether this degradation requires an external signal and how the antitoxin becomes released from TA complex under certain stress condition is not known. The more stable toxins come to action and inhibit protein synthesis by cleaving mRNA. *E. coli* strains lacking functional TA loci such as *mazEF* are less sensitive to a high temperature, oxidative stress and DNA damage [20]. The inhibitory effect of toxins on cell growth could be reversed if their cognate antitoxins are expressed at a later time [11]. Therefore toxins display a bacteriostatic rather than a bactericidal effect. TA loci have been suggested to play a role in the quality control of gene expression under growth-limiting conditions [1].

To find out whether *E. coli dinJ-yafQ* TA locus participates in bacterial stress responses, we tested the *E. coli* strain harboring a *dinJ-yafQ* deletion and its parent MG1655 for growth and viability under various stress conditions. Hovewer, starvation for glucose and nitrogen did not influence cell growth in these strains over the period of 25 hours (data not shown). Similarly, we have observed no differences in viability between *dinJ-yafQ* deletion strain and its parent strain subjected to heat and oxidative stress (see Materials and Methods). Therefore, the stress conditions when *dinJ-yafQ* TA loci becomes important, if any, remain to be elucidated.

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Escherichia coli dinJ-yafQ OPERONO TOKSINØ-ANTITOKSINØ SISTEMØ SAVYBËS

S a n t r a u k a

Bakterijø toksino-antitoksino (TA) sistemas koduoja genø poros, kurios sudaro operonà. Operono genai koduoja stabilø baltymà toksinà ir labilø baltymà antitoksinà. Antitoksinas neutralizuoja toksino poveiká làstelës augimui ir gyvybingumui sudarydamas su juo kompleksà. Toksino-antitoksino sistemos yra plaèiai paplitusios bakterijø ir archëjø chromosomose. *Escherichia coli* K-12 genomas koduoja keturias toksino-antitoksino sistemas ir *dinJ-yafQ* operonà, kuris, remiantis jo struktûrine organizacija bei koduojamø baltymø homologija, gali pasiþymëti toksino-antitoksino sistemos savybëmis. Šiame darbe klonavome *dinJ-yafQ* operono genus kartu ir atskirai á reguliuojamà, maþai kopijø turintá baltymø ekspresijos vektoriø ir tyrëme, kokià átakà turi DinJ YafQ baltymø sintezë bakterijø augimui ir gyvybingumui. YafQ baltymo sintezë stipriai inhibavo bakterijø augimà, bet neturëjo átakos jø gyvybingumui. DinJ baltymo sintezë kartu su YafQ baltymu, taip pat tik DinJ baltymo sintezë neturëjo átakos làsteliø augimui ir gyvybingumui. DinJ baltymas neutralizavo YafQ baltymo slopinantá poveiká augimui, ir tai liudija, kad *E. coli dinJ-yafQ* gali koduoti toksino-antitoksino sistemà. Palyginus *E. coli* DinJ ir YafQ baltymus su homologiðkais bakterijø ir archëjø baltymais, nustatyta, kad *E. coli* DinJ ir YafQ turi konservatyviø aminorûgðèiø, kurios yra svarbios baltymø tarpusavio sàveikai ir katalitiniam antitoksino baltymo aktyvumui.