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Bacterial Toxin-Antitoxin Gene System as Containment Control in Yeast Cells

P. KRISTOFFERSEN,[1] G. B. JENSEN,[2] K. GERDES,[3] AND J. PIŠKUR[1]*

Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby,[1] National Institute of Occupational Health, DK-2100 Copenhagen Ø,[2] and Department of Molecular Biology, Odense University, DK-5230 Odense M,[3] Denmark

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The potential of a bacterial toxin-antitoxin gene system for use in containment control in eukaryotes was explored. The *Escherichia coli relE* and *relB* genes were expressed in the yeast *Saccharomyces cerevisiae*. Expression of the *relE* gene was highly toxic to yeast cells. However, expression of the *relB* gene counteracted the effect of *relE* to some extent, suggesting that toxin-antitoxin interaction also occurs in *S. cerevisiae*. Thus, bacterial toxin-antitoxin gene systems also have potential applications in the control of cell proliferation in eukaryotic cells, especially in those industrial fermentation processes in which the escape of genetically modified cells would be considered highly risky.

Genetically modified microorganisms (GMMs) used in the biotechnological industry are normally kept physically closed off from their surroundings. The strains used are attenuated and will not survive very long if they escape into the environment. In recent years there has been a growing interest in the deliberate release of GMMs into the environment. GMMs suitable for release could, for example, be used for bioremediation of polluted soils, for biocontrol of fungicidal and insecticidal pests in agriculture, or as live vaccines in biomedicine. Upon release, such strains must be able to proliferate and compete with the indigenous strains present. However, to ensure safety, uncontrolled spread of GMMs into the environment must be prevented. Safety can be achieved through biological containment if the GMMs self-destruct by expression of killing genes after fulfilling their jobs. Several bacterial toxins are good candidates for use in bacterial containment systems, including membrane-destabilizing or pore-forming proteins (4, 15) and enzymes attacking the genetic material of the cell (1, 2, 7). The design and applications of active biological containment systems are reviewed and discussed in several publications (8, 9, 12, 13).

Recently, *relBE*, members of a new toxin-antitoxin gene family, have been found in *Escherichia coli* (5). To date, *relBE* homologues have been identified in a broad range of both gram-negative and gram-positive bacteria and in archaea (5, 6). The *relE* gene encodes a small (11-kDa) protein that is extremely toxic to bacterial cells, and the *relB* gene encodes an antitoxin of similar size that counteracts the cell killing activity of the RelE toxin (5, 6). The specific molecular targets of the RelE protein, as well as the physiological role of the RelE-RelB toxin-antitoxin system in bacteria, is still speculative (3, 5). So far, no *relBE* homologues have been found in eukaryotes.

In the study described here, we analyzed whether this toxin-antitoxin gene system also could be used to control proliferation of eukaryotic cells. For this purpose, we used *Saccharomyces cerevisiae* as a general model for eukaryotes, specifically fungi. We showed that expression of *relE* strongly inhibits the growth of yeast cells and that the products of *relE* and *relB* interact.

Strains and media. *E. coli* TOP10 (Invitrogen) was routinely used during vector constructions. The bacteria were maintained and grown in Luria-Bertani medium (14) supplemented with ampicillin (100 μg/ml). The yeast strain used was *S. cerevisiae* 281288DIV-36 (*MAT* **a** *his4-15 ura3-52 trp1*; Y493 from our laboratory collection). Yeast strains were transformed as described previously (11). Transformed yeast cells were grown in liquid or solid SC−ura (synthetic complete medium without uracil and with 2% glucose), SC−ura+gal (synthetic complete medium without uracil and with 2% galactose), SC−ura−met (synthetic complete medium without uracil and methionine and with 2% glucose), or SC−ura−met+gal (synthetic complete medium without uracil and methionine and with 2% galactose) (11). When necessary, the media were solidified by addition of agar to 2% (wt/vol).

Construction of a vector for expression of the RelE toxin in *S. cerevisiae*. The DNA manipulations were performed according to standard methods (14). All PCR amplifications (20 cycles consisting of 40 s of denaturation, 40 s of annealing, and 1 min of extension) were performed with Vent DNA polymerase (New England Biolabs) using a PTC-100 thermocycler (MJ Research Inc.). After agarose gel electrophoresis, amplified fragments were isolated using a DNA purification kit from Qiagen.

The coding region of the *relE* gene from *E. coli* was PCR amplified from pMG223 (5) by using the sense primer 5′-TA GGTACCATGGCGTATTTTCTGG-3′ and the antisense primer 5′-TGAATTCCTCGACTCAGAG-3′. *Kpn*I and *Eco*RI restriction enzyme recognition sites, which were added at the 5′ ends of the sense and antisense primers, respectively, are underlined. The PCR product was inserted into the *Kpn*I-*Eco*RI site of the polylinker of the yeast expression vector pYES2 (Invitrogen) to yield the plasmid pPK727.

Construction of a vector for expression of the RelE-RelB toxin-antitoxin in *S. cerevisiae*. A modified version of the pYES2 expression vector was constructed by removing the *GAL1* promoter and inserting the methionine (*MET25*) promoter from *S. cerevisiae*. P_{MET25} was amplified from pYC012 (10) by PCR using the forward primer 5′-AGACTAGTCCCG GGCTTAATTAAATAATATAC-3′ and the reverse primer

* Corresponding author. Mailing address: Department of Microbiology, Technical University of Denmark, Building 301, DK-2800 Lyngby, Denmark. Phone: (45) 4525 2518. Fax: (45) 4593 2809. E-mail: imjp@pop.dtu.dk.

5′-AGACTAGT*GGATCC*TGTATGGATGGGGGTA-3′. *Spe*I restriction enzyme recognition sites added at the 5′ ends of both primers are underlined, and the *Bam*HI site added in the reverse primer is in italics. P_{GAL1} was removed from pYES2 as an *Spe*I fragment, and the *Spe*I-digested PCR fragment was inserted into the *Spe*I site of this vector, yielding plasmid pPK908. Twenty-three nucleotides at the 5′ end of the PCR product were removed because of the presence of an *Spe*I restriction site in P_{MET25}. This deletion did not appear to have any significant effect on the promoter activity. The correct orientation of the inserted promoter was verified by restriction enzyme digestions.

The region of pPK727 including the *relE* gene under the control of the *GAL1* promoter and the *CYC1* transcriptional terminator was amplified by PCR with the following set of forward and reverse primers to introduce *Cla*I restriction sites (underlined): 5′-AGATCGATTACAGGGCGCGTGGGGA TGATC-3′ and 5′-AGATCGATAATACGCAAACCGCCTC TCCCC-3′. The amplified PCR product was inserted into the unique *Cla*I site of pPK908 to yield plasmid pPK988.

The coding region of the *relB* gene from *E. coli* was amplified from plasmid pBD2430 by PCR (5) using the sense primer 5′-TAGGTACCATGGGTAGCATTAACC-3′ and the antisense primer 5′-ATGGATCCTCAGAGTTCATCCAGC-3′. Each of these primers possesses a *Bam*HI site at its 5′ end (underlined). The amplified *relB* coding region was inserted into the *Bam*HI site of pPK988 downstream of the *MET25* promoter, yielding plasmid pPK1006. The orientation of *relB* was verified by restriction enzyme digestions, and the DNA sequence of the PCR product of *relB* was verified by DNA sequence analysis.

Expression of *relE* in *S. cerevisiae*. Yeast cells transformed with either plasmid pPK727 (containing *relE* under the control of the *GAL1* promoter) or the pYES2 vector were grown overnight in SC−ura, then diluted 1:100 in SC−ura or SC−ura+gal. Growth experiments were performed in 250-ml bottles with 20-ml cultures at 28°C with heavy agitation. The presence of glucose repressed the *relE* gene. Expression of *relE* from the *GAL1* promoter in pPK727 was induced by galactose when the SC−ura was replaced by SC−ura+gal. A clear inhibitory effect on cell growth in SC−ura+gal was observed for yeast cells containing the pPK727 plasmid, whereas no such inhibition was observed for yeast cells containing the empty pYES2 vector (Fig. 1). The same inhibitory effect was also observed when pPK727 was introduced into other yeast strains (data not shown).

Thus, the product of the *relE* gene is also active in *S. cerevisiae*. The *relE* gene could be maintained in yeast cells and induced when necessary, leading to cell death. However, the inhibition was not complete, despite the very peculiar appearance of the yeast cells, and it seemed that some of the cells were still growing, although extremely slowly (Fig. 1).

Expression of *relE-relB* in *S. cerevisiae*. Yeast cells transformed with plasmid pPK1006 (containing *relE* under the control of the *GAL1* promoter and *relB* under the control of the *MET25* promoter) were studied to see if there were interactions between the two proteins. The overnight culture was diluted 1:100 in four different growth media, SC−ura, SC−ura−met, SC−ura+gal, and SC−ura−met+gal. Growth experiments were performed in 250-ml bottles with 20-ml cultures at 28°C with heavy agitation. Expression of *relE* was induced by galactose (in SC−ura+gal and SC−ura−met+gal) but repressed by the presence of glucose. Expression of *relB* was induced in the absence of methionine (in SC−ura−met and SC−ura−met+gal) but repressed in the presence of methionine. Cells grown in SC−ura−met+gal showed higher growth rates than cells grown in the corresponding medium supplemented with methionine (Table 1). These results indicate that expression of *relB* counteracts the growth-inhibiting effect of the *relE* gene to some extent. A difference in doubling time was also observed for cells grown in SC−ura or SC−ura−met and can be explained by the fact that small amounts of the RelE toxin are synthesized also in the absence of galactose, due to a leaky activity of the uninduced *GAL1* promoter. This growth-inhibiting effect was counteracted by the action of the RelB protein in SC−ura−met (Table 1). The relatively small difference in doubling time between yeast cells grown in SC−ura−met+gal and those grown in SC−ura+gal suggested that either the level of *relB* expression was too low to fully counteract the toxic effect of the induced *relE* expression or the RelB-RelE interaction in the yeast cell was very weak.

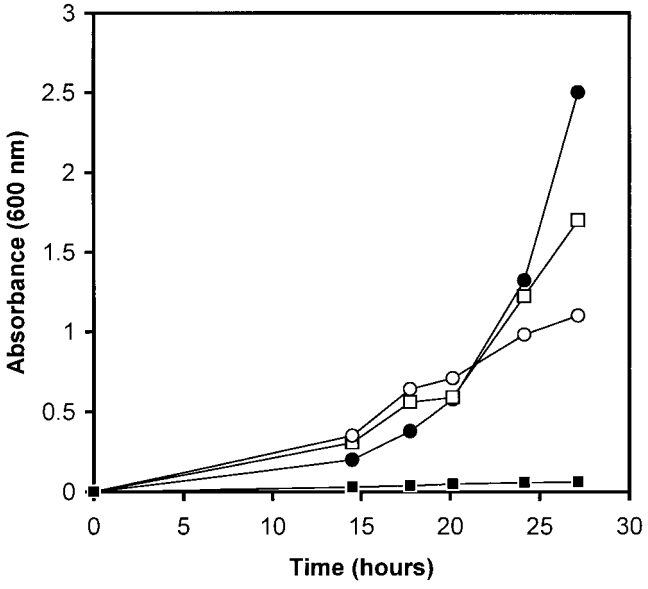

FIG. 1. Effect of *relE* expression, under the regulation of the *GAL1* promoter, on cell growth of *S. cerevisiae*. Cells harboring pPK727 were grown in SC−ura (□) or SC−ura+gal (■), and cells harboring pYES2 as a control were grown in SC−ura (○) or SC−ura+gal (●).

Conclusions. The data presented here clearly demonstrate that expression of the bacterial *relE* gene is also toxic to *S. cerevisiae* cells. The fact that the growth rates of *relB*-expressing cells are higher than those of non-*relB*-expressing cells suggests that both gene products function in *S. cerevisiae*. Thus, bacterial toxin-antitoxin systems can also be applied in eukaryotic cells. It is likely, even if so far it cannot be demonstrated directly, that the actions of the two genes occur at the protein

TABLE 1. Effect of expression of *relE* and *relB* on the growth rate of *S. cerevisiae* harboring plasmid pPK1006[a]

Growth medium	Induced gene(s)	Doubling time (h)
SC−ura−met	P_{MET25}-*relB*	3.5
SC−ura	None	5.5
SC−ura−met+gal	P_{MET25}-*relB*, P_{GAL1}-*relE*	13.0
SC−ura+gal	P_{GAL1}-*relE*	20.0

[a] pPK1006 carries the *relE* gene, induced in the presence of galactose, and the *relB* gene, induced in the absence of methionine.

level. The absence of a complete counteraction of the activity of *relE* by *relB* in SC−ura−met+gal could be partially reversed if a stronger promoter controls the *relB* gene, leading to overproduction of antitoxin compared to toxin. So far, it is not known whether several RelB molecules are required to counteract a single RelE molecule. Further experiments, in which the expression level of RelB is substantially higher than that of RelE, might demonstrate a total counteraction of RelE by RelB. Such data would support the hypothesis that the molecular targets for the RelE and RelB proteins in bacteria and *S. cerevisiae* are the same.

The *relE-relB* toxin-antitoxin genes, as well as similar toxin-antitoxin pairs, could be used as part of a containment system in genetically modified yeasts as well as other fungi. For example, the *relE* gene under the control of the glucose-repressed promoter could be used as a containment control in those industrial fermentation processes in which the escape of genetically modified yeast cells would be considered highly risky. Under fermentation conditions, the *relE* gene is suppressed by high levels of glucose, and if yeast cells escape from the fermentation tank they will self-destruct upon derepression of *relE* because of the extremely low levels of glucose in the environment. Because of the leakiness of the *GAL1* promoter, the *relB* gene could be constitutively expressed at a low level to ensure optimal growth under repressed conditions. Interestingly, it was previously demonstrated that expression of the *relE* gene in a mammalian cell line also led to inhibition of cell proliferation (K. Gerdes, M. Gotfredsen, H. Grøndlund, K. Pedersen, and P. Kristoffersen, U.S. patent application USSN 60/085067). Experiments analyzing the applications of the RelE-RelB toxin-antitoxin gene system for gene and cancer therapy are also in progress.

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