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In Vivo Interactions between Toxin-Antitoxin Proteins Epsilon and Zeta of Streptococcal Plasmid pSM19035 in *Saccharomyces cerevisiae*▽†

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The widespread prokaryotic toxin-antitoxin (TA) systems involve conditional interaction between two TA proteins. The interaction between the Epsilon and Zeta proteins, constituting the TA system of plasmid pSM19035 from *Streptococcus pyogenes*, was detected in vivo using a yeast two-hybrid system. As we showed using *Saccharomyces cerevisiae*, the Zeta toxin hybrid gene also exerts its toxic effects in a dose-dependent manner in eukaryotic cells. Analysis of mutant proteins in the two-hybrid system demonstrated that the N-terminal part of Zeta and the N-terminal region of Epsilon are involved in the interaction. The N-terminal region of the Zeta protein and its ATP/GTP binding motif were found to be responsible for the toxicity.

Toxin-antitoxin (TA) loci are very frequently found in prokaryotic genomes (for reviews, see references 6, 21, and 37). All these systems have similar genetic organization (in the form of an operon with the first cistron encoding an antitoxin and the second a toxin) and similar functioning features: (i) the presence of the antitoxin in excess to the toxin in bacterial cells; (ii) autoregulation on the transcriptional level; (iii) shorter half-life times of the antitoxins than of the toxins due to degradation by cellular proteases; and (iv) the formation of tight complexes between the antitoxins and inactivated toxins. TA systems present on low-copy-number plasmids enhance plasmid maintenance by postsegregational killing of plasmid-free cells (20, 26, 49). In contrast, the biological function of chromosomally located TA cassettes, often multiple, remains uncertain (25, 40, 41, 33, 44). Considering that many of the toxins affect protein synthesis, their function in stress response (6, 12, 19) and programmed cell death (1, 17, 23, 27, 28) was postulated. The recently revealed ability of several toxins to cleave mRNA (11, 38, 47, 48) put forward the hypothesis that the role of TA systems in the quality control of prokaryotic gene expression is similar to the eukaryotic nonsense-mediated mode of RNA decay (13, 2).

The Epsilon and Zeta proteins constitute one of the few characterized TA systems for gram-positive bacteria (22, 32, 50), discovered in the inc18 family pSM19035 plasmid in a clinical strain of *Streptococcus pyogenes* (3). This theta replicating, broad-range and low-copy-number 29-kb plasmid is stably maintained in gram-positive bacteria with DNA of low G+C content. The major stabilization function relies on the activity of the *epsilon* (ε) and *zeta* (ζ) genes acting as a post-segregational killing system (8, 50). This system is unique in both structural and functional features. Unlike other known TA systems, the ε antitoxin and ζ toxin genes are organized in one operon together with the ω gene, coding for a global transcriptional regulator of pSM19035 plasmid genes (15). Moreover, these two genes are not autoregulated by the antitoxin or by the TA complex, as is the case with the other TA operons. The product of the ω gene strongly represses transcription of the adjacent ε antitoxin and ζ toxin genes and its own transcription. The Omega protein also represses transcription of *copS* (a negative-copy control factor) and the δ gene (which encodes a homolog of ATPase ParA). Additionally, the product of the ω gene was shown to participate in the partition process of this plasmid, just like the ParB-like protein of a novel type of partitioning system (16). Compared to other TA toxins, Zeta is exceptionally large (287 versus ~100 amino acids). Zeta toxin is bactericidal for gram-positive *Bacillus subtilis* and bacteriostatic for *Escherichia coli*. Despite numerous experimental efforts (vide references 32 and 36), a cellular target of the Zeta toxin remains unknown.

TA modules are grouped into at least eight families based on sequence and structural and functional similarity (21). The ω-ε-ζ operon forms the distinctive one, with strict homologues in plasmids from the same inc18 family (4, 42), in their transposon derivatives (24, 34), in the chromosome of *Streptococcus pneumoniae* (30), and in the plasmid pVEF3 from *Enterococcus faecium* (43).

The structure of the complex of Zeta proteins inactivated by Epsilon proteins (ε_2-ζ_2) was solved by X-ray crystallography (35). In the heterotetramer, the two Epsilons are in contact as they are sandwiched between the monomers of Zeta. The N-terminal parts of the Epsilon protein(s) bind to the Zeta protein(s), closing the crevice containing the Walker A motif, which is essential for toxin activity. A significantly similar structure was shown for the homologous PezAT system of *S. pneumoniae* (30). Although the sequences between the systems

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TABLE 1. Plasmids used in this study

Plasmids	Description[a]	Primers used for amplification	Reference or source
pBT286	Vector pUC18 containing ω-ε-ζ genes		50
pACE 1	Vector pACYC184 containing ω-ε genes		50
pGAD424	*E. coli*/*S. cerevisiae*, Ap^r/*leu2*, ori ColE1/2μm, GAL4 activation domain		18
pGBT9	*E. coli*/*S. cerevisiae*, Ap^r/*trp1*,ori ColE1/2μm, GAL4 DNA binding domain		18
pGBT9umuD′	Positive control construct for THS		29
pGAD424umuD′	Positive control construct for THS		29
pGAD424-ε	Containing ε antitoxin gene (6609–6881)	ε_{start} and ε_{stop}	This work
pGBT9-ε	Containing ε antitoxin gene (6609–6881)	ε_{start} and ε_{stop}	This work
pGAD424-ζ	Containing ζ toxin gene (6883–7746)	ζ_{start} and ζ_{stop}	This work
pGBT9-ζ	Containing ζ toxin gene (6883–7746)	ζ_{start} and ζ_{stop}	This work
pGAD424-Δ8Nε	Deletion of ε antitoxin gene (6632–6881)	$\varepsilon_{\Delta 8N}$ and ε_{stop}	This work
pGAD424-Δ29Nε	Deletion of ε antitoxin gene (6696–6881)	$\varepsilon_{\Delta 29N}$ and ε_{stop}	This work
pGAD424-Δ5Cε	Deletion of ε antitoxin gene (6606–6863)	ε_{start} and $\varepsilon_{\Delta 5C}$	This work
pGAD424-Δ15Cε	Deletion of ε antitoxin gene (6606–6835)	ε_{start} and $\varepsilon_{\Delta 15C}$	This work
pGBT9-Δ12Nζ	Deletion of ζ toxin gene (7079–7746)	$\zeta_{\Delta 12N}$ and ζ_{stop}	This work
pGBT9-Δ28Nζ	Deletion of ζ toxin gene (6965–7746)	$\zeta_{\Delta 28N}$ and ζ_{stop}	This work
pGBT9-Δ66Nζ	Deletion of ζ toxin gene (6917–7746)	$\zeta_{\Delta 66N}$ and ζ_{stop}	This work
pGBT9-Δ27Cζ	Deletion of ζ toxin gene (6883–7660)	ζ_{start} and $\zeta_{\Delta 27C}$	This work
pGBT9-Δ87Cζ	Deletion of ζ toxin gene (6883–7480)	ζ_{start} and $\zeta_{\Delta 87C}$	This work
pGBT9-Δ138Cζ	Deletion of ζ toxin gene (6883–7327)	ζ_{start} and $\zeta_{\Delta 138C}$	This work
pGBT9-ζ_{Ploop^-}	$A_{7018}T$ and $A_{7019}T$ substitution mutant of ζ toxin gene (6883–7746)	ζ_{start}/Rζ_{mut} and Fζ_{mut}/ζ_{stop}	This work

[a] Position numbers (in parentheses) correspond to the coordinates of the pBT233 plasmid (8) sequence, EMBL accession no. X64695.

differ considerably (especially for the antidotes), the residues building the putative phosphoryltransferase fold are conserved in both toxins, Zeta and PezT. The unique structure of the ε_2-ζ_2 and PezA$_2$-PezT$_2$ complexes does not exhibit any similarity to other known three-dimensional structures of TA proteins. The estimated half-life time of the Epsilon protein in vivo is approximately 18 min, whereas the half-life of Zeta is more than 1 h (7).

The interaction between the toxin and antidote proteins is a prerequisite for the proper functioning of the proteic addiction system. To examine the interaction between the Epsilon and Zeta proteins in vivo, we employed the yeast two-hybrid system (THS). During the last decade, the THS, devised by Fields and Song (18), became a widely used and well-accepted technique to test the in vivo interaction of known proteins, define domains or amino acids critical for this interaction, and screen libraries that bind specific protein.

The main objective of this study was to investigate the interaction between the Epsilon and Zeta proteins in vivo and to analyze functioning of this TA system in eukaryotic cells.

MATERIALS AND METHODS

Yeast and bacterial strains, media, and growth conditions. Bacteria [*E. coli* DH5α *end*A *hsdR17 supE44 thi-1 recA1 gyrA relA* (ϕ80*lac*ZΔM15) Δ(*lacZYA-arg*F)*U169*] were grown in LB or SOB medium (Difco) (41) supplemented when necessary with an appropriate antibiotic(s): ampicillin (100 μg/ml) or tetracycline (30 μg/ml) at 37°C.

Yeast [*Saccharomyces cerevisiae* Y190 MATa *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3*::*GAL*→*lacZ LYS2*::*GAL*(*UAS*)→*HIS3 cyh*′] were grown in complete yeast extract-peptone-glucose or synthetic minimal yeast nitrogen base medium (Difco) supplemented with appropriate amino acids, adenine (40 μg/ml), and a source of carbon (3% or 0.5% glucose or 2% glycerol) at 28°C.

Transformation. The one-step transformation of yeast cells was performed as described by Chen et al. (9). One hundred μl of yeast cell suspension in transformation buffer (1.5 mg dithiothreitol, 80 μl 50% polyethylene glycol 4000, 20 μl 1 M LiAc, 50 μg single-stranded carrier DNA) was used for simultaneous transformation with 100 ng to 1 μg DNA of each plasmid. After 30 min of incubation at 45°C, transformants were recovered on yeast nitrogen base medium without selective amino acids (leucine and tryptophan) and supplemented with 3% of glucose. Plates were incubated at 28°C for up to 3 days.

For the transformation of *E. coli*, the standard calcium chloride method of Sambrook et al. (41) or electrotransformation with Gene-Pulser (Bio-Rad) was performed.

All plasmids used in this study are listed in Table 1.

DNA manipulations. Highly pure plasmid DNA was obtained using Nucleobond AX (Macherey Nagel) or the Wizard Plus Minipreps DNA purification system (Promega), following the manufacturer's instructions. Routine DNA recombinant techniques were performed as described by Sambrook et al. (41). Restriction enzymes and other enzymes were used according to suppliers' instructions. Reactions producing DNA fragments to be used in new constructs were performed with the proofreading *Tli* polymerase from Promega or the Expand high-fidelity PCR system from Boehringer Manheim.

Construction of plasmids for the THS. The Matchmaker THS from Clontech (10, 18), with the GAL4 transcriptional activator and *lacZ* as a reporter gene, was used to detect protein-protein interaction.

All fusions were constructed using specially designed primers for ε antitoxin and ζ toxin gene sequences expanded at the 5′ ends by the restriction enzyme recognition sites, i.e., sites for EcoRI and BamHI in the forward and reverse primers, respectively (see Table 2). These primers were used for cloning purposes. Both the pGAD424 and pGBT9 vectors were treated with the EcoRI and BamHI restriction enzymes followed by dephosphorylation with shrimp alkaline phosphatase before ligation.

The ε antitoxin and ζ toxin gene sequences and deletion mutants were PCR amplified from pBT286 plasmid DNA using specific primers (Table 2). After EcoRI and BamHI enzyme digestion, the obtained fragments were cloned into the pGAD424 and/or pGBT9 vectors. Constructs were introduced and propagated in *E. coli* cells.

The presence of the in-frame junction and the correctness of entire ε antitoxin and ζ toxin gene sequences in constructs were confirmed by sequencing with the appropriate primers, i.e., GAL4ad start and GAL4ad end or GAL4bd start and GAL4bd end and additionally, for the ζ toxin gene, $\zeta_{\Delta 66N}$ and $\zeta_{\Delta 138C}$ (see Table 2).

The plasmid pair pGAD424umuD′ and pGBT9umuD′ (29) was used as a positive control for interaction detection of prokaryotic proteins in the yeast THS.

TABLE 2. Sequences of primers used for construction and sequencing of mutant ε and ζ genes[a]

Primer	Sequence
ε_{start}	5′ GCC GAA TTC ATG GCA GTT ACG TAT GAA AAA ACA
ε_{stop}	5′ CTC GGA TCC TTA AGC CAC TTT CTC TTT ATT CAA
$\varepsilon_{\Delta 8N}$	5′ GCC GAA TTC TTT GAA ATA GAG ATC ATT AAC G
$\varepsilon_{\Delta 29N}$	5′ GCC GAA TTC TTG AAC CAT GAA TTA AAT
$\varepsilon_{\Delta 5C}$	5′ CTC GGA TCC TAT TCA AAA CTT GTT TTG
$\varepsilon_{\Delta 15C}$	5′ CTC GGA TCC CAT TGA CCG CCA ATA CTC ATG
ζ_{start}	5′ GCC GAA TTC ATG GCA AAT ATA GTC AAT TTT ACT
ζ_{stop}	5′ GCC GGA TCC TTA AAT ACC TGG AAG TTT AGG TGT
$\zeta_{\Delta 12N}$	5′ GCC GAA TTC GAG AAT CGC TTA AAT G
$\zeta_{\Delta 28N}$	5′ GCC GAA TTC GCG GTT GAA TCG CCA ACC G
$\zeta_{\Delta 66N}$	5′ GCC GAA TCC GAT ACC TTT AAA CAA CAG CAC CC
$\zeta_{\Delta 27C}$	5′ GCC GGA TCC TTC AGG TGT CTC TTG GTG
$\zeta_{\Delta 87C}$	5′ GCC GGA TCC ATC GCT AAA TAA GCC CGT
$\zeta_{\Delta 138C}$	5′ GCC GGA TCC GAT TTT AGG TAC TGC CAT GAC
$R\zeta_{mut}$	5′ CG CAA ACT GGT TAA CCC TGA CCC TGG
$F\zeta_{mut}$	5′ CCA GGG TCA GGG TTA ACC AGT TTG CG
GAL4ad start	5′ GCG TTT GGA ATC ACT ACA GG
GAL4ad end	5′ TGA ACG CCC CAA AAA GTC
GAL4bd start	5′ GAA GAG AGT AGT AAC AAA GG
GAL4bd end	5′ AAA ATC ATA AAT CAT AAG

[a] Restriction enzyme recognition sequences are underlined.

Site-directed mutagenesis by PCR mutation of Walker A motif. A two-step PCR was carried out to introduce a mutation into the definite site of the ζ toxin gene Walker A motif nucleotide sequence: two DNA fragments (together corresponding to the entire ζ toxin gene sequence), juxtaposed at the site of desired change, were generated using mutagenic forward ($F\zeta_{mut}$) or reverse ($R\zeta_{mut}$) (Table 2) primers and adequate primers (ζ_{stop} and ζ_{start}) (Table 2) for the ζ toxin gene ends, respectively. After purification, the two fragments were mixed at an equimolar ratio, denatured (94°C, 4 min), renaturated, and subjected to a T4 polymerase reaction in the presence of deoxynucleoside triphosphates. The obtained fragment was used in the second PCR amplification (the proofreading polymerase was used) with forward (ζ_{start}) and reverse (ζ_{stop}) primers (Table 2). In this procedure, the $A_{7018}A_{7019}$ nucleotide sequence was changed into TT, and the resulting new GTTAAC sequence, recognized by the HpaI restriction enzyme, was useful for controlling the mutant construction.

Assay for β-galactosidase activity. Preliminary screening for β-galactosidase activity was performed by testing for blue-color individual colonies on filters after a freeze/thaw cycle (45).

Yeast cellular extracts were prepared from 48-h cultures incubated in minimal medium supplemented with 2% ethanol (lacking selective leucine and tryptophan) at 28°C. The β-galactosidase activity expressed in vivo by *S. cerevisiae* was determined essentially as described by Rose et al. (39).

Growth drop test for yeast. Freshly grown yeast cells were suspended to 1×10^7 cells/ml and serially diluted (10×). Four μl of three consecutive dilutions were spotted on the yeast extract-peptone-glucose plate and incubated at 28°C.

Microscopic observations. DNA in living yeast cells was stained with 1 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) solution which was added to the culture. The cells were collected after 1 h of incubation, mixed with 1% agarose, and spread on an object slide. Preparations were studied using a Nicon Microphot-SA fluorescence microscope with a Plan Apochromat 100× differential interference contrast lens in combination with phase contrast. Images were captured using a cooled charge-coupled device ORCA ER (Hamamatsu) camera and processed with Photometrix software.

RESULTS

Detection of specific interaction between Epsilon and Zeta proteins in *Saccharomyces cerevisiae* cells. The formation of complexes by inactivating toxins with their cognate antidotes is the basis for the functioning of the TA systems. The toxicity of the Zeta protein is counteracted by the Epsilon protein in both *E. coli* and *B. subtilis* (50). In this study, the yeast THS was used to examine in vivo the interaction between the Epsilon and Zeta proteins of the streptococcal plasmid pSM19035.

To construct fusions of the GAL4 DNA binding or activation domain with the Epsilon or Zeta protein, DNA fragments encoding these proteins were PCR amplified from the initiation to the stop codon. The ligation with the pGAD424 or pGBT9 vector produced N-terminal fusions of these genes with GAL4 domains. Four constructs, pGAD424-ε, pGAD424-ζ, pGBT9-ε, and pGBT9-ζ, were separately introduced into *E. coli* DH5α competent cells. The ligation mixtures containing the ζ toxin open reading frame were transformed into the DH5α strain already harboring pACE1 (a plasmid carrying the ε antitoxin gene), which would protect the host cells from the possible toxic effects of the hybrid Zeta protein.

The functionality of the Zeta protein fused to the GAL4 domains was confirmed in the cotransformation experiment described previously for the pCUZ1 and pCUE1 plasmids (50). The plasmids with the ζ toxin fusion gene were cotransformed with a compatible plasmid, pACE1, with 100% efficiency, while for empty vectors (i.e., not providing Epsilon in *trans*: pGAD424 or pGBT9), only about a 6% cotransformation frequency was observed. This result indicates that the Zeta protein, extended on its N terminus by amino acid sequences from the GAL4 activation or binding domain, retains its toxicity against *E. coli* cells.

Combinations of two plasmid constructs (one always based on pGAD424 and the other on pGBT9) were introduced into the yeast strain Y190 in a one-step transformation procedure. Although approximately the same amounts of DNA of different plasmid pairs were used, the transformation efficiencies varied significantly and were dependent on the fused gene. While the efficiency of cotransformation with the pGAD424-ζ and pGBT9-ε pair was at the same level as that of the control transformation with the THS vectors, when pGAD424-ζ was used together with the pGBT9 vector, the number of cotransformants dropped about 100-fold. The same situation was observed for the opposite composition of the hybrids (i.e., pGBT9-ζ and pGAD424). It was extremely difficult to obtain double transformants using these plasmids with both being fused to the ζ toxin gene. Only in the case of highly competent yeast, showing very efficient control transformation, were a few small colonies of double transformants with pGBT9-ζ and pGAD424-ζ obtained. (see Fig. S1 in the supplemental material.)

Toxicity of Zeta toward yeast cells. (i) Transformant viability. The growth of cotransformants was compared by a drop test (Fig. 1). After 2 days of incubation at 28°C, transformants

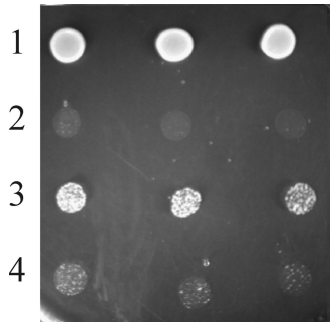

FIG. 1. Growth of yeast cotransformants carrying ζ toxin gene cloned into pGAD424 or pGBT9 vector: dilution test (5×10^6 cells were plated with 10× and 100× dilutions). Lane 1, pGAD424-ε and pGBT9-ζ; lane 2, pGAD424-ζ and pGBT9-ζ; lane 3, pGAD424 and pGBT9-ζ; lane 4, pGAD424-ζ and pGBT9.

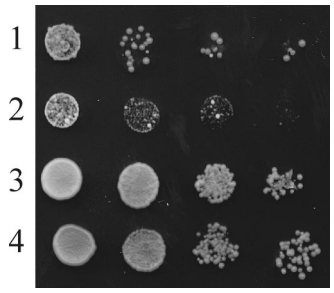

FIG. 2. Effect of ζ toxin gene dose on the growth of Y190 transformants (2.5×10^6 cells were plated with 10×, 100×, and 1,000× dilutions). Lane 1, pGAD424-ζ and pGBT9; lane 2, pGAD424-ζ; lane 3, pGAD424-ε; lane 4, pGAD424-ζ and pGBT9-ε.

with coexisting ε antitoxin and ζ toxin genes grew well (lane 1), whereas double ζ toxin gene hybrid transformants failed to grow at all (lane 2). For the cotransformants carrying one plasmid with a fused ζ toxin gene and the other being an empty vector, growth retardation was observed (lanes 3 and 4). This growth retardation was particularly evident for the plasmid pair consisting of pGAD424-ζ and pGBT9. Similar results were obtained by growing yeast cotransformants in liquid medium (data not shown).

(ii) Gene dosage effects. Both THS vectors carry the same 2μm yeast plasmid origin of replication, which determines their copy number. The plasmid copy number (~100) remains the same regardless of whether one type of plasmid or two different plasmids are propagated in the cell. In other words, the total copy number of 2μm origins is approximately 100. This makes studies of gene dosage effect (~100 versus ~50) by transforming yeast with a given gene carried on one or two 2μm-based plasmids possible (5).

The drop growth test performed for one-plasmid transformants confirmed the strong effect of the ζ toxin gene hybrid on yeast cells (Fig. 2). Its presence in the maximal dose greatly inhibited yeast growth (lane 2). Lowering the ζ toxin gene hybrid dose by cotransformation with a second plasmid (empty vector; lane 1) improved growth, although not to the level of the control (ζ toxin gene absent; lane 3). The comparison of the growth of yeast cells carrying the ζ toxin gene at a reduced dose (lane 1) with that of cells carrying the ζ toxin gene at a similar dose but also carrying ε (lane 4) indicated that the latter grew much better. The results of these observations indicate that the Zeta protein is toxic for yeast in a dose-dependent manner and that the toxic effects are alleviated by the presence of Epsilon in the same cell.

Microscopic examination of cells transformed with ζ toxin gene fusion plasmids grown in the presence of DAPI revealed a high proportion of deformed, probably dead cells, which did not permit the accumulation of the fluorescent dye, leaving them yellow (obscure in the photograph). The differences in DAPI staining between cotransformants with various pairs of THS plasmids are shown in Fig. 3.

Specific interaction between Epsilon and Zeta proteins in THS. The preliminary detection of interaction between the Epsilon and Zeta proteins was done by using the qualitative filter test for β-galactosidase production. In this test, the activation of the transcription of the reporter gene is manifested by the blue color of transformant colonies. This was followed by a quantitative β-galactosidase activity assay. A summary of the results obtained in the THS for every combination of fused ε antitoxin and ζ toxin genes is presented in Table 3.

The blue color of the colony and β-galactosidase activity were correlated and detected only when pairs of THS plasmids contained the ε antitoxin and ζ toxin genes. Any other combination (except for the positive control) did not reveal the restoration of a functional Gal4 activator. As can be noticed from the β-galactosidase activity measurements, the hybrid containing the ζ toxin gene fused to the region encoding the

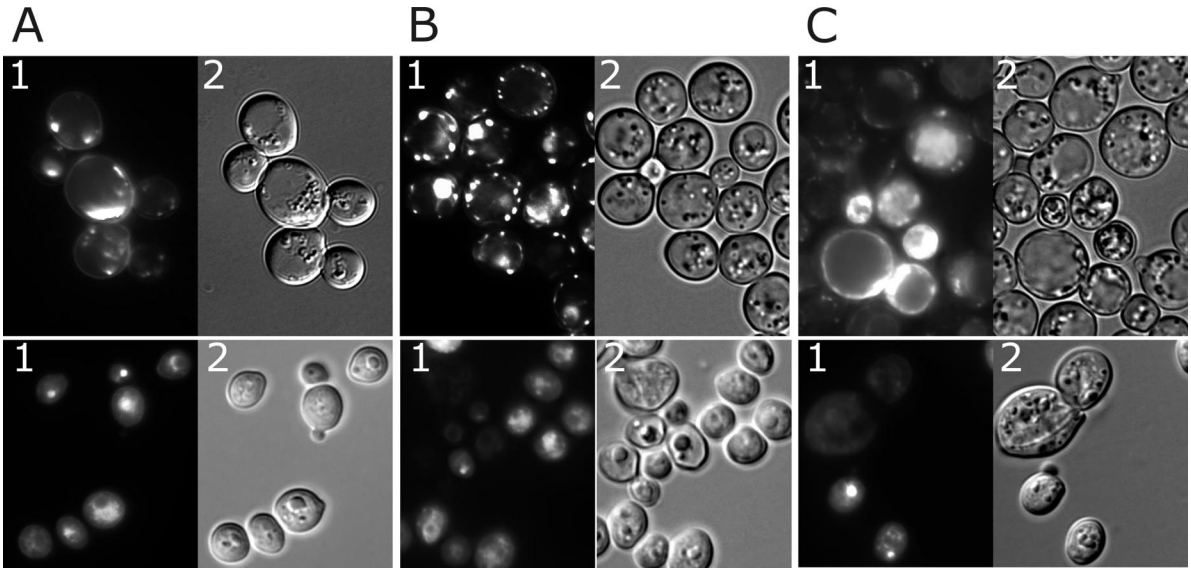

FIG. 3. Morphology of Y190 cells transformed with THS hybrid plasmids. pGAD424-ε and pGBT9-ζ (A), pGAD424 and pGBT9-ζ (B), or pGAD424-ζ and pGBT9-ζ (C), stained with DAPI, are shown. Panels 1 show fluorescence; panels 2 show Nomarski contrast (differential interference contrast). Upper and lower panels show in vivo staining and staining after ethanol fixation, respectively.

TABLE 3. Interaction of hybrid Epsilon and Zeta proteins in THS

Pair of plasmids	Colony color	β-Galactosidase activity[a]
pGAD424 and pGBT9	White	<1
pGAD424-ζ and pGBT9-ε	Blue	7.9 ± 1.9
pGAD424-ε and pGBT9-ζ	Blue	295 ± 75.4
pGAD424-ε and pGBT9-ε	White	<1
pGAD424-ζ and pGBT9-ζ	ND	ND
pGAD424-ε and pGBT9	White	<1
pGAD424-ζ and pGBT9	White	<1
pGAD424 and pGBT9-ε	White	<1
pGAD424 and pGBT9-ζ	White	<1
pGAD424umuD′ and pGBT9umuD′	Blue	49 ± 22.8

[a] β-Galactosidase specific activity in nanomoles of *O*-nitrophenyl galactoside hydrolyzed per minute per milligram of protein; average of three measurements ± standard deviation. ND, not done.

TABLE 4. Interaction of mutated Epsilon or Zeta proteins in THS

Pair of plasmids[a]	Colony color	β-Galactosidase activity[b]	Zeta toxicity
pGAD424-ε and pGBT9-ζ	Blue	319 ± 95	+
pGAD424-Δ8Nε and pGBT9-ζ	Blue	205 ± 69	+
pGAD424-Δ29Nε and pGBT9-ζ	White	<3	+
pGAD424-Δ5Cε and pGBT9-ζ	Blue	392 ± 94	+
pGAD424-Δ15Cε and pGBT9-ζ	White	<1	+
pGAD424-ε and pGBT9-Δ12Nζ	White	<1	−
pGAD424-ε and pGBT9-Δ28Nζ	White	<3	−
pGAD424-ε and pGBT9-Δ66Nζ	White	<7	−
pGAD424-ε and pGBT9-Δ27Cζ	Blue	302 ± 84	+
pGAD424-ε and pGBT9-Δ87Cζ	Blue	299 ± 43	−
pGAD424-ε and pGBT9-Δ138Cζ	Blue	275 ± 33	−
pGAD424-ε and pGBT9-ζ_{Ploop^-}	Blue	169 ± 32	−

[a] Abbreviations: N, amino-terminal end; C, carboxy-terminal end; Ploop$^-$, K46L substitution.

[b] β-Galactosidase specific activity in nanomoles of *O*-nitrophenyl galactoside hydrolyzed per minute per milligram of protein; average of three measurements ± standard deviation.

binding domain activated transcription much more efficiently (~40 times) than with the other combination (i.e., the fusion of the ζ toxin gene with the region coding for the activation domain).

Analysis of mutant Epsilon and Zeta proteins in THS. A series of amino- and carboxy-terminal deletions of both proteins were constructed with the intention of identifying the regions of the Epsilon and Zeta proteins responsible for the interaction with each other (Fig. 4). A stronger interaction occurs when the ε antitoxin gene is fused to the activation domain and the ζ toxin gene to the binding domain sequence. Therefore, this combination was chosen for the construction of the deletion fusions. In all cases, fragments of the ε or ζ toxin gene, deleted from the carboxy- or amino-terminal end, were prepared by PCR using specific primers. Fragments of the ε antitoxin gene were cloned into pGAD424 and ζ toxin gene fragments into the pGBT9 vector. The standard procedure for transformation of *E. coli* cells and identification of correct clones was employed. When ζ toxin gene deletions were ligated to pGBT9, the ligation mixtures were introduced into either DH5α or DH5α harboring the pACE1 plasmid. This allowed the determination of the toxicity of a given mutant Zeta protein.

For the cotransformation of the yeast reporter strain, all combinations of pairs were matched in such a way that only one fusion plasmid contained the deletion mutant whereas the other carried intact coding sequence. Pairs with one "empty" vector (i.e., pGAD424 and pGBT9-Δζ) were introduced into yeast cells to exclude the possibility of transcription activation by fragments of one of the studied proteins only (data not shown).

As with the complete fusion proteins, cotransformants with plasmid pair combinations with a deletion mutant were screened after selection for their ability to express β-galactosidase by the filter assay and by measuring β-galactosidase activity in the solution. The pair pGAD424-ε with pGBT9-ζ was used as a reference. The results obtained using THS for the mutant Epsilon and Zeta proteins are shown in Table 4. Again, β-galactosidase activity correlated with the corresponding colony color.

The results summarized in Table 4 indicate that the amino-terminal part of the Zeta protein is critical for interaction with the Epsilon protein. Deletion of 12 amino acid residues led to a loss of interaction with the intact Epsilon protein, whereas deletions of up to 138 amino acids from the carboxy-terminal end did not disturb the interaction. A 15-amino-acid deletion at the carboxy-terminal end or a 29-amino-acid deletion at the amino-terminal end of the Epsilon protein makes the interaction with the intact Zeta protein impossible.

The pattern of toxicity for the deletion mutants of the Zeta protein reveals that in this case, also, its N terminus has a critical role. As can be judged from the ability to transform *E. coli* cells in the absence of the ε-antitoxin-gene-encoding plas-

FIG. 4. Graphical representation of Epsilon and Zeta protein mutants constructed in this study. Grey indicates the ability of the protein to interact in a yeast THS. The position of mutated Walker A sequences in the ζ toxin gene is marked "P-loop." Lengths of bars correspond to the dimensions of represented proteins. Toxicity of the Zeta protein is indicated by a plus sign.

mid with ζ toxin gene mutant fusion plasmids, removing as little as 12 N-terminal amino acids abolishes the toxicity of the Zeta protein. The deletion of 27 carboxy-terminal amino acids does not disturb the Zeta protein toxicity (nor the ability to interact with the Epsilon protein), whereas deletion of 87 or more amino acids results in a loss of toxicity (the ability to interact with the Epsilon protein is retained).

Attempts to obtain a deletion of five amino acids from the N terminus of the Zeta protein were also undertaken, but in spite of repeated cloning procedures, they failed. At the same time, the control experiments with the use of the same vector preparation and other ζ toxin gene deletions and the test for self-ligation of the PCR ζ toxin gene product were successful. Therefore, a possible explanation for the failure to clone the ζ toxin gene devoid of five codons is that its product, while retaining toxicity, is unable to interact with the Epsilon protein. So, there are no conditions under which such a construct could be maintained in the cell.

The analysis of the ζ toxin gene open reading frame product in silico revealed only one structural motif that is an ATP binding Walker A motif between residues 40 and 47. Mutation in this motif (leading to the substitution K46L; see Methods) abolished the toxicity of Zeta, while its ability to interact with Epsilon was only slightly reduced.

DISCUSSION

In the current study, we examined in vivo interaction between the Epsilon and Zeta proteins of the plasmid TA system of gram-positive bacteria. The yeast THS was employed to detect and analyze specific interactions of these proteins. For *E. coli*, it has been previously shown (7) that the proteins Epsilon and Zeta, overexpressed from a plasmid containing the ε-antitoxin–ζ-toxin genes, copurified as a stable inactive heterotetrameric ε_2-ζ_2 complex. The crystallographic data for this complex (35) revealed a dumbbell-shaped structure with two ε-ζ halves (I and II), in which the Zeta protein(s) interacts with the Epsilon protein(s) but not with other Zeta proteins. With the THS, only two proteins involved in the ε-ζ dimer were assayed.

The data presented in Table 4 are in good agreement with the data from the study of the crystal structure of the ε_2-ζ_2 complex (35). In the crystal, the two proteins interact mainly via the first N-terminal helix of Epsilon, starting at position number 9 of the protein, which is consistent with the interaction pattern seen in the truncated Epsilon mutant proteins in our study. However, the deletion of eight amino acids in the N-terminal end of Epsilon already results in a decrease in the strength of its interaction with Zeta protein. The C-terminal part of Epsilon is not involved in Zeta binding, and this is confirmed by the mutant Δ5C Epsilon protein's ability to interact with toxin. An observed increase in the β-galactosidase activity for the pGAD424-Δ5Cε and pGBT9-ζ plasmid pair compared to that for pGAD424-ε and pGBT9-ζ lies within the experimental error of both measurements. It may also reflect an increased strength of interaction resulting from the lower molecular weight of the mutant Δ5C Epsilon protein, lessening the disadvantageous entropic contribution of losing degrees of freedom during complex formation. The deletion of as many as 15 residues (out of 90) probably already affects folding of the entire protein and consequently its interactions.

Similarly, the pattern of interaction of the truncated Zeta protein is consistent with its crystal structure. The interaction with Epsilon is affected by N-terminal deletions but not by C-terminal deletions, where as much as 138 residues can be deleted and no interaction pattern changes are observed. For this alpha/beta protein, the C-terminal helical part can probably be deleted without an effect on the rest of the protein structure, since it constitutes a separate folding unit. Our study therefore confirms that the structure seen in the crystal is similar to the one existent in vivo.

In contrast to the structure of the ε_2-ζ_2 heterotetramer and in vivo observations of Epsilon-protein-overproducing *E. coli* cells (7), we did not detect interaction between pGAD424-ε and pGBT9-ε. Notably, low β-galactosidase activity for the pGBT9-ε and pGAD424-ζ pair indicates that the binding domain of the transcriptional activator fused to the Epsilon protein is not able to bind DNA efficiently. This, together with the very short in vivo lifetime of the antidote (7), probably leads to weak interaction below the detection level in our measurements.

The experiments with the use of the yeast THS confirm that Epsilon prevents the action of Zeta by interacting with this toxin protein. The analysis of mutant (mainly truncated) proteins reveals that the N-terminal part of the Zeta protein is involved in both toxicity and binding of the antidote. This is manifested by our inability to clone in *E. coli*(pACE1) the truncated ζ toxin gene encoding the protein in which five N-terminal amino acids are absent. Most likely, in this construct the mutated ζ toxin gene encodes a protein that retains toxicity while its ability to interact with the antidote is abolished. Further deletions in the N terminus of Zeta affect both toxicity and Epsilon binding. Also, the Walker A motif is located in the N-terminal part of Zeta. Destruction of this motif in the mutant protein eliminates only its toxicity without affecting its ability to interact with the antidote.

The use of *S. cerevisiae* as a host for in vivo experiments with bacterial proteins gave us the unique opportunity for simultaneous observation of the effects of the bacterial Zeta toxin on eukaryotic cells. The physiological effects of Zeta overproduction in yeast have not been studied yet. The yeast two-hybrid plasmids are 2μm-origin-of-replication-based vectors, and as a result of transformation with both, the corresponding fusion proteins are produced in one cell at similar quantities. The importance of proper stoichiometry between the antidote Epsilon protein and the poison Zeta protein has been demonstrated with bacterial cells (50). In its natural genetic context, in gram-positive cells, this system is very efficient and bactericidal. In contrast, in gram-negative *E. coli*, its functioning is bacteriostatic and less effective in plasmid stabilization. The overexpression of the Zeta protein in *B. subtilis* leads to massive cell death, and an overdose of the antitoxin Epsilon destroys the plasmid stable maintenance.

Plate growth tests, together with microscopic examination of yeast cells exposed to the activity of chimerical Zeta proteins, indicate that here the toxic effects may be weaker and are ζ toxin gene dose dependent. Weaker effects of Zeta on eukaryotic cells than on prokaryotic ones may, of course, reflect the evolutionary distance between those microorganisms. How-

ever, it should be noted that only hybrid proteins, having either Gal4 DNA binding or the transactivation domain fused to the N terminus of Zeta, were used in the yeast cells. In *E. coli*, the THS vectors (pGAD424 or pGBT9) carrying those *GAL4*::ζ toxin hybrid genes use the pUC origin and replicate as high-copy-number plasmids. Nevertheless, the toxic effects of chimerical Zeta proteins are counteracted by Epsilon encoded by a moderate-copy-number vector, pACYC184. In contrast, when the wild-type ζ toxin gene is present on pUC-series vectors, its activity cannot be counteracted by the ε antitoxin gene present on pACYC184 (data not shown). This indicates that manipulations at the N-terminal region of the Zeta protein most likely result in a weakening of its biological activity, at least in *E. coli* cells.

Recently a PezAT system homologous to ω-ε-ζ was found on the chromosome of a closely related pathogenic strain of *Streptococcus pneumoniae* (30). Its molecular and structural characterization showed significant similarity in the functioning and three-dimensional structure of the toxin-antidote complex. The authors investigated interaction between the PezA and PezT proteins in vivo in a bacterial THS. Interestingly, the interaction (manifested by the growth of an indicator *E. coli* strain on selective media) could be detected only for one pairwise combination. This resembles the big difference observed in the levels of reporter gene activity in dependence on which the THS vector ζ toxin gene was cloned. A similar inequity has often been described (also by our THS-positive control authors) and probably reflects alterations in folding of the fused proteins. Similar to the case with the ζ toxin gene, mutations in Walker A motif residues of *pezT* abolished PezT lethality.

Kristoffersen et al. (31) have expressed bacterial *relE-relB* TA genes in yeast cells and demonstrated that induced expression of the *relE* gene is highly toxic to *S. cerevisiae*. The growth inhibition effect of RelE was counteracted to some extent by induction of RelB production. The authors attribute this inefficient protection of the host cells by the antidote RelB protein to the differences in the effectiveness of the two promoters used or a very weak RelE-RelB interaction in yeast cells. It has also been shown that when overexpressed in a human osteosarcoma cell line, this toxin induced apoptosis (46).

The Kid toxin of plasmid R1 was shown to inhibit proliferation of yeast, frog embryo cells, and human cells. It was shown that the death of HeLa cells upon expression of the Kid toxin is caused by the activation of the apoptosis pathway (14).

The TA systems constitute a mechanism for regulated bacterial cell killing. When used in eukaryotic cells, the elements of these systems provide some potential clinical applications. The fine-tuning of this mechanism was proposed as an opportunity for cancer gene therapy, with the toxin acting as an antitumor agent and the antidote being used to antagonize its action in normal human cells (14). Our study has accomplished a first step toward utilizing the ε antitoxin and ζ toxin genes for such purposes. We have established that these components of the TA system of plasmid pSM19035 function in eukaryotes, as shown for *Saccharomyces cerevisiae*. The consequences of Zeta-protein toxicity in *S. cerevisiae* are similar to those in prokaryotes, i.e., Zeta inhibits cell proliferation/causes cell death, while Epsilon neutralizes Zeta's toxicity; Epsilon alone does not affect the viability of yeast cells. Importantly, we have demonstrated that the ε antitoxin and ζ toxin genes may function under independent transcriptional control, irrespective of the ω gene's regulation. We have also described the regions of the Zeta and Epsilon proteins essential for their operation, which opens the possibility of designing even smaller "drugs" than the full-length ε antitoxin and ζ toxin genes. In conclusion, our findings indicate that further investigation should focus on developing a similar system that would work on human cells.

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