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pSM19035-encoded ζ toxin induces stasis followed by death in a subpopulation of cells

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The toxin–antitoxin operon of pSM19035 encodes three proteins: the ω global regulator, the ε labile antitoxin and the stable ζ toxin. Accumulation of ζ toxin free of ε antitoxin induced loss of cell proliferation in both *Bacillus subtilis* and *Escherichia coli* cells. Induction of a ζ variant (ζY83C) triggered stasis, in which *B. subtilis* cells were viable but unable to proliferate, without selectively affecting protein translation. In *E. coli* cells, accumulation of free ζ toxin induced stasis, but this was fully reversed by expression of the ε antitoxin within a defined time window. The time window for reversion of ζ toxicity by expression of ε antitoxin was dependent on the initial cellular level of ζ. After 240 min of constitutive expression, or inducible expression of high levels of ζ toxin for 30 min, expression of ε failed to reverse the toxic effect exerted by ζ in cells growing in minimal medium. Under the latter conditions, ζ inhibited replication, transcription and translation and finally induced death in a fraction (~50 %) of the cell population. These results support the view that ζ interacts with its specific target and reversibly inhibits cell proliferation, but accumulation of ζ might lead to cell death due to pleiotropic effects.

INTRODUCTION

Toxin–antitoxin (TA) systems were initially found on low-copy-number plasmids and were shown to play a role in post-segregational killing (PSK) of bacterial cells that no longer carried the plasmid. Upon loss of the plasmid, the higher rate of turnover of the antitoxin by a cellular protease resulted in accumulation of the toxin unbound to the antitoxin, and selective killing or inhibition of proliferation of plasmid-free cells (Alonso *et al.*, 2006; Engelberg-Kulka & Glaser, 1999; Engelberg-Kulka *et al.*, 2004; Gerdes, 2000; Hayes, 2003; Zielenkiewicz & Ceglowski, 2001). With few exceptions, the TA systems of plasmids, Bacteria and Archaea share common functional and organizational characteristics. The antitoxin gene, which usually precedes that of the toxin, regulates transcription of the TA operon either alone or as a complex bound to the toxin complex (Anantharaman & Aravind, 2003; Engelberg-Kulka & Glaser, 1999; Gerdes *et al.*, 1997; Gerdes, 2000; Pandey & Gerdes, 2005; Zielenkiewicz & Ceglowski, 2001). Generally, the labile antitoxins (72–90 aa long) and stable toxins (90–130 aa long) are small proteins. The DNA-binding motifs associated with different antitoxins can be categorized into four different subfamilies (Anantharaman & Aravind, 2003). However, the toxins, which were initially identified in plasmid F (CcdAB), R1 or R100 (Kis/Kid-PemIK), in a *Salmonella dublin* virulence plasmid (VapBC), in P1 (Phd/Doc), RK2 (ParDE), Rts1 (HigBA) and P307 (RelBE), defined seven different families of TA systems (Anantharaman &

This paper is dedicated to the memory of Piotr Ceglowski, who contributed so much to the advancement of pSM19035 biology.

Abbreviations: Ap, ampicillin; BM, Belitsky medium; Cm, chloramphenicol; Em, erythromycin; DAPI, 4′,6′-diamino-2-phenylindole; EM, electron microscopy; FM, fluorescence microscopy; Km, kanamycin; LB, Luria–Bertani; PSK, post-segregational killing; PCD, programmed cell death; Rf, rifampicin; TA, toxin–antitoxin; VBNC, viable but non-culturable; wt, wild-type.

A table of supplementary data is available with the online version of this paper.

Aravind, 2003; Pandey & Gerdes, 2005). With few exceptions (e.g. CcdB, ParE), the toxins of these families are either known or predicted to act on RNA and function as regulators of translation (Anantharaman & Aravind, 2003; Christensen *et al.*, 2003; Gerdes *et al.*, 2005; Muñoz-Gomez *et al.*, 2005; Pandey & Gerdes, 2005; Pedersen *et al.*, 2003). The existence of TA systems in the chromosome of prokaryotic and archaeal organisms, and the demonstration that the growth inhibition caused by expression of the toxin can be reversed by subsequent expression of the cognate antitoxin, suggested that TA systems might have a role in inducing a viable but non-culturable (VBNC) state under physiological conditions that might otherwise compromise cell viability (Alonso *et al.*, 2006; Gerdes, 2000; Gerdes *et al.*, 2005). Previously, it was shown that inhibition of translation by expression of the RelE or MazF toxins induced bacteriostasis and that this could be fully reversed by the action of the antitoxins RelB or MazE, respectively (Gerdes *et al.*, 2005; Pedersen *et al.*, 2002). Later it was shown that the reversion of the toxic effects of RelB or MazE is only possible during a certain time window after expression of the antitoxin-free toxins, otherwise programmed cell death (PCD) ensues (Amitai *et al.*, 2004; Hazan *et al.*, 2004; Sat *et al.*, 2003).

One orphan family of TA systems, which is evolutionarily unrelated to the seven previously described TA families (see above), is encoded by plasmids of the Inc18 group and comprises ζ toxin, ε antitoxin as well as a third component (ω regulator) (Brantl *et al.*, 1990; Ceglowski *et al.*, 1993a, b; de la Hoz *et al.*, 2000; Fig. 1). The ω protein exists as a dimer in solution (ω_2) (Misselwitz *et al.*, 2001) and belongs to the ribbon–helix–helix family of transcriptional regulators (Murayama *et al.*, 2001; Weihofen *et al.*, 2006; Welfle *et al.*, 2005). Protein ω_2 is a global regulator of plasmid copy number, accurate plasmid segregation, TA expression and conjugational transfer (Camacho *et al.*, 2002; de la Hoz *et al.*, 2000, 2004). Regulation of transcription by ω_2 occurs through specific interactions with its cognate DNA-binding sequences upstream of the $\omega\varepsilon\zeta$ promoter (P_ω) comprising seven unspaced copies of a 7 bp repeat present in both the direct and inverted orientation (de la Hoz *et al.*, 2000, 2004; Weihofen *et al.*, 2006). In contrast to the other TA families neither the ε antitoxin (90 aa long, 10 kDa) nor the ζ toxin (287 aa long, 32 kDa) is involved in the control of its own expression and they are expressed from the regulated P_ω and a low-activity and constitutive (maintenance), P_ε, promoter (de la Hoz *et al.*, 2000, Fig. 1). The cytotoxic effects of the elongated monomeric ζ protein are counteracted by the dimeric ε (ε_2) antitoxin that forms a stable $\varepsilon_2\zeta_2$ heterotetramer complex (Camacho *et al.*, 2002; Meinhart *et al.*, 2003; Fig. 1). Interactions between ε_2 and ζ are primarily mediated by the C-terminal domain of ε (Meinhart *et al.*, 2003).

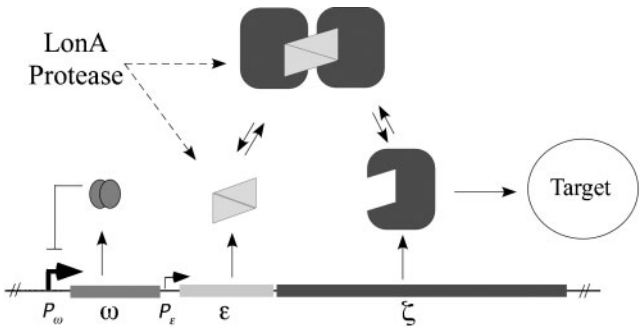

Fig. 1. Physical map of the pSM19035-encoded $\omega\varepsilon\zeta$ operon. Genes are indicated by bars, non-coding DNA by thin lines, and the gene products (ω_2, ε_2 and ζ) and their stoichiometry denoted. The bent arrows denote the direction of transcription, and P_ω and P_ε are indicated. The broken arrows indicate that the LonA protease degrades ε either in its free state or in a complex with ζ. The ω_2 regulatory circuit is indicated.

Previously it was shown that ζ protein has a significantly lower thermodynamic stability than ε_2 protein in both the free and the complex state (Camacho *et al.*, 2002). Proteolytic studies indicate that ζ protein is more stable in the $\varepsilon_2\zeta_2$ complex than in the free state (Camacho *et al.*, 2002). *In vivo* studies, however, reveal a short half-life of the ε antitoxin ($\sim$18 min) and a long lifetime of the ζ toxin ($>$60 min) (Camacho *et al.*, 2002). When transcription or translation of plasmid-borne ε and ζ genes is inhibited a short lag period precedes the rapid reduction in c.f.u. and during this interval degradation of the unstable ε_2 antitoxin is observed (Camacho *et al.*, 2002; Fig. 1).

The crystal structure of the biologically non-toxic $\varepsilon_2\zeta_2$ protein revealed that the tetrameric $\varepsilon_2\zeta_2$ complex contains ε_2 sandwiched between two ζ monomers (Meinhart *et al.*, 2003). Site-directed mutagenesis suggested that free ζ may act as a phosphotransferase using ATP to phosphorylate an as-yet-unidentified substrate, but the mechanism of action and specific target site remain to be elucidated. In $\varepsilon_2\zeta_2$, the toxin activity of ζ is inhibited because the N-terminal helix of the antitoxin ε blocks the ATP-binding site (Meinhart *et al.*, 2003). A toxin similar to ζ has also been identified in the chromosome of *Streptococcus pneumoniae* (Meinhart *et al.*, 2003).

It has been proposed previously that TA loci might serve two different functions: (i) to halt cell proliferation under stress conditions that lead to a VBNC state (Gerdes *et al.*, 2005), or (ii) to induce PCD in a subpopulation of cells in order to provide nutrients for the survivors (Engelberg-Kulka *et al.*, 2004). The purpose of this study was to determine whether ζ induces reversible stasis and if one of these hypotheses applies also to the orphan $\varepsilon\zeta$ TA system.

METHODS

Bacterial strains, plasmids and media. All bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) medium or a minimal medium [M9, S7 or Belitsky minimal (BM) medium] supplemented, when necessary, with the appropriate amino acid (50 μg ml^{-1}) and antibiotic(s)

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
***B. subtilis* strains**		
YB886	*trpC2 metB5 sigB37 attSPβ xin-1*	Yasbin *et al.* (1980)
YB-pX	+ *recA4 amyE*::*xylR-P*$_{xylA}$	Zielenkiewicz & Ceglowski (2005)
YB-pXζ	+ *recA4 amyE*::*xylR*::*P*$_{xylA}$-ζ [pBT322-2]	Zielenkiewicz & Ceglowski (2005)
BG687	+ *amyE*::*xylR-P*$_{xylA}$	This work
BG873	+ *amyE*::*xylR-P*$_{xylA}$ (*met*$^+$)	This work
BG689	+ *amyE*::*xylR*::*P*$_{xylA}$ζ*Y83C*	This work
BG871	+ *amyE*::*xylR*::*P*$_{xylA}$ζ*Y83C* (*met*$^+$)	This work
BG673	+ *clpC*::*spc*	This work
BG677	+ *clpE*::*spc*	This work
BG675	+ *clpP*::*spc*	This work
BG669	+ *lonA*::*cat*	This work
BG671	+ *clpX*::*ery*	This work
***E. coli* strains**		
CC118	(Δ*ara leu*) *araD lacX*74 *galE galK phoA thi-1 rpsE spoB argE recA1*	Bio-Rad
XL-1 Blue	[F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^R)] *lac endA1 gyrA96 thi-1 hsdR17 supE44 relA1 recA1*	Stratagene
Plasmids*		
pBT233-7	Rep$_{Inc18}$, ωεζ operon	Ceglowski *et al.* (1993b)
pBT233-2	Rep$_{Inc18}$, ω and ε genes	Ceglowski *et al.* (1993b)
pBT346	Rep$_{ColE1}$, Rep$_{60}$, *P*$_{cat}$-ωεζ, Em^R, Cm^R	Ceglowski *et al.* (1993b)
pCB539	Rep$_{ColE1}$, Rep$_{60}$, *P*$_{cat}$ εζ-GFP, Em^R, Cm^R	This work
pCB297	Rep$_{p15A}$, *P*$_{lacO}$-ε gene, Ap^R	Camacho *et al.* (2002)
pCB298	Rep$_{ColE1}$, Rep$_{60}$, *P*$_{cat}$-ωζ genes, Em^R, Cm^R	Camacho *et al.* (2002)
pFus2	Rep$_{pMB1}$, *araC-P*$_{araBAD}$, Km^R	Lemonnier *et al.* (2000)
pKL147	Rep$_{ColE1}$, *dnaX-gfp*, Amp^R	Lemon & Grossman (1998)
pCB635	Rep$_{pMB1}$, *araC-P*$_{araBAD}$-ζ gene, Km^R	This work

*Rep$_{pMB1}$, Rep$_{p15A}$ and Rep$_{ColE1}$ are *E. coli*-compatible replicons. The compatible replicons of *B. subtilis* plasmids are Rep$_{Inc18}$ (a pSM19035 derivative) and Rep$_{60}$ (a pTA1060 derivative).

[for *Escherichia coli* 100 μg ampicillin (Ap) ml^{-1}, 50 μg kanamycin (Km) ml^{-1}, 50 μg erythromycin (Em) ml^{-1} or 15 μg chloramphenicol (Cm) ml^{-1} and for *Bacillus subtilis* 5 μg Em ml^{-1}, 60 μg spectinomycin (Sp) ml^{-1} or 5 μg Cm ml^{-1}].

Strain and plasmid constructions. Purified plasmid DNA was prepared using a Qiagen plasmid kit following the manufacturer's instructions. *B. subtilis* chromosomal DNA was isolated as previously described (Alonso *et al.*, 1988). Transformation of *E. coli* was performed according to the standard calcium chloride method and transformation of competent *B. subtilis* cells with chromosomal DNA or ligated plasmid DNA was performed as previously described (Alonso *et al.*, 1988). The *ery*, *spc* and *cat* genes confer resistance to Em, Sp and Cm, respectively. Plasmid-borne *clpX*::*ery*, *clpC*::*spc*, *clpE*::*spc*, *clpP*::*spc* and *lonA*::*cat* (a gift from Dr Tarek Msadek, Institut Pasteur, Paris, France, and Dr Uli Gerth, Ernst-Moritz-Arndt-Universität Greifswald, Greifswald, Germany) were linearized and integrated into the chromosome via a double cross-over event, with selection for the selectable marker to generate strains BG671, BG673, BG677, BG675 and BG669 (see Table 1) and correct insertion was confirmed by PCR analysis.

B. subtilis YB-pXZ *recA*4 [xylose repressor (XylR)-xylose regulated promoter (*P*$_{xylA}$)-ζ gene-*cat* gene] or (YB-pX) *recA4* [XylR-*P*$_{xylA}$-*cat*] bearing pBT233-2 were a gift from Piotr Ceglowski (see Zielenkiewicz & Ceglowski, 2005). A spontaneous ζ variant was isolated in which a point mutation (A to G in codon 83) results in a tyrosine to cysteine substitution (ζY83C). Recently an identical mutant was independently obtained in screens for clones surviving ζ overproduction (Nowakowska *et al.*, 2005).

The XylR-*P*$_{xylA}$-*cat* or XylR repressor-*P*$_{xylA}$-ζY83C-*cat* cassette was transferred to YB886, generating strains BG687 and BG689, respectively, and to YB886 (Met$^+$), generating strains BG873 and BG871, respectively (Table 1).

Plasmids pBT233-2, pBT233-7 (Ceglowski *et al.*, 1993b), pBC297, pBC298 (Camacho *et al.*, 2002) and pFUS2 (Lemonnier *et al.*, 2000) have been described previously. For the construction of the pCB635-borne ζ gene under the control of the arabinose-regulated promoter (*P*$_{araBAD}$) a PCR-amplified ζ gene was placed under the transcriptional control of *P*$_{araBAD}$. By site-directed mutagenesis an *Xho*I site was inserted just before the stop codon of the ζ gene on pBT346, to generate pBT346-*Xho*I. The *Xho*I–*Sph*I DNA fragment containing the *gfpmut1* gene (Lemon & Grossman, 1998) was fused to *Xho*I/*Pvu*II-cleaved pBT346-*Xho*I to generate pCB539. The PCR amplified ε gene (from the ribosome-binding site up to the stop codons) was cloned into *Eco*RI/*Hin*dIII-cleaved pLEX (Diederich *et al.*, 1994). pCB298 was constructed by deleting the 196 bp *Sna*BI–*Bsp*HI DNA segment within the coding region of the ε gene.

Measurement of the half-life of the ε protein in protease-deficient *B. subtilis* cells. *B. subtilis* strains BG671, BG673, BG677, BG675 or BG669 (Table 1) bearing pSM19035-derived plasmids (pBT233-7-borne ωεζ operon or pBT233-2-borne ω and ε genes) (Ceglowski *et al.*, 1993b) were grown to mid-exponential phase in rich medium (LB) with aeration at 37 °C (under this condition the cell doubling time is 30±2 min). Rf (50 μg ml^{-1}) was added, and samples were collected at different time intervals. Aliquots of the cells were plated and the rest of the culture lysed. The cell extracts were separated, blotted to Hybond PVDF and Western blotted as previously described (Camacho *et al.*, 2002). Rabbit polyclonal antiserum against ε protein was used to detect the presence of the ε protein (Camacho *et al.*, 2002).

Assay for studies on the effect of ζ expression on the viability of *B. subtilis* or *E. coli* cells. *B. subtilis* BG689 cells containing the ζY83C variant or ζ-free BG687 cells were grown to 4×10^7–1×10^8 cells ml^{-1} in S7 minimal medium, 0·5 % xylose was then added, and samples were collected at different times and plated on LB medium without xylose unless otherwise indicated.

B. subtilis BG689 cells containing the ζY83C variant or ζ-free BG687 cells were grown to $\sim5\times10^8$ cells ml^{-1} in S7 minimal medium. Xylose was then added to 0·5 % and the cultures divided into five aliquots to avoid clonal selection. The selection for forward mutations was carried out by plating on solid agar medium containing Rf (10 μg ml^{-1}) (Rf^R mutants, spontaneous mutation frequency $3\cdot6\times10^{-8}$, $P<0\cdot0001$) or 0·5 % xylose (e.g. ζ^R or absence of the ζ cassette).

E. coli CC118 cells carrying pCB297 containing the ε gene under the control of an IPTG-dependent promoter and pCB298 containing ω and ζ genes (Camacho *et al.*, 2002) were grown in rich medium (LB) to $\sim7\times10^7$ cells ml^{-1}. IPTG was then removed by washing the cells with pre-warmed LB medium and growth was allowed to continue. At different times IPTG was added, samples were collected and plated on LB agar containing 0·2 % glucose and IPTG to a final concentration of 0·5 mM.

E. coli CC118 cells carrying plasmids pCB297 and pCB635 were grown to $\sim4\times10^7$ cells ml^{-1} in M9 minimal medium supplemented with 2 % (v/v) glycerol, 0·2 % glucose and 10 μM IPTG. Arabinose (0·2 %) was added to induce ζ expression. When indicated, 1 mM IPTG was added (to induce ε expression) and samples were taken at different times and plated on LB medium with 0·2 % glucose.

Rate of DNA, RNA and protein synthesis. *E. coli* CC118 cells bearing plasmids pCB297 and pCB635 were grown at 37 °C in M9 minimal medium plus 2 % glycerol and 0·2 % glucose to $\sim5\times10^7$ cells ml^{-1} then arabinose was added to a final concentration of 0·2 % (to induce ζ expression). Samples of 0·5 ml were taken at the time points indicated and added to 2·5 μCi [6-^{3}H]thymidine (DNA synthesis), 2·5 μCi [5-^{3}H]uridine (RNA synthesis) or 2·5 μCi L-[4,5-^{3}H]leucine (protein synthesis) [1 μCi = 37 kBq]. In addition, a sample was taken for enumeration of viable bacteria. After 1 min of incorporation, samples were chased for 2–3 min with 10 μg ml^{-1} of unlabelled thymidine, uridine or leucine, respectively. The samples were then incubated with lysozyme (2 μg ml^{-1}) for 2 min, and then cold TCA (added to a final concentration of 20 %) for 60 min on ice before centrifugation at 20 000 ***g*** for 30 min at 4 °C. Pellets were washed with 200 μl cold TCA 20 % and with 200 μl cold 96 % ethanol; finally, the precipitate was trapped on nitrocellulose filters, which were then dried and transferred to scintillation vials. Radioactivity was measured in a liquid scintillation counter and used to calculate the amount of radioactivity incorporated at each time point.

Fluorescence and electron microscopy. Exponentially growing *B. subtilis* BG689 or BG687 cells were obtained by inoculating overnight cultures in fresh LB medium and grown to $\sim5\times10^7$ cells ml^{-1} at 37 °C. At time zero, xylose was added, samples taken at different times, the cells fixed and the DNA stained with 4′,6′-diamino-2-phenylindole (DAPI) (0·2 μg ml^{-1}) for nucleoid visualization as described by Carrasco *et al.* (2004). To analyse membrane integrity, cells were stained with the membrane-permeant SYTO 9 and the membrane-impermeant propidium iodide, and examined by fluorescence microscopy (FM) as previously described (Carrasco *et al.*, 2004). SYTO 9, which stains all bacteria with green fluorescence, and propidium iodide, which stains 'membrane-compromised' bacteria with red fluorescence, were purchased from Molecular Probes (Leiden) and used as described previously (see Sanchez *et al.*, 2005). For electron microscopy (EM) sectioning, cells were fixed with glutaraldehyde, treated with osmium tetroxide and embedded in Spurr's low-viscosity medium (Carrasco *et al.*, 2004).

2D gel electrophoresis, image analysis and protein identification. Strains BG873 and BG871 were grown in BM medium (Stulke *et al.*, 1993) up to $\sim1\times10^8$ cells ml^{-1}. The proteins were then labelled with 10 μCi L-[^{35}S]methionine ml^{-1} for 5 min before (control) and at different times (10, 30 and 60 min) after 0·5 % xylose addition. L-[^{35}S]Methionine incorporation was stopped by the addition of 1 mg Cm ml^{-1} and an excess of unlabelled L-methionine (10 mM) on ice. The cells were disrupted by ultrasonic treatment, and the soluble protein fraction was separated from the cell debris by centrifugation. Incorporation of L-[^{35}S]methionine was measured by precipitation of aliquots of protein extracts with 10 % TCA on filter papers, as described previously (Bernhardt *et al.*, 1999). The protein content was determined using the Bradford assay (Bradford, 1976), and 80 μg of the L-[^{35}S]methionine-labelled protein extract was separated by 2D-PAGE using non-linear immobilized pH gradients (IPG) in the pH range 4–7 (Amersham Biosciences) and a Multiphor II apparatus (Amersham Pharmacia Biotech) as described previously (Bernhardt *et al.*, 1999). The gels were dried on filter paper, exposed to Phosphor screens (Molecular Dynamics) and detected with a PhosphorImager SI instrument (Molecular Dynamics). The image analysis was performed with the Decodon Delta 2D software (http://www.decodon.com), which is based on dual-channel image analysis (Bernhardt *et al.*, 1999). For identification of the proteins by mass spectrometry, non-radioactive protein samples of 200 μg were separated by preparative 2D-PAGE. The resulting 2D gels were fixed in 40 % (v/v) ethanol/10 % (v/v) acidic acid and stained with colloidal Coomassie brilliant blue (Amersham Biosciences). Spot cutting, tryptic digestion of the proteins and spotting of the resulting peptides onto the MALDI-targets (Voyager DE-STR, PerSeptive Biosystems) were performed using the Ettan Spot Handling Workstation (Amersham-Biosciences), according to the standard protocol described previously (Eymann *et al.*, 2004). The MALDI-TOF-TOF measurement of spotted peptide solutions was carried out on a Proteome-Analyser 4700 (Applied Biosystems) as described previously (Eymann *et al.*, 2004).

Transcriptome analysis. In these experiments the toxin effect of wt ζ was reversed by expression of ε antitoxin, which was under control of an IPTG-inducible promoter. *E. coli* XL-1 Blue cells containing both the plasmid pCB298, which provided constitutive expression of ω and ζ, and the plasmid pCB297, carrying the ε gene under the control of an IPTG-dependent promoter (Camacho *et al.*, 2002), were grown in LB medium up to $\sim2\times10^7$ cells ml^{-1} and then the IPTG was removed by washing the cells twice with pre-warmed LB medium before resuspending the cells in fresh medium to give up to $\sim1\times10^7$ cells ml^{-1}. The culture was then split into two equal volumes; 30 ml aliquots, representing zero time samples, were removed for RNA stabilization and subsequent isolation using the SV total RNA isolation system (Promega) according to the method described at www.ifr.bbsrc.ac.uk/safety/microarrays/protocols.html. IPTG was added to one of the cultures and then growth of both

cultures was continued. Cells were harvested at 10, 40 and 50 min time points for RNA stabilization and subsequent isolation. The total RNA concentrations were checked for their integrity and yield using UV spectrometry and an Agilent 21000 bioanalyser (Agilent Technologies) according to the recommended protocol. Transcriptome analysis by microarray hybridization using the *E. coli* microarray previously described (Anjum *et al.*, 2003) was undertaken according to the method of Mohedano *et al.* (2005). At least two biological replicates and two technical replicates (hybridizations) were included in the analysis for each time point. The subsequent data were initially analysed using a modified version of the expression analysis tool described by Pearson *et al.* (2003). Mean fluorescence intensities of differentially expressed genes in the zero time control and ζ toxin-induced samples were compared by regression analysis of the fluorescence intensity curve (at 10, 40 and 50 min) and scored as being differentially expressed if $P<0{\cdot}1$ for the F test. The numbers of genes affected by induction of ζ toxin were calculated for several different categories of stress response; see Results.

RESULTS AND DISCUSSION

Expression of the ζY83C toxin inhibits cell proliferation

Previously, it was shown that depletion of the ε_2 antitoxin causes ~10 000-fold reduction in the plating efficiency of wt *B. subtilis* cells bearing pBT233-7-borne $\omega\varepsilon\zeta$ genes, whereas the plating efficiency was not affected when cells carried only the pBT233-2-borne ω and ε genes (Camacho *et al.*, 2002). A strong reduction in the plating efficiency of *B. subtilis* wt, Δ*clpC*, Δ*clpE* or Δ*clpP* cells bearing pBT233-7-borne $\omega\varepsilon\zeta$ genes was observed upon exposure to 50 μg Rf ml^{-1} for 120 min (Fig. 2a). However, upon addition of 50 μg Rf ml^{-1} no reduction in the plating efficiency of *B. subtilis* Δ*lonA* cells bearing the pBT233-7-borne $\omega\varepsilon\zeta$ operon was observed (Fig. 2a) and the level of ε protein remained constant at least during the first 120 min (data not shown). The plating efficiency in Δ*clpX* cell showed an intermediate phenotype (Fig. 2a). Thus it is likely that depletion of the ε_2 antitoxin is compromised in the absence of the LonA protease, and to a minor extent in the absence of the ClpX chaperone.

The wt ζ toxin cannot be cloned in wt *E. coli* or *B. subtilis* cells in the absence of the ε_2 antitoxin (Sitkiewicz *et al.*, 1999). To study the effect of the ζ toxin in the absence of the ε_2 antitoxin a spontaneous ζ variant (consisting of a Tyr to Cys substitution at codon 83, ζY83C) was isolated from *B. subtilis* YB-pXZ *recA4* cells bearing a plasmid-borne ε gene (pBT322-2) after xylose induction. The DNA of the YB-pXζY83C *recA4* strain was used to transform wt YB886 competent cells, free of pBT322-2, to generate strain BG689. Similarly, a control strain (BG687) containing the cassette, but lacking the ζY83C gene, was constructed.

Under repressed conditions BG689 cells could be grown in the absence of the ε antitoxin gene. In the presence or absence of 0·5 % xylose (the inducer of P_{xylA}), the BG687 control strain had a doubling time and plating efficiency similar to the non-induced BG689 strain containing a single copy of the ζY83C gene integrated into the chromosome (data not shown).

Previously, it was shown that exponentially growing YB886 cells ($\sim 1\times10^8$ cells ml^{-1}) harbouring $\omega\varepsilon\zeta$ genes

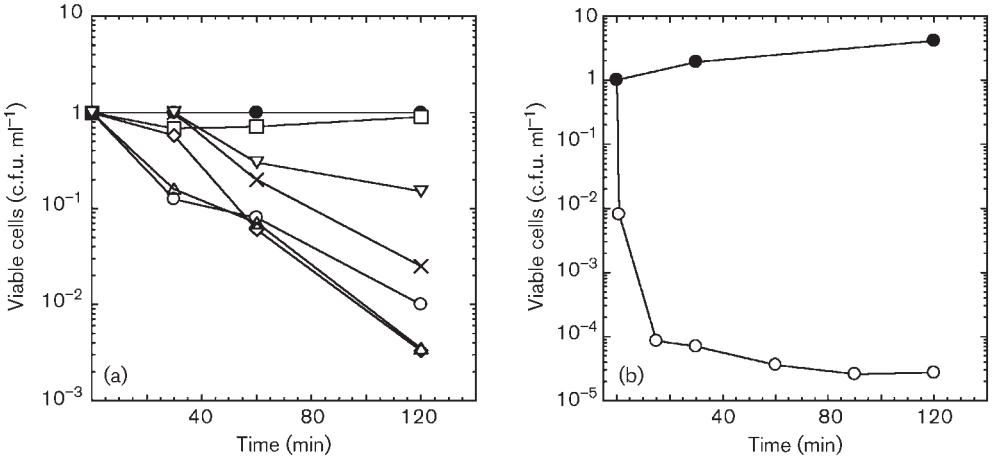

Fig. 2. The wt ζ or ζY83C toxin inhibits c.f.u. in *B. subtilis* cells. (a) Δ*lonA* (□), Δ*clpX* (▽), Δ*clpC* (×), Δ*clpE* (◇), Δ*clpP* (△) or wt (○) cells bearing pBT233-7-borne $\omega\varepsilon\zeta$ genes or pBT233-2-borne $\omega\varepsilon$ genes were grown in S7 medium. The behaviour of cells bearing pBT233-2-borne $\omega\varepsilon$ genes was analysed in wt (●), Δ*lonA*, Δ*clpX*, Δ*clpC*, Δ*clpE* or Δ*clpP* without significant differences; hence only the former is shown. Rf (50 μg ml^{-1}) was added at time zero and the number of c.f.u. determined. Survival curves represent means from at least three independent experiments. (b) The effect of ζY83C expression on c.f.u. was measured. BG689 cells were grown exponentially in S7 medium (●). To half of the culture 0·5 % xylose was added (○) to induce ζY83C transcription (time zero). To determine c.f.u., samples were withdrawn and various time points and appropriate dilutions spread on LB plates.

on pBT233-7 ($\sim$16 copies cell^{-1}) or pDB101 (1–2 copies cell^{-1}) produced $\sim$700 $\varepsilon_2\zeta_2$ and $\sim$50 $\varepsilon_2\zeta_2$ complexes per cell, respectively, and in both cases the half-life of ζ protein was longer than 60 min (Camacho *et al.*, 2002; data not shown). The ability to induce expression of the ζY83C gene fused to P_{xylA} was analysed by Western immunoblotting. With this gene as a single copy in the chromosome, the amount of induced ζY83C protein reached maximal levels 60 min after addition of xylose (0·5 %) and this was sufficient to halt cell proliferation (see Fig. 2b). When *B. subtilis* BG689 cells were grown to $\sim 1 \times 10^8$ cells ml^{-1}, and induced with 0·5 % xylose for 60 min, $\sim$300 ζY83C proteins were present per cell, but upon exposure to 50 μg Rf ml^{-1}, to halt de novo synthesis, the half-life of ζY83C was approximately twofold shorter compared to that of ζ toxin (e.g. pBT233-7 bearing cells) (Camacho *et al.*, 2002; data not shown).

B. subtilis BG689 cells were grown in S7 minimal medium to $\sim 5 \times 10^7$ cells ml^{-1} and expression of the ζY83C gene was induced by addition of 0·5 % xylose. An exponential decay in the number of c.f.u. ($\sim$10 000-fold reduction) was observed within the first 15 min after addition of xylose, compared to the uninduced strain (Fig. 2b). A similar reduction in the plating efficiency was previously reported for the wt ζ toxin ($\sim$8000-fold reduction in c.f.u. 120 min after addition of Rf; Camacho *et al.*, 2002) and for ζY83C ($\sim$7000-fold reduction in c.f.u. 120 min after Rf addition; our unpublished results) after depletion of the plasmid-encoded ε_2 antitoxin or after accumulation of ε-free ζ toxin ($\sim$5000-fold reduction in c.f.u. 120 min after addition of xylose; Zielenkiewicz & Ceglowski, 2005). It is likely, therefore, that (i) the ε_2 antitoxin neutralizes the toxic effect of both ζ and ζY83C toxins, (ii) traces of ε_2 efficiently delayed the toxic effects of ζ (see Fig. 2) and ζY83C toxins (data not shown), and (iii) the activity of both ζ and ζY83C toxins triggers cell stasis with similar efficiency, suggesting that the target site of the ζ variant (ζY83C) is the same as that of native ζ.

The small fraction of cells (2000–4000 cells ml^{-1}) that still formed colonies after induction of ζY83C expression (Fig. 2b) did not genetically acquire resistance to the toxin, as they regrew a new population that was just as sensitive to ζY83C as the parental strain. However, when BG689 cells ($\sim 5 \times 10^8$ cells ml^{-1}) were grown and plated in the presence of 0·5 % xylose (i.e. with constant exposure to the toxic action of ζY83C) few colonies were recovered (data not shown). Analysis of the surviving clones revealed that 85 % of them were still sensitive to the toxic effect exerted by ζY83C, and $\sim$14 % had DNA rearrangements on the ζY83C expression cassette. The remaining fraction ($1{\cdot}1 \times 10^{-7}$, $P<0{\cdot}0001$) was still sensitive to the reintroduction of a new plasmid-borne ζ gene, suggesting that none carried a mutation in the ζ target site. Recently, 28 clones that survived the effects of ζ expression were shown to contain deletions, insertions or point mutations in the ζ gene (Nowakowska *et al.*, 2005). It is unlikely, therefore, that any of the surviving clones recovered from our screens carried a mutation in the ζ target.

Production of the ζY83C toxin compromises the cell membrane of a small fraction of the cell population

To determine whether the 10 000-fold reduction in c.f.u. induced by expression of the ζY83C protein (Fig. 2b) correlated with a bacteriolytic or bacteriostatic state, BG689 cells (at $\sim 5 \times 10^7$ cells ml^{-1}) induced at 0·5 % xylose to express ζY83C protein for 60 min were stained with SYTO 9 (which stains all bacteria, green fluorescence) and with propidium iodide (which stains 'membrane-compromised' bacteria, red fluorescence). In the presence or absence of inducer $\sim$3 % of BG687 control cells (lacking the ζY83C gene) or BG689 cells in the absence of inducer were positively stained with propidium iodide after 60 min (see Sanchez *et al.*, 2005). In the presence of inducer, however, the proportion of propidium-iodide-stained BG689 cells increased to $\sim$17 % of the total SYTO 9-stained cells (Fig. 3a). The proportion of propidium-iodide-stained cells remained constant for at least 120 min. The fact that the c.f.u. count was reduced $\sim$10 000-fold, but fewer than 20 % of the cells were stained with propidium iodide, suggested that expression of ζ toxin mainly induced stasis. When cells expressing the ζY83C toxin were analysed by EM, defects in the cell morphology (e.g. 'holes' in the peptidoglycan layer) were observed in $\sim$18 % of the observed cells when compared to control cells ($\sim$2 %) (data not shown). To address whether ζ interacts with the cell membrane and/or cell wall a hybrid ζ-GFP variant was constructed. YB886 cells bearing the plasmid-borne *ωεζ-gfp* genes (five copies per cell) were grown up to $\sim 5 \times 10^7$ cells ml^{-1} and Rf was added. After 30 min of Rf addition the ε_2 antitoxin was degraded and the accumulation of ε-free ζ-GFP triggered the 10 000-fold reduction of c.f.u., suggesting that the ζ-GFP protein was active (data not shown). We failed, however, to detect the accumulation of ζ or ζ-GFP protein in the cell membrane or cell wall using anti-ζ polyclonal antibodies, immunogold labelling and EM, or ζ-GFP and FM techniques, respectively (data not shown). Hence, our data do not support the hypothesis that cell membrane and/or cell wall integrity was the direct target of ζ action.

Expression of the ζY83C toxin does not affect chromosomal segregation

To investigate any potential changes in chromosome dynamics the morphology of the nucleoid was analysed. Previously it was shown that absence of DAPI-staining material (anucleate cells) is rare ($<$ 0·1 %) in wt cells (Britton *et al.*, 1998). *B. subtilis* BG689 cells were grown in minimal medium up to $\sim 5 \times 10^7$ cells ml^{-1}, xylose was added to one half of the culture and 60 min after addition the nucleoids were stained with DAPI. The cells were fixed and visualized by FM. From the non-induced [no xylose (−Xyl) control] culture, absence of DAPI-stained material was observed in $\sim$0·3 % of total cells (Fig. 3b). When

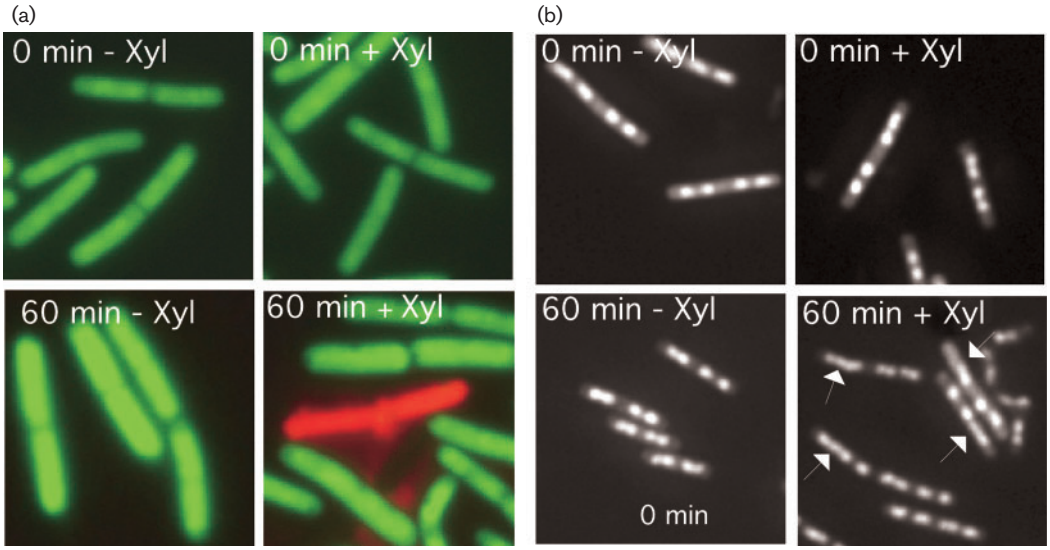

Fig. 3. Effect of ζY83C on membrane permeability (a) or nucleoid segregation (b). BG689 cells were grown exponentially in S7 medium. To half of the culture 0·5 % xylose was added to induce ζY83C transcription (time zero). Cells at time zero and 60 min (with or without xylose, +Xyl or −Xyl) were fixed, stained with SYTO 9 and propidium iodide and analysed by FM (a), or stained with DAPI and analysed by FM to visualize the nucleoid (b). The arrows denote the absence of the nucleoid or an uneven number of nucleoids.

ζY83C expression was induced, the length of individual cells was either marginally affected or unaltered when compared to cells of the non-induced control (Fig. 3b, 60 min +/− Xyl). Additionally, ζY83C expression increased the number of cells without DAPI-stained material to ∼4 % of total cells or a 13-fold increase when compared to the non-induced control. Thus it is likely that nucleoid segregation was not the primary defect, at least during the first 120 min of exposure to ζY83C toxin. Recently it was shown that when cells overexpressing ζ toxin were growing in rich medium cell length was reduced and absence of DAPI-stained material increased up to ∼15 % of total cells after 120 min of induction (Zielenkiewicz & Ceglowski, 2005).

Expression of ζY83C toxin triggers stasis without gross inhibition of protein translation

Previously it was shown that RelE and MazF (Kid) are toxins that inhibit protein translation in response to nutritional stress (Gerdes *et al.*, 2005). RelE cleaves mRNAs that are positioned at the ribosomal A-site (Pedersen *et al.*, 2003), whereas the ribosome requirement for MazF (Kid) mRNA cleavage is not obvious (Muñoz-Gomez *et al.*, 2005; Zhang *et al.*, 2003). To determine whether ζY83C protein affects protein translation, different experiments were performed. Concomitant with the inhibition of cell growth (see Fig. 2b) the ζY83C toxin reduced incorporation of radiolabelled thymidine (DNA synthesis), uridine (RNA synthesis) or leucine (protein synthesis) by less than threefold, within a 60 min window (data not shown). Thus the bulk synthesis of DNA, RNA or proteins did not seem to be grossly affected by the action of the ζY83C toxin.

To confirm that ζY83C did not markedly affect protein translation a proteomic analysis of cell expressing ζY83C was performed during a 60 min interval. The proteomic system was optimized for the measurement of methionine incorporation. Hence, *met*$^{+}$ variants of BG687 (BG873) and BG689 (BG871) were constructed (Table 1) and used to confirm that the loss of the *metB*5 marker did not affect activity. Indeed, upon induction of ζY83C expression with 0·5 % xylose for 60 min the increase in the OD_{500} of the culture was halted and a >1000-fold reduction in c.f.u. was measured. In contrast, growth of the BG873 control strain was unaffected by addition of xylose and the plating efficiency increased twofold (data not shown).

B. subtilis BG873 or BG871 cells were grown in BM medium to $\sim 1 \times 10^{8}$ cells ml^{-1} and xylose was added at different times. Then [^{35}S]methionine was added for 5 min and autoradiograms of the labelled proteins in strains BG873 (control) and BG871 (expressing ζY83C) were compared with the untreated control before and after addition of xylose. At 10 min after xylose addition, as expected XylA (xylose isomerase) was induced (data not shown) and after 60 min some pyrimidine metabolic proteins, GyrB, the catabolite control protein (CcpA), TufA-F2 fragments as well as other proteins (red spots in Fig. 4) were induced in both strains. Repression of MetE, Hag, ClpP, a TufA-F1 fragment, and some other oxidative-stress-responsive proteins (SodA and those belonging to the PerR regulon, i.e. AhpC, AhpF, KatA) (all labelled in green) were repressed in both strains 30 min (data not shown) and 60 min after addition of xylose (Fig. 4). From the ∼700 proteins that we could identify in the Coomassie-stained cytoplasmic

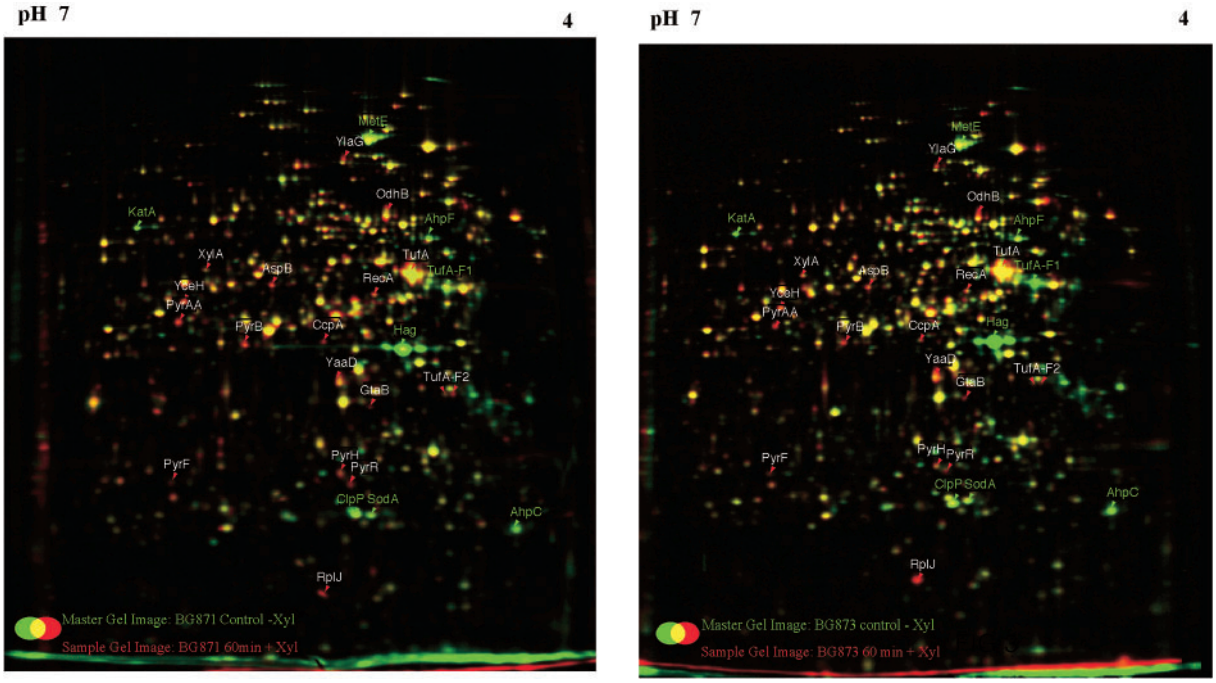

Fig. 4. Analysis of global protein synthesis of *B. subtilis* BG871 expressing ζY83C (a) and the control strain BG873 (b) before (green image) and 60 min after the addition of 0·5 % xylose (Xyl) (red image). Cytoplasmic proteins were labelled with L-[^{35}S]methionine and separated by 2D-PAGE as described in Methods. The pH gradient is indicated. Image analysis of the autoradiograms was performed using the Decodon Delta 2D software. Proteins that are synthesized at increased or decreased levels upon xylose addition are indicated by red spots (white labels) or green spots and labels, respectively.

proteome (or ∼40 % of all theoretically expressed proteins in the pH range 4–7) we failed to detect any difference between the strains (Fig. 4). From these results we can conclude that (i) upon xylose addition the expression of a few abundant proteins was modified even in the absence of the ζY83C toxin, and (ii) under conditions of ζY83C expression that lead to cell stasis (see Fig. 2b), protein synthesis was not grossly distorted (Fig. 4). Thus in contrast to RelE and MazF (Kid) (see above), the ζY83C toxin did not have major effects on protein translation.

Expression of the ε antitoxin reverses the toxic effect exerted by the ζ toxin

Previously it was shown that (i) the ζ toxin was active in *B. subtilis*, *E. coli* and even *Saccharomyces cerevisiae* cells (Sitkiewicz *et al.*, 1999), and (ii) repression of the ε gene, transcribed from a pCB297-borne ε gene under the control of a strong hybrid LacI-regulated promoter, led to a reduction of c.f.u. of *E. coli* cells bearing pCB298-borne ωζ genes (transcribed from P_{ω}, which is constitutively expressed) (Camacho *et al.*, 2002). This system was therefore established in *E. coli*, where a larger number of tools for regulated gene expression are available, so that reversal of the effects of the ζ toxin, by IPTG induction of the antitoxin ε, could be investigated.

E. coli CC118 cells bearing plasmids pCB297 (∼15 copies per cell) and pCB298 (∼200 copies per cell), were grown in M9 medium to a density of $\sim 7 \times 10^6$ cells ml^{-1}, then IPTG was washed out and the culture split into two aliquots (Fig. 5, denoted by a filled arrow). One aliquot was incubated without IPTG (to repress ε expression) while 1 mM IPTG was added back to the second aliquot after 60 min to induce ε expression. In the absence of IPTG the OD_{500} ceased to increase after ∼100 min (data not shown) and the number of c.f.u. decreased >700-fold after 360–420 min, when compared to the IPTG control culture (ε expressed) (Fig. 5).

To determine whether the toxic effect of ζ overexpression (∼200 copies of the ζ gene per cell) can be reversed by ε antitoxin expression, *E. coli* CC118 cells bearing plasmids pCB297 and pCB298 were grown in M9 medium to $\sim 5 \times 10^7$ cells ml^{-1}, IPTG was washed out and the culture split into two aliquots (Fig. 5, denoted by a filled arrow). As before, one aliquot was incubated without IPTG and to the other one 1 mM IPTG was added to induce expression of ε. Thereafter at 60 min intervals the culture without IPTG was again divided into two aliquots, and IPTG was added to one aliquot to induce ε expression (Fig. 5, denoted by an empty arrow). Within the first 240 min of exposure to the toxic effect of ζ the expression of ε_2 antitoxin (IPTG readded after wash) reversed the reduction in c.f.u. (Fig. 5). This

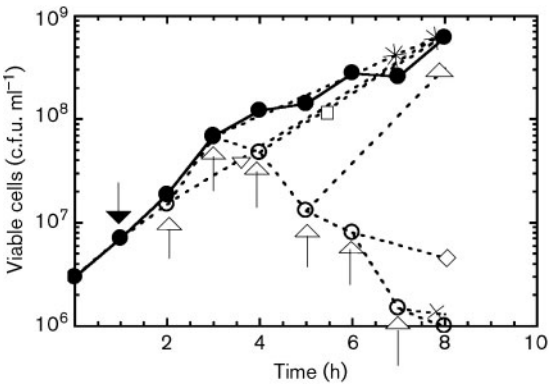

Fig. 5. The ε antitoxin partially reverses ζ toxin activity. *E. coli* CC118 cells bearing pBT297-borne P_{lacO}-ε and pBT298-borne P_{cat}-ωζ genes were grown in M9 medium (●). IPTG was removed by washing (time zero, denoted by a filled arrow) and the culture further incubated. At various time points samples were withdrawn and split in two aliquots. To one aliquot 1 mM IPTG was added to induce ε expression (empty arrows) at 60 (⋆), 120 (▽), 180 (□), 240 (△), 300 (◇) or 360 (×) min and samples were further incubated. To determine c.f.u., samples were withdrawn at various time points and spread on LB plates supplemented with 1 mM IPTG, 15 μg Cm ml^{-1} and 50 μg Ap ml^{-1}.

suggested that ζ-induced stasis was a reversible state because the number of c.f.u. recovered after ε expression even after 240 min of ζ action. Similar results were observed with the RelBE or MazEF TA systems, but here cells were growing in rich media (Pedersen *et al.*, 2002). However, when the cells were exposed for more than 240 min to the toxic effects of ζ toxin (i.e. incubation without IPTG) induction of ε expression, by addition of 1 mM IPTG, did not allow the recovery of the number of c.f.u. (Fig. 5). Similar results were observed with the MazEF system (Amitai *et al.*, 2004; Engelberg-Kulka *et al.*, 2004).

To determine the proportion of cells that after 240 min exposure to ζ action were incapable of proliferation on nutrient agar from those with a compromised membrane ('metabolically inactive'), the cells were stained with SYTO 9 and propidium iodide. About 22 % of SYTO 9-stained cells were also stained with propidium iodide (data not shown). It is likely, therefore, that expression of ζ elicits bacteriostasis that might be reversed by production of its cognate ε antitoxin, whereas the remaining fraction (~20 %) of the cells, which were stained with propidium iodide, might die.

Effect of ζ induction on gene expression

To gain insight into the molecular mechanism(s) that govern the VBNC or the cell death state, due to expression of ζ toxin, the pattern of gene expression was analysed at an early time of ζ action to avoid any secondary effect. Furthermore, to avoid a gratuitous induction of the RelE and/or MazF toxins a *relA recA* background was selected (see Engelberg-Kulka & Glaser, 1999; Godoy *et al.*, 2006). *E. coli* XL-1 Blue cells bearing plasmids pBT297 and pBT298 were grown in LB medium to ~1×10^7 cells ml^{-1}. The culture was split into two aliquots: one remained as it was (control strain, with inducible ε and constitutive ωζ expression) and IPTG was removed from the other one by washing (to deplete the antitoxin ε), and both cultures were incubated further. In the previous section it was shown that from 60 to 80 min after removal of IPTG a reduction in c.f.u. was observed, indicating that these time points should reveal early effects of the ζ toxin on the transcriptome. Duplicate or triplicate samples were harvested at 10, 40 and 50 min after removal of IPTG for RNA isolation and hybridization to spotted amplicon microarrays of the *E. coli* genome (Anjum *et al.*, 2003). RNA samples at 10, 40 and 50 min post-depletion time points were compared with their 'time zero' RNA sample. This approach allowed both for comparison of data from different time points within an experiment, and also for comparison of data from similar independent experiments. We compared the transcripts altered by depletion of the ε antitoxin (and thus accumulation of ε-free ζ toxin) with those of a control strain lacking the ζ toxin by regression analysis of the mean fluorescence intensities over time (i.e. at 10, 40 and 50 min after removal of IPTG). This was necessary to identify genes that were altered due to specific effects of the ζ toxin rather than wash-out of IPTG using fresh medium. Accumulation of ζ toxin in the background inhibits cell growth, reduces the number of c.f.u. and alters translation of ~70 genes at 50 min post-repression of ε expression. Previously, it was shown that seven regulatory proteins (namely CRP, IHF, FNR, Fis, ArcA, H-NS and Lrp) are sufficient for directly modulating the expression of 51 % of the genes in *E. coli* (Martinez-Antonio & Collado-Vides, 2003). The rate of transcription of these seven global regulatory proteins was not significantly affected when compared to the control strain (expressing the ε antitoxin), suggesting that the global control of basal level gene expression by altering the chromosome structure is not the main target of ζ.

The ζ toxin significantly altered ($P<0{\cdot}1$) the transcription of only 26 essential genes (*cdsA*, *dapB*, *dfp*, *hisS*, *infB*, *lgt*, *murE*, *nadB*, *nrdA*, *pyrG*, *proC*, *pth*, *rpoB*, *rpsB*, *rplD*, *rplJ*, *secD*, *thrS*, *tktA*, *topA*, *trpS*, *tsf*, *tufA*, *ychF*, *yejE* and *yciL*). We see no obvious link between these essential genes and expression of other members of their respective pathways as they were not uniformly affected upon accumulation of ζ.

The ζ-induced VBNC state resembles the loss of culturability observed when bacteria enter stationary phase. During stationary phase various regulatory networks are activated (Nystrom, 1999). The lists of genes affected by production of ζ toxin, in the absence of the ε antitoxin, were therefore compared with several known categories of stress-response genes to see whether specific stress pathways such as starvation, stationary phase, SOS response, etc., were associated with the mechanism of action of the ζ toxin (Table 2). Previously it was shown that stress-induced stasis relies to a

large extent on a single regulator, RpoS (Hengge-Aronis, 1993; Nystrom, 2003). As shown in Table 2 and the supplementary data (Table S1, available with the online version of this paper), the level of *rpoS* and *rpoS*-controlled genes (Hengge-Aronis, 1993), starvation-induced stasis genes (Nystrom, 2003) or oxidative stress genes were not uniformly affected (less than 16 % of known response genes) upon accumulation of ζ protein in the absence of ε antitoxin. Transcription of the starvation-induced stasis genes *relA* and *dnaK*, the oxidative stress genes *rpoE* and *arcA*, the peroxidase dismutase genes *sodA* and *sodB*, or catalase genes *katE* and *katG* was not significantly altered upon ζ toxin accumulation, but inhibition of *spoT* expression, which might cause accumulation of ppGpp, was observed (Table 2 and Table S1). Similarly, expression of the *recA* gene was not affected and only 22 % of known SOS genes were altered by accumulation of ζ toxin, indicating that cell death cannot be simply attributed to the accumulation of un-repaired double-strand breaks (Table 2, Table S1). Furthermore, an increase in the rate of mutations by stress-induced stasis was not observed. This is consistent with (a) the lack of filamentation upon ζ induction (see above), and (b) the hypothesis that protein and/or DNA oxidation could not be the main reason of the observed cell death. It is likely, therefore, that ζ expression was not affecting any pathway specifically and that the ζ toxin seems to have pleiotropic effects. The ζ-exerted effect on many of the genes associated with known stress-induced pathways cannot lead to PCD and is only affecting a relatively small proportion of the genes in each pathway (i.e. 6·7–22·7 %).

We also analysed the list of genes that affected TA systems by accumulation of ζ toxin. We observed that ζ altered transcription of ∼12·5 % of putative TA or cell killing genes (Table 2, Table S1), but induction or repression of bona fide cell-killing systems upon ζ accumulation was not observed.

Table 2. Comparison of genes affected by ζ expression with known stress-response genes

Stress response	No. of genes evaluated*	Genes altered by toxin (%)†
TA systems and cell killing	8	12·5
Stationary phase‡	110	15·5
Starvation	19	10·5
SOS	22	22·7
Radiation	13	15·3
Detoxification	20	0
Aerobic/anaerobic	22	9·1

*Number of genes associated with stress response and included in the regression analysis. References of the relevant genes can be obtained from http://genolist.pasteur.fr/Colibri/.

†Based on the number of genes for which data were available for each time point after toxin induction.

‡Includes RpoS-regulated and other stationary-phase genes.

Excess of ζ can be partially reversed by ε expression

To investigate whether the reversible effect of ζ was dose or time dependent a new plasmid system, in which the expression of the ζ protein could be controlled, was constructed. The pCB635-borne ζ gene, under the control of the strong AraC regulated (P_{araBAD}) promoter (∼20 copies per cell) and pCB297 (∼15 copies per cell) were used. We assumed that the presence of ∼20 copies of the ζ gene per cell under the control of a strong promoter (pBT635-borne P_{araBAD}-ζ gene) should lead to high overexpression. Indeed, high expression of the ζ gene from pCB635 led to accumulation of amounts of ζ toxin that can be easily detected by Coomassie blue stained SDS-polyacrylamide gels.

E. coli CC118 cells bearing pCB297 and pCB635 were grown in M9 minimal medium supplemented with 0·2 % glucose and with the minimal amount of IPTG (0·05 mM) compatible with cell growth to $\sim 4 \times 10^7$ cells ml^{-1}, then arabinose (0·2 %) was added to induce ζ expression (Fig. 6a, filled arrow). After arabinose addition OD_{500} increased during the first 30 min and then decreased (Fig. 6a). The number of c.f.u. decreased ∼1000-fold after 30 min and >10 000-fold after 90 min exposure to ζ action when compared with the non-induced control (Fig. 6b).

At 30, 60, 90, 120 and 180 min after the induction of ζ protein, samples were withdrawn and split into two aliquots. To one aliquot 1 mM IPTG and 0·2 % glucose were added to induce ε expression and to reduce ζ expression (Fig. 6, empty arrows). After 30 min of ε induction the OD_{500} increased, and the number of c.f.u. completely recovered (Fig. 6a, b). However, when cells were exposed to the ζ toxin for 60 min or more, expression of ε only partially reversed the inhibitory effect on c.f.u. (∼100-fold recovery) (Fig. 6b). A comparison of the data shown in Figs 5 and 6 suggested that (i) the bacteriostatic effect of the ζ toxin and the time window for ε_2 reversal of ζ toxicity were dose-dependent, and (ii) killing of part of the population might take place. These dual effects could be explained if we assumed that the VBNC state is a 'physiological adaptation' but under prolonged conditions of stasis under normal or moderate overexpression, the enzymes required for macromolecular synthesis may be depleted (normal decay), the DNA damaged and the energy supply exhausted. However, when ζ was overexpressed the period of time in which bacteriostasis is reversible was markedly reduced.

The ζ toxin inhibits replication, transcription and translation

Previously it was shown that cells exposed to MazF toxin for a long period reach a point of no return with subsequent cell death and it was postulated that this may correlate with the synthesis of new product(s) leading to PCD (see Engelberg-Kulka *et al.*, 2004). Alternatively, the partial reversion by ε_2 of the growth-arrested cells exposed to moderate or high concentrations of ζ could be attributed to a pleiotropic

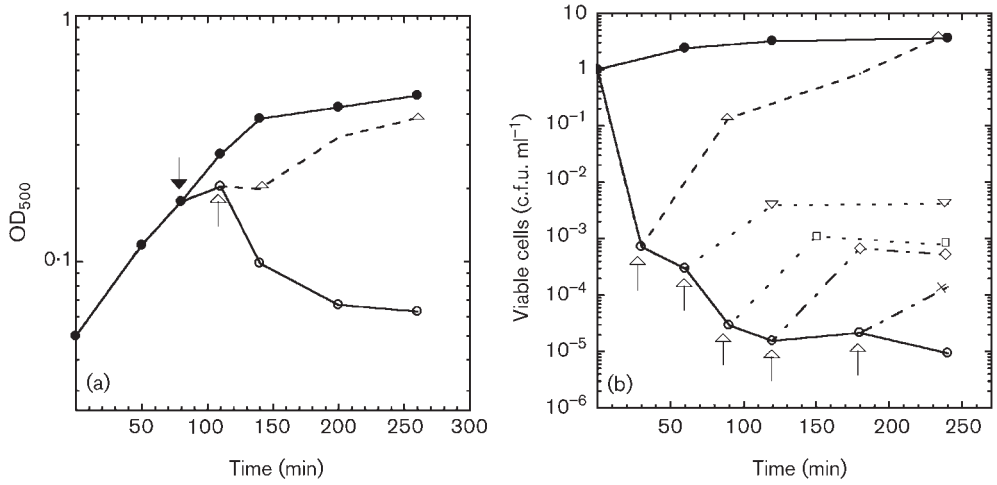

Fig. 6. Overexpressing wt ζ inhibits c.f.u. in *E. coli* cells and ε fails to reverse the ζ effect. *E. coli* CC118 cells bearing pBT297-borne P_{lacO}-ε and pCB635-borne P_{araBAD}-ζ genes were grown in M9 supplemented with 0·2 % glucose and 0·05 mM IPTG (●). Arabinose (0·2 %) was added (time zero, denoted by a filled arrow) to induce ζ expression (○). (a, b) At various time points samples were withdrawn and split in two aliquots. To one of them 1 mM IPTG (empty symbols), to induce ε expression, was added at 30 (△), 60 (▽), 90 (□), 120 (◇) or (×) 180 min and samples were further incubated. (b) To determine c.f.u. samples were withdrawn at various time points and spread on LB plates supplemented with 1 mM IPTG, 100 μg Ap ml^{-1} and 35 μg Km ml^{-1}.

effect. To investigate the physiological state of the cell a pulse–chase experiment was performed in *E. coli* CC118 cells bearing pCB297 and pCB635 that were grown in M9 minimal medium with 0·2 % glucose to $\sim 5 \times 10^7$ cells ml^{-1}, after which time arabinose (0·2 %) was added to induce high levels of ζ expression (time zero). At different times after induction of ζ protein expression the incorporation of radiolabelled material (over a 1 min time window) into freshly synthesized DNA, RNA and proteins was measured. At 60 min after induction of ζ the physiological state of the cells was altered, because incorporation of radioactivity during the 1 min pulse into DNA and RNA was reduced by ~50-fold, but protein synthesis was reduced ~6-fold (Fig. 7). Similar results for protein synthesis were observed when the well-defined protein synthesis inhibitor Cm (50 μg ml^{-1}) was added (data not shown). Under starvation-induced stasis, protein synthesis was also observed even in the absence of exogenous nutrients (Matin, 1991). This is consistent with the hypothesis that ongoing protein synthesis is a prerequisite for ε_2 reversal of the effects elicited by ζ.

Since, DNA, RNA and protein synthesis are inhibited (this work) and ζ may act as phosphotransferase using NTP to phosphorylate an as-yet-unidentified substrate (Meinhart *et al.*, 2003) we hypothesized that ζ toxin might exert a pleiotropic effect on the physiological state of the cells leading to killing of part of the cell population.

Overproduction of ζ leads to death of a subpopulation of cells

To determine whether ζ exerted a pleiotropic effect leading to cell death, the change in the morphology of the bacteria

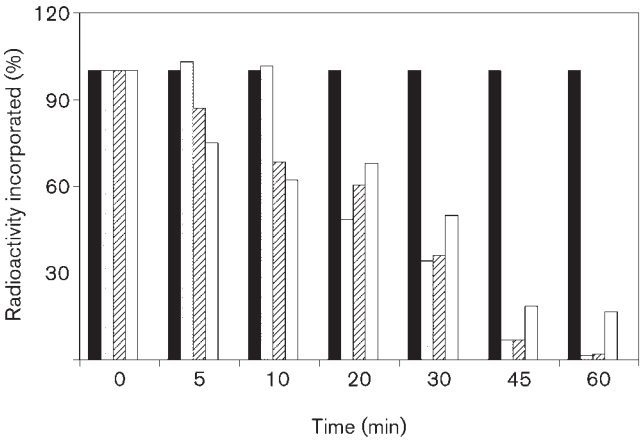

Fig. 7. Effect of ζ overproduction on cell physiology. *E. coli* CC118 cells bearing pBT297-borne P_{lacO}-ε and pCB635-borne P_{araBAD}-ζ genes were grown in M9 supplemented with 0·2 % glucose and 0·05 mM IPTG. At time zero the culture was divided into two aliquots and arabinose (0·2 %) was added to one sample to induce ζ expression. At various time points samples were withdrawn and 2·5 μCi [6-^{3}H]thymidine (DNA synthesis, stippled bars), 2·5 μCi [5-^{3}H]uridine (RNA synthesis, hatched bars) or 2·5 μCi L-[4,5-^{3}H]leucine (protein synthesis, white bars) added. The black bars denote DNA, RNA or protein synthesis in the absence of ζ induction, taken as 100 %. After a 1 min pulse of radioactivity incorporation, samples were chased for 2 min with an excess of unlabelled thymidine, uridine or methionine; cells were then lysed, the DNA, RNA or proteins precipitated and incorporated radioactivity measured in a scintillation counter.

was studied by EM. *E. coli* CC118 cells bearing pCB297 and pCB635 were grown in LB with 0·2 % glucose to $\sim 5 \times 10^7$ cells ml^{-1}, then 0·2 % arabinose was added to induce high ζ overexpression (time zero). At 60 or 90 min after addition of arabinose the cells were harvested and embedded for EM studies. As revealed in Fig. 8(d), induction of high ζ overexpression led to clumping of the cytoplasm, loss of membrane integrity and the presence of ghost cells (cells which have no cytoplasm but still have a cell wall) as compared to control cells. At 60 min, an increase in cytoplasmic clumping was seen and about 50 % of the cells (276 total cells analysed) appeared as ghosts. The irreversible loss of membrane integrity in up to 50 % of total cells revealed that a fraction of the cell population dies. We assumed that collapse of the membrane potential caused the irreversible loss of membrane integrity and cell death. This is consistent with our failure to detect the accumulation of the active ζ-GFP fusion on the cell membrane (data not shown).

Recently it was proposed that TA systems provide bacteria with a system for altruistic PCD in which part of the population is sacrificed to enable the rest to survive on the nutrients leaking out of the dead siblings (Aizenman *et al.*, 1996). Prolonged exposure to constitutive expression of the 200 copies (Fig. 5) or high overexpression of the 20 copies of the ζ gene (Fig. 6) led to death of a fraction (20–50 %) of the cell population.

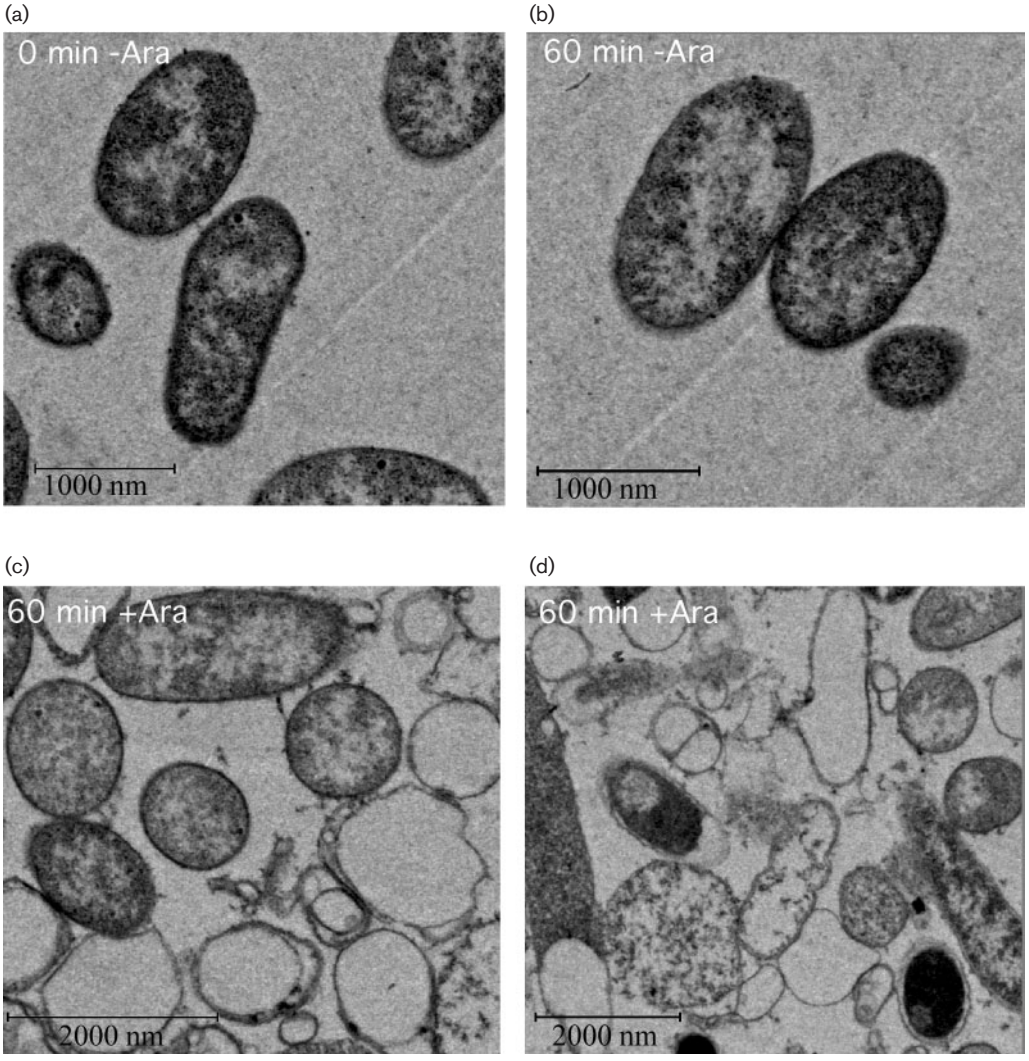

Fig. 8. Electron micrographs of *E. coli* cells overproducing ζ. *E. coli* CC118 cells bearing pBT297-borne P_{lacO}-ε and pCB635-borne P_{araBAD}-ζ genes were grown in M9 supplemented with 0·2 % glucose and 0·05 mM IPTG. At time zero arabinose (0·2 %) was added to induce ζ expression. (a) Cells at time zero either in the presence or absence of arabinose (−Ara); only the latter condition is shown. (b) Cells after 60 min of incubation in the absence of arabinose. (c, d) Cells after 60 min of incubation with 0·2 % arabinose.

Conclusions

This work shows that different outcomes can be expected from exposure of bacteria to different amounts of the ζ toxin. At 'normal' levels ζ or ζY83C toxin induces a VBNC state in the majority of the cells and 'killing' of a fraction of the population (~20 %), whereas at very high levels of ζ 'killing' of ~50 % of the cell population occurs. It is likely that ζ triggers stasis and the nutrients released by the fraction of the dead siblings allow cells to survive the stress condition with ε antitoxin expression reversing the ζ-exerted shutdown.

Unlike RelE, MazF or Kid (Muñoz-Gomez *et al.*, 2005; Pedersen *et al.*, 2003; Zhang *et al.*, 2003), expression of ζY83C appeared not to affect synthesis of the bulk of proteins in *B. subtilis* cells, and high overexpression of ζ did not selectively impair protein synthesis in *E. coli* cells. Chromosomal segregation, DNA topology and cell division were not the main target of ζY83C expression as described for the CcdB toxin. It was shown that CcdB uncouples replication and cell division by directly inhibiting the action of DNA gyrase (Bernard & Couturier, 1992; Miki *et al.*, 1984). It is likely, therefore, that under physiological ζ concentrations (i.e. one copy of the induced ζY83C) the toxin provides a control mechanism that triggers a VBNC state to help bacteria to adjust the rates of intracellular metabolic processes (DNA, RNA or protein synthesis is reduced less than threefold within a 60 min time window) under adverse environmental conditions.

In *E. coli* high non-physiological concentrations of the ζ gene (i.e. 200 copies expressed from a constitutive promoter – 'overexpression' – or from 20 copies expressed by a strong promoter – 'high overexpression') induced a VBNC state that was reversed by the expression of the ε antitoxin during a given time window. Bacteriostasis induced by 'overexpression' of ζ was fully reversible by subsequent expression of the ε antitoxin during a time window of 240 min, suggesting that this TA system serves as a checkpoint control for cellular processes that should be downregulated in growth-arrested cells, rather than as an initiator of PCD (Gerdes *et al.*, 2005). However, after prolonged exposure to ζ, expression of ε cannot reverse the growth-arrested state. 'High overexpression' of ζ reduced the time window of partial reversibility by the ε antitoxin to 60 min. At non-physiological concentrations of ζ the nutritional stress and growth arrest might trigger an orchestrated organized PCD as previously postulated (Engelberg-Kulka & Glaser, 1999). In this elaborate strategy for cell death a fraction of the population dies, releasing nutrients (altruistic principle) to be used by the sibling cells to overcome the growth arrest. However, the inhibition of bulk RNA or protein synthesis at physiological levels or moderate excess of ζ protein was not observed and the presence of a supra-regulator, controlling many different stress response systems, was not obvious. Alternatively, the prolonged exposure to 'overexpressed' ζ or short exposure to 'highly overexpressed' ζ toxin might exert pleiotropic effects via secondary targets with a subsequent loss of proofreading and/or erroneous incorporation of residues in RNA, DNA or proteins. The death of a subpopulation allows the remaining cells, upon ε expression, to recover from the growth-arrested mode by using nutrients released from their dead siblings.

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