pSM19035-encoded \( \zeta \) toxin induces stasis followed by death in a subpopulation of cells

Virginia S. Lioy,1 M. Teresa Martín,1 Ana G. Camacho,1 Rudi Lurz,2 Haike Antelmann,3 Michael Hecker,3 Ed Hitchin,4 Yvonne Ridge,4 Jerry M. Wells4,5 and Juan C. Alonso1

1Department of Microbial Biotechnology, Centro Nacional de Biotecnología, CSIC, 28049 Madrid, Spain
2Max-Planck-Institut für molekulare Genetik, D-14195 Berlin, Germany
3Institut für Mikrobiologie, Ernst-Moritz-Arndt-Universität, D-17487 Greifswald, Germany
4Department of Food Safety Science, BBSRC Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich Research Park, Colney, Norwich NR4 7UA, UK
5University of Amsterdam, Swammerdam Institute of Life Sciences, 1018 WV Amsterdam, The Netherlands

The toxin–antitoxin operon of pSM19035 encodes three proteins: the \( \alpha \) global regulator, the \( \varepsilon \) labile antitoxin and the stable \( \zeta \) toxin. Accumulation of \( \zeta \) toxin free of \( \varepsilon \) antitoxin induced loss of cell proliferation in both Bacillus subtilis and Escherichia coli cells. Induction of a \( \zeta \) variant (\( \zeta^{Y83C} \)) 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 \( \zeta \) toxin induced stasis, but this was fully reversed by expression of the \( \varepsilon \) antitoxin within a defined time window. The time window for reversion of \( \zeta \) toxicity by expression of \( \varepsilon \) antitoxin was dependent on the initial cellular level of \( \varepsilon \).

After 240 min of constitutive expression, or inducible expression of high levels of \( \zeta \) toxin for 30 min, expression of \( \varepsilon \) failed to reverse the toxic effect exerted by \( \zeta \) in cells growing in minimal medium. Under the latter conditions, \( \zeta \) inhibited replication, transcription and translation and finally induced death in a fraction (~50%) of the cell population. These results support the view that \( \zeta \) interacts with its specific target and reversibly inhibits cell proliferation, but accumulation of \( \zeta \) 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.

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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., 2003; Pedersen 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 unrelated to the seven previously described TA families (see above). The crystal structure of the biologically non-toxic monomeric ε2ζ complex contains ε2 and ζ hetero-tetramer complex (Camacho et al., 2002; Meinhart et al., 2003; Fig. 1). The cytotoxic effects of the elongated monomeric ζ protein are counteracted by the dimeric ε (ε2ζ) antitoxin that forms a stable ε2ζ hetero-tetramer complex (Camacho et al., 2002; Meinhart et al., 2003; Fig. 1). Interactions between ε2 and ζ are primarily mediated by the C-terminal domain of ε (Meinhart et al., 2003). Previously it was shown that ζ protein has a significantly lower thermodynamic stability than ε2 protein in both the free and the complex state (Camacho et al., 2002). Proteolytic studies indicate that ζ protein is more stable in the ε2ζ complex than in the free state (Camacho et al., 2002). In vivo studies, however, reveal a short half-life of the ε antitoxin (~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 ε2 antitoxin is observed (Camacho et al., 2002; Fig. 1).

The crystal structure of the biologically non-toxic ε2ζ2 protein revealed that the tetrameric ε2ζ2 complex contains ε2 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 ε2ζ2, 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 εζ 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

<table>
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<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<td><strong>B. subtilis</strong> strains</td>
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<td>Y8886</td>
<td>trpC2 metB5 sigB37 attSP β xin-1</td>
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<td>YB-pX</td>
<td>+ recA4 amyE::xylR-P_{spC}</td>
<td>Ziekenkiewicz &amp; Ceglowski (2005)</td>
</tr>
<tr>
<td>YB-pX;</td>
<td>+ recA4 amyE::xylR::P_{xylA};ζ</td>
<td>Ziekenkiewicz &amp; Ceglowski (2005)</td>
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<td>BG687</td>
<td>+ amyE::xylR-P_{spC}</td>
<td>This work</td>
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<td>BG783</td>
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<td>BG689</td>
<td>+ amyE::xylR::P_{xylA};ζ Y83C</td>
<td>This work</td>
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<tr>
<td>BG871</td>
<td>+ amyE::xylR::P_{xylA};ζ Y83C (met&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>BG673</td>
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<tr>
<td>BG677</td>
<td>+ clpE::spc</td>
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<td>BG675</td>
<td>+ clpP::spc</td>
<td>This work</td>
</tr>
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<td>BG669</td>
<td>+ lonA::cat</td>
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<td>+ clpX::ery</td>
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<td>Stratagene</td>
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<tr>
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<td>[P&lt;sup&gt;+&lt;/sup&gt; proAB lacZAM15 Tn10 (Tet&lt;sup&gt;+&lt;/sup&gt;)] lac endA1 gycA96 thi-1 hsdR17 supE44 relA1 recA1</td>
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<td><strong>Plasmids</strong></td>
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<td>pBT233-7</td>
<td>Rep&lt;sub&gt;Inc18&lt;/sub&gt;, ωɛ&lt;sup&gt;+&lt;/sup&gt; operon</td>
<td>Ceglowski et al. (1993b)</td>
</tr>
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<td>Ceglowski et al. (1993b)</td>
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<td>Ceglowski et al. (1993b)</td>
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<td>pCB298</td>
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<td>Camacho et al. (2002)</td>
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<td>pFus2</td>
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<td>Lemonnier et al. (2000)</td>
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<td>Lemon &amp; Grossman (1998)</td>
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<td>pCB635</td>
<td>Rep&lt;sub&gt;PM1&lt;/sub&gt;, araC-P&lt;sub&gt;araBAD&lt;/sub&gt;-ζ gene, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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</tbody>
</table>

*Rep<sub>PM1</sub>, Rep<sub>InSA</sub> and Rep<sub>ColE1</sub> are E. coli-compatible replications. The compatible replications of B. subtilis plasmids are Rep<sub>Inc18</sub> (a pSM19035 derivative) and Rep<sub>pol</sub> (a pTA1060 derivative).*

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

### 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::ε, clpC::ε, clpE::ε, clpP::ε 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 recA4 [xylrose repressor (XylR)-xylrose regulated promoter (P_{xylA})-ζ gene-cat gene] or (YB-pX) recA4 [XylR-P_{xylA};ζ 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_{stat}-cat or XylR repressor-P_{xylA};ζY83C-cat cassette was transferred to Y888, generating strains BG673 and BG689, respectively, and to Y886 (Met<sup>+</sup>), generating strains BG873 and BG871 (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 XhoI site was inserted just before the stop codon of the ζ gene on pBT346, to generate pBT346-Xhol. The Xhol--SpeI DNA fragment containing the gfpmt1 gene (Lemon & Grossman, 1998) was fused to Xhol-PvuI-cleaved pBT346-Xhol to generate pCB539. The PCR amplified ε gene (from the ribosome-binding site up to the stop codons) was cloned into EcoRI/HindIII-cleaved pLEX (Diederich et al., 1994), pCB298 was constructed by deleting the 196 bp StuI-BspHI DNA segment within the coding region of the ε gene.
Measurement of the half-life of the $\epsilon$ 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 or pBT233-2-borne $\omega$ and $\epsilon$ genes) (Ceglowski et al., 1993b) were grown to mid-exponential phase in rich medium (LB) with agitation 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 antisera against $\epsilon$ protein was used to detect the presence of the $\epsilon$ protein (Camacho et al., 2002).

Assay for studies on the effect of $\zeta$ expression on the viability of B. subtilis or E. coli cells. B. subtilis BG689 cells containing the $\zeta$Y83C variant or $\zeta$-free BG687 cells were grown to $\sim 5 \times 10^8$ 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 aliquots to avoid clonal selection. The selection for forward mutations was performed on LB medium without xylose unless otherwise indicated. BG689 cells containing the $\zeta$-free cells were obtained by inoculating E. coli BM10 cells (control) and at different times (10, 30 and 60 min) after 0.5% xylose addition. 1-[35S]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 1-[35S]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 1-[35S]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) acetic 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-Analyzer 4700 (Applied Biosystems) as described previously (Eymann et al., 2004).

Transcriptome analysis. In these experiments the toxic effect of wt $\zeta$ was reversed by expression of $\epsilon$ 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 $\omega$ and $\zeta$, and the plasmid pCB297, carrying the $\epsilon$ gene under the control of an IPTG-dependent promoter (Camacho et al., 2002), were grown in LB medium up to $\sim 5 \times 10^9$ 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 $\sim 1 \times 10^8$ 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.ije.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 2100 bioanalyzer (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 < 0.1 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 ε2 antitoxin causes ~10 000-fold reduction in the plating efficiency of wt B. subtilis cells bearing pBT233-7-borne oε2 genes, whereas the plating efficiency was not affected when cells carried only the pBT233-2-borne o and ε genes (Camacho et al., 2002). A strong reduction in the plating efficiency of B. subtilis wt, ΔclpC, ΔclpP or ΔclpP cells bearing pBT233-7-borne oε2 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 ΔlonA cells bearing the pBT233-7-borne oε2 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 ΔclpX cell showed an intermediate phenotype (Fig. 2a). Thus it is likely that depletion of the ε2 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 ε2 antitoxin (Sitkiewicz et al., 1999). To study the effect of the ζ toxin in the absence of the ε2 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 PxylA), 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 (~1 × 10⁸ cells ml⁻¹) harbouring oε2 genes

![Fig. 2](image-url)
on pBT233-7 (~16 copies cell\(^{-1}\)) or pDB101 (1–2 copies cell\(^{-1}\)) produced ~700 \(\varepsilon_2\zeta\) and ~50 \(\varepsilon_2\zeta\) complexes per cell, respectively, and in both cases the half-life of \(\zeta\) protein was longer than 60 min (Camacho et al., 2002; data not shown). The ability to induce expression of the \(\zeta\)Y83C gene fused to \(P_{\text{yclA}}\) was analysed by Western immunoblotting.

With this gene as a single copy in the chromosome, the amount of induced \(\zeta\)Y83C 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 \(~1 \times 10^8\) cells ml\(^{-1}\), and induced with 0.5% xylose for 60 min, ~300 \(\zeta\)Y83C proteins were present per cell, but upon exposure to 50 \(\mu\)g Rf ml\(^{-1}\), to halt de novo synthesis, the half-life of \(\zeta\)Y83C was approximately twofold shorter compared to that of \(\zeta\) 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 ~5 \times 10^7 cells ml\(^{-1}\) and expression of the \(\zeta\)Y83C gene was induced by addition of 0.5% xylose. An exponential decay in the number of c.f.u. (~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 \(\zeta\) toxin (~8000-fold reduction in c.f.u.) 120 min after addition of Rf; Camacho et al., 2002) and for \(\zeta\)Y83C (~7000-fold reduction in c.f.u.) 120 min after Rf addition; our unpublished results) after depletion of the plasmid-encoded \(\varepsilon_2\) antitoxin or after accumulation of \(\varepsilon\)-free \(\zeta\) toxin (~5000-fold reduction in c.f.u.) 120 min after addition of xylose; Zielienkiewicz & Ceglowski, 2005). It is likely, therefore, that (i) the \(\varepsilon_2\) antitoxin neutralizes the toxic effect of both \(\zeta\) and \(\zeta\)Y83C toxins, (ii) traces of \(\varepsilon_2\) efficiently delayed the toxic effects of \(\zeta\) (see Fig. 2) and \(\zeta\)Y83C toxins (data not shown), and (iii) the activity of both \(\zeta\) and \(\zeta\)Y83C toxins triggers cell stasis with similar efficiency, suggesting that the target site of the \(\zeta\) variant (\(\zeta\)Y83C) is the same as that of native \(\zeta\).

The small fraction of cells (2000–4000 cells ml\(^{-1}\)) that still formed colonies after induction of \(\zeta\)Y83C expression (Fig. 2b) did not genetically acquire resistance to the toxin, as they regrew a new population that was just as sensitive to \(\zeta\)Y83C as the parental strain. However, when BG689 cells (~5 \times 10^6 cells ml\(^{-1}\)) were grown and plated in the presence of 0.5% xylose (i.e. with constant exposure to the toxic action of \(\zeta\)Y83C) 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 \(\zeta\)Y83C, and ~14% had DNA rearrangements on the \(\zeta\)Y83C expression cassette. The remaining fraction (1.1 \times 10^{-7}, \(P<0.0001\)) was still sensitive to the reintroduction of a new plasmid-borne \(\zeta\) gene, suggesting that none carried a mutation in the \(\zeta\) target site. Recently, 28 clones that survived the effects of \(\zeta\) expression were shown to contain deletions, insertions or point mutations in the \(\zeta\) gene (Nowakowska et al., 2005). It is unlikely, therefore, that any of the surviving clones recovered from our screens carried a mutation in the \(\zeta\) target.

**Production of the \(\zeta\)Y83C 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 \(\zeta\)Y83C protein (Fig. 2b) correlated with a bacteriolytic or bacteriostatic state, BG689 cells (at ~5 \times 10^7 cells ml\(^{-1}\)) induced at 0.5% xylose to express \(\zeta\)Y83C 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 ~3% of BG687 control cells (lacking the \(\zeta\)Y83C 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 ~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 ~10 000-fold, but fewer than 20% of the cells were stained with propidium iodide, suggested that expression of \(\zeta\) toxin mainly induced stasis. When cells expressing the \(\zeta\)Y83C toxin were analysed by EM, defects in the cell morphology (e.g. ‘holes’ in the peptidoglycan layer) were observed in ~18% of the observed cells when compared to control cells (~2%) (data not shown). To address whether \(\zeta\) interacts with the cell membrane and/or cell wall a hybrid \(\zeta\)-GFP variant was constructed. YB886 cells bearing the plasmid-borne \(\omega\varepsilon_2\zeta\)-gfp genes (five copies per cell) were grown up to ~5 \times 10^7 cells ml\(^{-1}\) and Rf was added. After 30 min of Rf addition the \(\varepsilon_2\) antitoxin was degraded and the accumulation of \(\varepsilon\)-free \(\zeta\)-GFP triggered the 10 000-fold reduction of c.f.u., suggesting that the \(\zeta\)-GFP protein was active (data not shown). We failed, however, to detect the accumulation of \(\zeta\) or \(\zeta\)-GFP protein in the cell membrane or cell wall using anti-\(\zeta\) polyclonal antibodies, immunogold labelling and EM, or \(\zeta\)-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 \(\zeta\) action.

**Expression of the \(\zeta\)Y83C 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 ~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 ~0.3% of total cells (Fig. 3b). When
Y83C 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, Y83C 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 Y83C toxin. Recently it was shown that when cells overexpressing Y 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 Y83C 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-Gómez et al., 2005; Zhang et al., 2003). To determine whether Y83C protein affects protein translation, different experiments were performed. Concomitant with the inhibition of cell growth (see Fig. 2b) the Y83C 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 Y83C toxin.

To confirm that Y83C did not markedly affect protein translation a proteomic analysis of cell expressing Y83C 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 metB5 marker did not affect activity. Indeed, upon induction of Y83C expression with 0.5% xylose for 60 min the increase in the OD500 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 ~1×10^8 cells ml^{-1} and xylose was added at different times. Then [35S]methionine was added for 5 min and autoradiograms of the labelled proteins in strains BG873 (control) and BG871 (expressing Y83C) 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
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 \( \text{f} \)Y83C toxin, and (ii) under conditions of \( \text{f} \)Y83C 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 \( \text{f} \)Y83C toxin did not have major effects on protein translation.

Expression of the \( \varepsilon \) antitoxin reverses the toxic effect exerted by the \( \zeta \) toxin

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

\( E. \text{coli} \) CC118 cells bearing plasmids pCB297 (~15 copies per cell) and pCB298 (~200 copies per cell), were grown in M9 medium to a density of ~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 \( \varepsilon \) expression) while 1 mM IPTG was added back to the second aliquot after 60 min to induce \( \varepsilon \) 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 (\( \varepsilon \) expressed) (Fig. 5).

To determine whether the toxic effect of \( \zeta \) overexpression (~200 copies of the \( \zeta \) gene per cell) can be reversed by \( \varepsilon \) antitoxin expression, \( E. \text{coli} \) CC118 cells bearing plasmids pCB297 and pCB298 were grown in M9 medium to ~5 \times 10^6 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 back to induce \( \varepsilon \) expression (Fig. 5, denoted by an empty arrow). Thereafter at 60 min intervals the culture without IPTG was again divided into two aliquots, and IPTG was added to one aliquot to induce \( \varepsilon \) expression (Fig. 5, denoted by an empty arrow). Within the first 240 min of exposure to the toxic effect of \( \zeta \) the expression of \( \varepsilon_2 \) antitoxin (IPTG readded after wash) reversed the reduction in c.f.u. (Fig. 5).
IPTG was added to induce expression of the gene of interest. The culture was further incubated. At various time points samples were withdrawn and split in two aliquots. To one aliquot 1 mM IPTG was added to induce expression (empty arrows) at 60 min, 120 min, 180 min, 240 min, 300 min 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.

Fig. 5. The ε antitoxin partially reverses ζ toxin activity. E. coli CC118 cells bearing pBT297-borne P_{lac}εβ and pBT298-borne P_{car}ζ 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 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.

suggest 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 x 10⁷ cells ml⁻¹. The culture was split into two aliquots: one remained as it was (control strain, with inducible ε and constitutive ζ 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 ampiclon 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 ε antitoxin (i.e. incubation without IPTG) induction of ζ 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<0.1) the transcription of only 26 essential genes (cdaA, dapB, djp, hisS, infB, lgt, murE, nadB, nrdA, pyrG, proC, pth, rpoB, rpsB, rplD, rplJ, sccD, thrS, tktA, topA, trpS, tsf, tufA, ychE, yjeE 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

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

* 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}) 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 ~4 × 10⁷ cells ml⁻¹, then arabinose (0·2 %) was added to induce ζ expression (Fig. 6a, filled arrow). After arabinose addition OD₅₀₀ 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 OD₅₀₀ 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 ε₂ 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 ε₀ of the growth-arrested cells exposed to moderate or high concentrations of ζ could be attributed to a pleiotropic
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 \( \zeta \) expression (time zero). At different times after induction of \( \zeta \) protein expression the incorporation of radio-labelled material (over a 1 min time window) into freshly synthesized DNA, RNA and proteins was measured. At 60 min after induction of \( \zeta \) the physiological state of the cells was altered, because incorporation of radioactivity during the 1 min pulse into DNA and RNA was reduced by \( \sim 50 \)-fold, but protein synthesis was reduced \( \sim 6 \)-fold (Fig. 7). Similar results for protein synthesis were observed when the well-defined protein synthesis inhibitor Cm (50 \( \mu \)g 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 \( \varepsilon_2 \) reversal of the effects elicited by \( \zeta \).

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

**Overproduction of \( \zeta \) leads to death of a subpopulation of cells**

To determine whether \( \zeta \) exerted a pleiotropic effect leading to cell death, the change in the morphology of the bacteria
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 \( \zeta \) 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 \( \zeta \) 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 \( \zeta \)-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 \( \zeta \) gene (Fig. 6) led to death of a fraction (20–50 \%) of the cell population.

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**Fig. 8.** Electron micrographs of *E. coli* cells overproducing \( \zeta \). *E. coli* CC118 cells bearing pBT297-borne \( \text{P}_{\text{lacO}}^{-}\zeta \) and pCB635-borne \( \text{P}_{\text{araBAD}}^{-}\zeta \) 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 \( \zeta \) 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 κ toxin. At ‘normal’ levels κ or ςY83C 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 κ ‘killing’ of ~50% of the cell population occurs. It is likely that κ triggers stasis and the nutrients released by the fraction of the dead siblings allow cells to survive the stress condition with κ antitoxin expression reversing the κ-exerted shutdown.

Unlike RelE, MazF or Kid (Muñoz-Gomez et al., 2005; Pedersen et al., 2003; Zhang et al., 2003), expression of ςY83C appeared not to affect synthesis of the bulk of proteins in B. subtilis cells, and high overexpression of κ did not selectively impair protein synthesis in E. coli cells. Chromosomal segregation, DNA topology and cell division were not the main target of ςY83C 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 κ concentrations (i.e. one copy of the induced ςY83C) 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 κ 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 κ antitoxin during a given time window. Bacteriostasis induced by ‘overexpression’ of κ was fully reversible by subsequent expression of the κ 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 κ, expression of κ cannot reverse the growth-arrested state. ‘High overexpression’ of κ reduced the time window of partial reversibility by the κ antitoxin to 60 min. At non-physiological concentrations of κ 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 κ protein was not observed and the presence of a supraregulator, controlling many different stress response systems, was not obvious. Alternatively, the prolonged exposure to ‘overexpressed’ κ or short exposure to ‘highly overexpressed’ κ 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 κ expression, to recover from the growth-arrested mode by using nutrients released from their dead siblings.

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