Acta Biochimica Polonica

Vol. 52 No. 1/2005

99–107

QUARTERLY

Characterization of *Bacillus subtilis* clones surviving overproduction of Zeta, a pSM19035 plasmid-encoded toxin✪

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Received: 15 January, 2005; accepted: 02 March, 2005

Key words: plasmid stable maintenance, postsegregational killing, toxin, Gram positive bacteria

The postsegregational killing system of pSM19035 plasmid consists of the proteins Zeta and Epsilon, a toxin and an antidote, respectively. Zeta mutants were isolated with the use of *Bacillus subtilis* strain with the ζ gene under control of an inducible promoter integrated into the chromosome. Results of mutant analysis point to the amino terminal part of the Zeta protein as being responsible for the toxicity.

Large plasmids possess mechanisms ensuring their stable inheritance in bacterial population. Apart from the partition and multimer resolution systems which enhance equal distribution of plasmid copies into daughter cells, systems relying on postsegregational killing (PSK) of plasmid-free cells are also frequent. The manner of PSK action is based on the activity of a stable toxin that is prevented by a labile antidote, both expressed from a single operon. Whereas all known toxins are proteins, the antidote can be of a proteic or RNA nature. After the plasmid loss *de novo* synthesis of plasmid-encoded products ceases, the unstable antidote is degraded by cellular proteases and the toxin performs its action; therefore cells that do not contain the plasmid are eliminated from the population or their growth is inhibited. The PSK systems have been extensively studied in Gram negative bacteria (Zielenkiewicz & Cegłowski, 2001; Hayes, 2003), however, some examples in Gram positive bacteria have also been described (Weaver *et al.*, 1996; Grady & Hayes, 2003).

✪This work was supported by the State Committee for Scientific Research (KBN, Poland) grant No. 6 P04B 008 20

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Abbreviations: aa, amino acid; BCIP, 5-bromo-4-chloro3-indolylphosphate; NBT, nitroblue tetrazolium; ORF, open reading frame; PSK, postsegregational killing; RBS, ribosome-binding site.

The precise mechanisms of toxin action and their specific targets are characterized in few cases only. The *Escherichia coli* sex factor F-encoded toxin, CcdB, as well as RK2-encoded ParE act on gyrase, in the former case binding to the GyrA subunit (Bernard & Couturier, 1992). Another toxin, Kid of the R1 plasmid was shown to inhibit DnaB-dependent replication (Ruiz-Echevarria *et al.*, 1995; Jiang *et al.*, 2002). Recent investigations ascribe to the PemK toxin, a Kid homologue present on the R100 plasmid, the function of a sequence-specific endonuclease (Hargreaves *et al.,* 2002). The PSK systems are abundant also on prokaryotic chromosomes (Pedersen & Gerdes, 1999; Hayes, 2003). Their function has not been elucidated although possible roles in general stress response, bacterial apoptosis, genome shuffling or cell cycle arrest have been postulated (Christensen *et al.*, 2001; Hayes, 2003).

Much less is known about the PSK systems in plasmids of Gram positive bacteria. The proteic *axe-txe* stability system of the pRUM plasmid from a clinical isolate of *Enterococcus faecium* has recently been described. The cassette codes for two small proteins, Axe being the antidote and Txe the toxin. The system stabilizes plasmids in the original host, in *Bacillus thuringiensis* and even in the Gram negative *E. coli* (Grady & Hayes, 2003).

The 29 kb low copy number plasmid pSM19035 isolated from *Streptococcus pyogenes* is stably maintained in a broad range of Gram positive bacteria of low G+C content in their chromosomal DNA. Two regions (SegA and SegB) involved in the plasmid stability have been identified. While the SegA region encodes a site-specific recombinase, the SegB region assures better-than-random plasmid segregation (Cegłowski et *al.*, 1993a; 1993b). It consists of four genes organized into two transcription units: δ and ω-ε-ζ (de la Hoz *et al.*, 2000). Although the protein product of gene δ reveals significant homology to a family of ATPases involved in active plasmid partitioning, most of the stabilization function relies on the remaining part of the SegB region (Cegłowski *et al.*, 1993b). The ω-ε-ζ operon constitutes the PSK system with the ε and ζ genes encoding an antidote and a toxin, respectively (Zielenkiewicz & Cegłowski, in press). The ω gene product represses the expression of this operon, moreover, it plays a role of the global regulator of several transcription units in the pSM19035 plasmid (de la Hoz *et al.*, 2000).

The ω-ε-ζ operon was shown to stabilize plasmids in the Gram positive *Bacillus subtilis* and also in the Gram negative *E. coli* in a less efficient manner. The ε and ζ genes were separated and shown to act *in trans* with respect to each other. The toxic effects of the ζ gene expression were counteracted by proper expression of ε. In *B. subtilis*, overproduction of Zeta, the ζ gene product, was shown to provoke massive cell death. In cells constantly overproducing the antidote Epsilon protein, the ω-ε-ζ operon failed to act as a stabilization cassette. This indirectly indicates that, like in other proteic PSK systems, the antidote is less stable than the toxin (Zielenkiewicz & Cegłowski, in press). The difference in the stability of Zeta and Epsilon has been shown *in vivo* and *in vitro* (Camacho *at al.*, 2002).

The ε gene encodes the 90 aa protein Epsilon that prevents the toxic action of Zeta. Zeta, composed of 287 amino acids, is much larger than other PSK-encoded toxins of usual length of about 100 aa (Gerdes, 2000). A homology search of Zeta and Epsilon sequences with those present in public databases did not show any significant identity to proteins of known function. The only meaningful motifs present in the N-terminal region of Zeta are the ATP/GTP binding motifs Walker A and Walker B (Cegłowski *et al.*, 1993a).

Plasmids of the *inc*18 family, pRE25 and pIP501, contain entire or truncated ζ genes almost identical to that of pSM19035, also a member of the same family (Brantl *et al.*, 1990; Schwarz *et al.*, 2001). The crystal struc-

ture of Zeta complexed with its antidote Epsilon ($Epsilon_2Zeta_2$) has been solved (Meinhart *et al.*, 2003). The structure is unique among the PSK toxins crystalysed.

The aim of this work was to identify the regions of the Zeta protein important for its toxic action. Therefore we performed systematic studies of *B. subtilis* mutants obtained after prolonged exposition to Zeta toxin production.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. The *Bacillus subtilis* strains YB886 (Friedman & Yasbin, 1983), YBZ01 (Zielenkiewicz & Cegłowski, in press), *Escherichia coli* K-12 DH5α (Hanahan, 1983) and DH5α_{pACE1} with pACYC184 bearing the ε gene (Zielenkiewicz, 2001) were used. The strain YBZ01 overproducing the Zeta toxin was constructed by integration of the *xyl* promoter-repressor expression cassette (Kim *et al.*, 1996) fused to the intact ζ gene sequence into the chromosomal *amy*E locus of YB886. The recipient strain contained the pAT18 vector (Trieu-Cuot *et al.*, 1991) bearing the ε gene, the source of the antidote Epsilon (Zielenkiewicz, 2001). The vector pGBT9 (yeast Two Hybrid System, CLONTECH) was used for cloning the ζ gene sequence(s). *B. subtilis* and *E. coli* were grown in LB medium supplemented with appropriate antibiotics: chloramphenicol (5 μg/ml, *B. subtilis*) or ampicillin (100 μg/ml, *E. coli*) when required.

Strategy for B. subtilis mutant isolation. YBZ01 cells were grown in LB at 37°C to A_{600} = 0.1 and than D-xylose was added to 0.5% final concentration. At the time of the lowest A_{600} (1–2 h of incubation) samples were plated on D-xylose supplemented LB and incubated at 37°C overnight. Growing colonies were collected and preserved as spore strips.

Cloning of ζ gene. Chromosomal DNA from clones of YBZ01 growing in the presence of xylose was isolated and purified as described (Burdett, 1982). This DNA served as a template for amplification of the ζ genes sequences by PCR using oligonucleotides: ForwardZeta 5′-GCC GAA TTC ATG GCA GTT ACG TAT GAA AAA ACA-3′ and ReverseZeta 5′-GCC GGA TCC TTA AAT ACC TGG AAG TTT AGG TGT-3′. A mixture of Taq and Pfu polymerases was used (kind gift of J. Willert, MPI, Berlin, Germany). The purified fragments were cloned into pGBT9 vector.

Evaluation of Zeta protein toxicity. Mutant-derived ζ gene DNA was PCR amplified and ligated to the pGBT9 vector cut with *Eco*RI and *Bam*HI (MBI Fermentas). The ligation mixture was used for simultaneous transformation (Sambrook *et al.*, 1989) of competent DH5α and DH5α_{pACE1} *E. coli*. Equal volumes of both bacterial suspensions were plated on LB supplemented with ampicillin. If only DH5α transformants appeared, the cloned ζ gene sequence was considered altered.

ζ gene sequencing and sequence analysis. Nucleotide sequences were determined using ABI Prism 377 with BigDye Terminator Cycle Sequencing Kit (ver 1.0, Applied Biosystems). The sequencing was carried out using as the template the pGBT9-derived plasmids (approx. 500 ng) and primers: GAL4BDstart 5′-GAA GAG AGT AGT AAC AAA GG-3′ and GAL4BDend 5′-AAA ATC ATA AAT CAT AAG-3′ (approx. 5 pmol) or PCR generated DNA fragments of *B. subtilis* mutant chromosomal DNA with primers ForwardZeta and ReverseZeta (approx. 5 pmol). Sequence analysis was carried out using the GCG programs (Wisconsin Package).

Preparation of cell extracts from B. subtilis. Cell extracts were prepared according to Silhavy *et al.* (1984) from cultures grown overnight in LB with chloramphenicol and 0.5% D-xylose at 37°C. Pellets of 2 ml samples were collected and incubated at 37°C with 2 mg/ml of lysozyme for 30 min before boiling.

Western blot analysis. Ten-microlitre samples were run on 12% SDS polyacrylamide gels and electroblotted onto nitrocellulose membrane (Schleicher & Schuell) as described by Sambrook *et al.* (1989). Anti-Zeta rabbit serum (kindly provided by J.C. Alonso, 1:5000 dilution) was used as the primary antibody. Class II anti-rabbit alkaline phosphatase-conjugated antibody (Promega, 1:7500 dilution) detection was performed using NBT and BCIP. The chromatic reaction was stopped by the addition of EDTA when the intensity of the band reach maximum.

RESULTS AND DISCUSSION

The *B. subtilis* strain YBZ01 used in this study contains a single copy of the ζ gene under the control of the xylose inducible p_{xylA} promoter (Fig. 1). To protect the cells from the possible toxic action of Zeta, the strain also contained a plasmid carrying the gene coding for the antidote Epsilon.

However, it should be stressed here that due to the tight control of ζ gene expression, the strain YBZ01 does not require the presence of the plasmid with the ε gene. After xylose addition to the growing culture rapid induction of Zeta production is observed resulting in massive cell death. As can be seen in Fig. 2, prolonged incubation of the culture subjected to induction of Zeta production leads to growth recovery of the very few surviving cells. In the course of four independent growth experiments, we collected 247 colonies of YBZ01 plated at the time of the lowest A_{600} on xylose-supplemented LB. Eighty-eight were subjected to further analysis.

We expected to obtain a set of diverse mutants in the Zeta protein in positions important for its toxicity. However, the ability of cells to grow in the presence of xylose could also be due to two other possible phenomena: mutations in the p_{xylA} promoter or in genes responsible for xylose uptake resulting in the inability of those cells to overproduce (or produce at all) Zeta, or mutations in the gene(s) responsible (directly or indirectly) for the production of the cellular target for Zeta action. The surviving clones were again subjected to the xylose induction procedure to confirm their lack of response to the inducer. None of them appeared to respond to the induction in the sense that no drop in their growth could be observed. Characterization of the collected *B. subtilis* clones employed verification of the toxic effectiveness of Zeta expressed form their chromosomes and nucleotide sequence determination. The test of Zeta toxicity relied on the different transformation efficiency with cloned ζ gene into *E. coli* cells bearing or not a plasmid providing the Epsilon antidote. Only mutated (or weakened) Zeta protein expressed from the vector would lead to approximately the same number of transformants in both *E. coli* strains (with or without the ε gene).

The ζ gene of the first 44 "survivors" was PCR amplified, cloned, transformed into *E. coli* and sequenced. As the sequencing of 17 out of the mutants showed the prevalence of nonsense or frameshift mutations, in the next 44 mutants the production of Zeta was detected immunologically with anti-Zeta rabbit serum. Only in the case of three clones was the presence of the Zeta protein confirmed, and all three showed remarkably low-

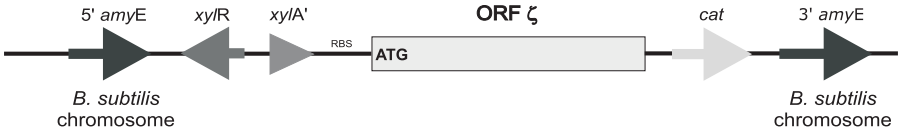

Figure 1. Schematic representation of the region surrounding the inserted ζ gene in *B. subtilis* YBZ01 chromosome.

Gene names are marked as well as a position of the RBS.

ered toxicity against *E. coli* cells. The sequence of these three ζ genes and of eight additional clones being the representative of the four experiments mentioned was established.

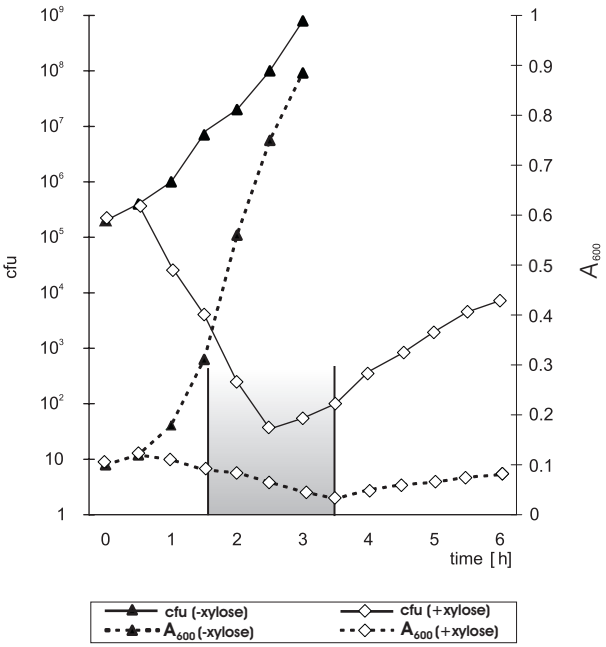

Figure 2. Selection of Zeta mutants of *B. subtilis* cells.

The inducer (0.5% xylose) was added when the A_{600} reached 0.1. Samples of cells growing in xylose presence were taken 1–2 h after induction (gray area). Measurements of A_{600} values (decimal scale) and cfu counts (logarithmic scale), represented by dashed or continuous lines, respectively, are presented for the culture growing without (triangles) and with xylose (open squares).

The results of sequence analysis of the ζ gene of 28 "survivors" are shown in Table 1. The mutations found could be grouped into 15 classes (named **a** to **o**): the most frequent class is represented by 9 cases, the next two classes by 3 or 4 cases and the remaining 12 by only a single representative. The prevalent mutation which was sequenced in 14 clones is a deletion of one A in the AAAAAAA tract beginning at position 78 that results in a frameshift and translation termination after 51 triplets; in 5 clones this mutation is accompanied by additional change(s). Moreover, in 8 classes of mutations (21 clones, 75% of total) deletion or insertion events lead to the frameshift resulting in stop codon formation and production of truncated proteins. In two cases nucleotide substitutions create the stop codon TAA at the beginning of the ζ gene (class **l** and **m**). In 5 classes the substitution A248G is present, in two as a single one, in the next two concurrent with the C73T or G355A transitions and in one with insertion of two codons ACAGGA after A357. In the case of class **f** mutant the Zeta protein was not detected, similarly as in one of the two A248G single mutants (class **e**). However, in the second A248G clone (class **d**), as well as in class **h** and **i** mutants the presence of Zeta protein was confirmed immunologically (Fig. 3). Moreover, the level of the Zeta protein detected in class **d** mutant does not differ from that in the induced YBZ01 strain. Nevertheless, the ζ gene from all three mutants cloned in the pGBT9 vector could be transformed into *E. coli* DH5α cells. This indicates that the Zeta proteins analysed are not toxic or their toxicity is considerably lowered. The fact that two identical A248G mutants differ in Zeta production allows us to suggest that class **e** and possibly class **f** mutants are defective in the xylose induction system (in the p_{xylA} promoter or repressor protein encoded by the *xyl*R gene) or in xylose uptake.

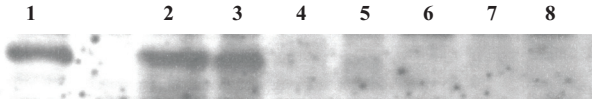

Figure 3. Immunological detection of Zeta protein produced by *B. subtilis* YBZ01 mutants.

Whole cell extracts were separated on SDS/PAGE and blotted onto nitrocellulose membrane, Western blot analysis was performed with anti-Zeta rabbit serum as primary antibody. Lanes contain extracts of xylose induced cells: 1, YBZ01; 2, class **k** mutant; 3, class **d** mutant; 4, 5, class **a** mutant; 6, 7, mutants not sequenced. In lane 8 the cell extract of uninduced YBZ01 is a negative control.

In the course of experiments no chromosomal mutant giving resistance to Zeta toxin

Table 1. Mutations found in the ζ gene.

The presence of Zeta was immunologically detected as described in Material and Methods. Zeta toxicity was tested *via* transformation of cloned ζ gene into *E. coli* DH5α and DH5α_{pACE1} cells. Ability to transform both strains with the same efficiency is denoted (–). The mutation number denotes nucleotide position in the ζ gene relative to ATG.

Class	Mutation	Presence of Zeta	Zeta toxicity	No. of clones
a	ΔA78 (7A→6A)	ND	NA	9
b	ΔA78 (7A→6A), Ins. G after A32, A111C	ND	ND	1
c	ΔA78 (7A→6A), A248G	–	ND	4
d	A248G	+	–	1
e	A248G	–	ND	1
f	A248G, C73T	–	ND	1
g	A248G,G355A	+	–	1
h	A248G, ΔA255 (5A→4A)	–	–	1
i	A248G, Ins. T after C104	–	–	1
j	A248G, Ins. G after G182	–	–	1
k	A248G, Ins. ACAGGA after A357	+	–	1
l	G4T, C5A	ND	NA	1
m	C31T	ND	NA	1
n	Δ(T46-C97)	ND	NA	1
o	ΔA492	ND	ND	3

NA, not applicable; ND, not determined.

was found among all the "survivors" tested. The probable reason is the selection procedure employed: the lack of a randomly mutagenizing agent and the constant pressure caused by the presence of the toxin. The presence of a toxin as strong as Zeta, leads to the appearance of cells with inactivated ζ gene as the most frequent event.

It is noteworthly that all the sequence changes found in the ζ gene localized to 5′ terminal fragment of the coding sequence (last mutation found is in position 492 out of the total 864 nucleotides), see Fig. 4. So, the mutations manifest in the N-terminal part of Zeta, which in turn may mean that this is the region important for the toxicity of this protein. It needs to be reminded here that the NTP binding Walker motif A is located between amino acids 39 and 47 (Cegłowski *at al.*,1993a). We found out that the sequence changes within the motif lead to the complete abolishment of Zeta toxicity (unpublished results). Altogether, this encourages us to propose that the amino terminus of Zeta is responsible for its toxicity.

The class **d** mutant A248G results in the substitution Tyr83Cys – the level of Zeta production is not affected but the toxicity is considerably lowered. Inspection of the Epsilon$_2$Zeta$_2$ complex crystal (Meinhart *et al.*, 2003) allows one to speculate about the possible role of the Tyr83Cys substitution. Its surface localization in the N-terminal Zeta part which forms the complex with Epsilon suggests an involvement in interaction with a cellular target. The class **g** and **k** mutations result in the substitution Gly119Arg and insertion of two amino acids TyrGly after Gly119, respectively. Mutation of the buried glycine at position 119 located between two secondary

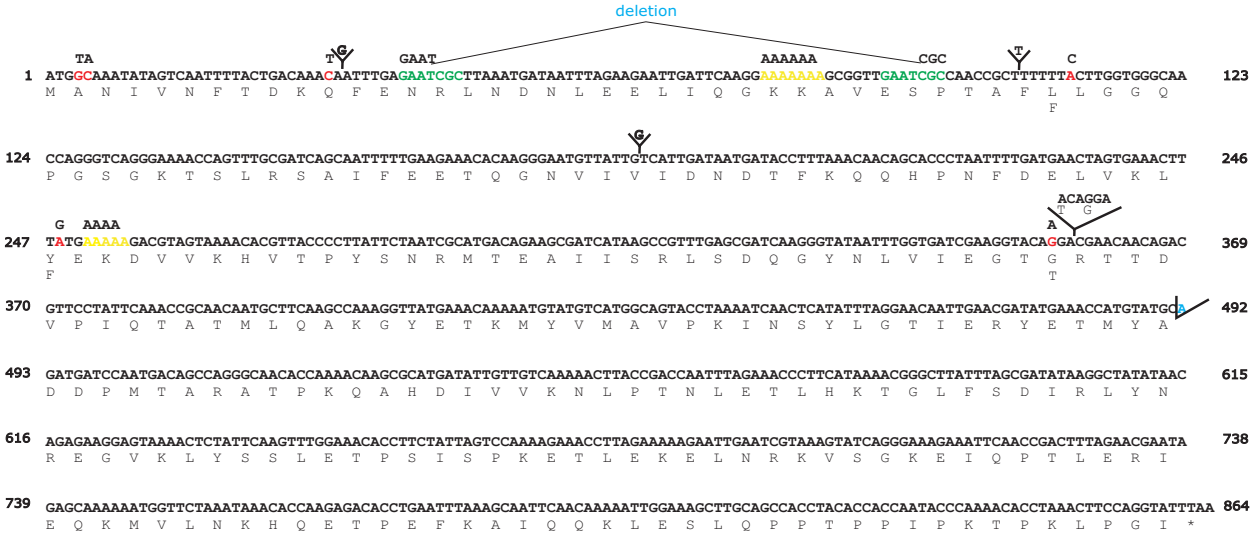

Figure 4. Graphic representation of mutations found in the ζ gene sequence.

All mutations found are marked above the sequence: substitutions in red, deletions in blue, in green – repeated sequences where a large deletion took place. Oligo(A) tracts where deletions occurred are in yellow. Amino acids resulting from nucleotide substitutions are marked below the sequence. Number 1 denotes the first nucleotide of ζ coding sequence.

structure elements probably affect the folding of the whole molecule.

Little is known about the precise interactions between the PSK toxins and their targets. For the best characterized CcdB of *E. coli* sex factor F it was shown that three carboxy terminal amino acids are directly involved in GyrA binding and thus in toxicity (Bahassi *et al.,* 1995). In the case of Doc of P1 phage and Kid of R1 plasmids, mutants have been isolated that were non-toxic but retained the ability to co-autoregulate their operons. The gene sequences were changed in diverse positions: for Doc in the middle part of the protein (positions 66, 70, 76 of 126 aa) and for Kid at both extremities of the molecule (18 and 85 of 110 aa) (Magnuson & Yarmolinsky, 1998; Santos-Sierra *et al.,* 2003). However, despite being different at the amino acid level, the CcdB and Kid toxins exhibit similar overall folding (Hargreaves *et al.,* 2002). In contrast the structure of Zeta does not resemble any structure deposited in public databases.

The unique characteristics of Zeta (a broad range of Gram positive hosts, lack of homology to other known sequences, strong toxicity) encourages us to perform a more detailed analysis of regions involved in its particular functions: toxicity and interactions with the antidote Epsilon. The research concerning this PSK system originating from pathogenic Gram positive bacteria could in perspective give a raise to novel antibacterial agents with a potential for use in human therapy.

REFERENCES

Bahassi EM, Salmon MA, Van Melderen L, Bernard P, Couturier M. (1995) F plasmid CcdB killer protein: ccdB gene mutants coding for non-cytotoxic proteins which retain their regulatory functions. *Mol Microbiol.*; **15**: 1031–7.

Bernard P, Couturier M. (1992) Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J Mol Biol.,* **226**: 735–45.

Brantl S, Behnke D, Alonso JC. (1990) Molecular analysis of the replication region of the conjugative *Streptococcus agalactiae* plasmid pIP501 in *Bacillus subtilis*. Comparison with plasmids pAM beta 1 and pSM19035. *Nucleic Acids Res.*; **18**: 4783–90.

Camacho AG, Misselwitz R, Behlke J, Ayora S, Welfle K, Meinhart A, Lara B, Saenger W, Welfle H, Alonso JC. (2002) *In vitro* and *in vivo* stability of the epsilon2zeta2 protein complex of the broad host-range *Streptococcus pyogenes* pSM19035 addiction system. *Biol Chem.*; **11**: 1701–13.

Cegłowski P, Boitsov A, Chai S, Alonso JC. (1993a) Analysis of the stabilization system of pSM19035-derived plasmid pBT233 in *Bacillus subtilis*. *Gene.*, **136**: 1–12.

Cegłowski P, Boitsov A, Karamyan N, Chai S, Alonso JC. (1993b) Characterization of the effectors required for stable inheritance of *Streptococcus pyogenes* pSM19035-derived plasmids in *Bacillus subtilis*. *Mol Gen Genet.*; **241**: 579–85.

Christensen SK, Mikkelsen M, Pedersen K, Gerdes K. (2001) RelE a global inhibitor of translation is activated during nutritional stress. *Proc Natl Acad Sci USA.*, **98**: 14328–33.

Friedman BM, Yasbin ER. (1983) The genetics and specificity of the constitutive excision repair system of *Bacillus subtilis*. *Mol Gen Genet.*, **190**: 481–86.

Gerdes K. (2000) Toxin-antitoxin modules may regulate synthesis of macromolecules during nutritional stress. *J Bacteriol.*, **182**: 561–72.

Grady R, Hayes F. (2003) Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug resistant, clinical isolate of *Enterococcus faecium*. *Mol Microbiol.*; **47**: 1419–32.

Hanahan D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol.*, **166**: 557–80.

Hargreaves D, Santos-Sierra S, Giraldo R, Sabariegos-Jareno R, de la Cueva-Mendez G, Boelens R, Diaz-Orejas R, Rafferty JB. (2002) Structural and functional analysis of the kid toxin protein from *E. coli* plasmid R1. *Structure* (Camb).; **10**: 1425–33.

Hayes F. (2003) Toxins-antitoxins: plasmid maintenance programmed cell death and cell cycle arrest. *Science.*, **301**: 1496–9.

de la Hoz AB, Ayora S, Sitkiewicz I, Fernandez S, Pankiewicz R, Alonso JC, Cegłowski P. (2000) Plasmid copy-number control and better-than-random segregation genes of pSM19035 share a common regulator. *Proc Natl Acad Sci USA.*, **97**: 728–33.

Jiang Y, Pogliano J, Helinski DR, Konieczny I. (2002) ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Escherichia coli* gyrase. *Mol Microbiol.*, **44**: 971–9.

Kim L, Mogk A, Schumann W. (1996) A xylose-inducible *Bacillus subtilis* integration vector and its application. *Gene.*, **181**: 71–6.

Magnuson R, Yarmolinsky MB. (1998) Corepression of the P1 addiction operon by Phd and Doc. *J Bacteriol.*; **180**: 6342–51.

Meinhart A, Alonso JC, Strater N, Saenger W. (2003) Crystal structure of the plasmid maintenance system epsilon/zeta: functional mechanism of toxin zeta and inactivation by epsilon 2 zeta 2 complex formation. *Proc Natl Acad Sci USA.*; **100**: 1661–6.

Pedersen K, Gerdes K. (1999) Multiple hok genes on the chromosome of *Escherichia coli*. *Mol Microbiol.*, **32**: 1090–102.

Ruiz-Echevarria MJ, Gimenez-Gallego G, Sabariegos-Jareno R, Diaz-Orejas R. (1995) Kid a small protein of the parD stability system of plasmid R1 is an inhibitor of DNA replication acting at the initiation of DNA synthesis. *J Mol Biol.*; **247**: 568–77.

Sambrook J, Fritsch EF, Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual,* 2 edn. Cold Spring Harbor Laboratory Cold Spring Harbor NY.

Santos-Sierra S, Lemonnier M, Nunez B, Hargreaves D, Rafferty J, Giraldo R, Andreu JM, Diaz-Orejas R. (2003) Non-cytotoxic variants of the Kid protein that retain their auto-regulatory activity. *Plasmid.*, **50**: 120–30.

Silhavy TJ, Berman ML, Enquist LW. (1984) *Experiments with Gene Fusions*. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Schwarz FV, Perreten V, Teuber M. (2001) Sequence of the 50-kb conjugative plasmid pRE25 from *Enterococcus faecalis* RE25. *Plasmid.*; **46**: 170–87.

Trieu-Cuot P, Carlier C, Poyart-Salmeron C, Courvalin P. (1991) Shuttle vectors containing a multiple cloning site and a lacZ alpha gene for conjugal transfer of DNA from *Escherichia coli* to gram-positive bacteria. *Gene.,* **102**: 99–104.

Weaver KE, Jensen KD, Colwell A, Sriram SI. (1996) Functional analysis of the *Enterococcus faecalis* plasmid pAD1-encoded stability determinant par. *Mol Microbiol.*; **20**: 53–63.

Zielenkiewicz U. (2001) Role of genes *epsilon* and *zeta* of plasmid pSM19035 in its stable maintenance in bacterial cells. *Ph.D. Thesis*; Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland.

Zielenkiewicz U, Cegłowski P. (2001) Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems. *Acta Biochim Polon.*; **48**: 1003–23.

Zielenkiewicz U, Cegłowski P. (2005) The toxin-antitoxin system of the streptococcal plasmid pSM19035. *J Bacteriol.*; in press.