Introduction of a mini-gene encoding a five-amino acid peptide confers erythromycin resistance on *Bacillus subtilis* and provides temporary erythromycin protection in *Proteus mirabilis*

Svetlana I. Novikova a,b, Anastassia M. Bushueva a, Lesya A. Truchuk a, Galina E. Konstantinova a, Anna V. Serkina a, Christian Hoischen c, Johannes Gumpert c, Galina G. Chestukhina a, Alexander Mankin d, Alexei B. Shevelev a,*

a V.M. Stepanov Laboratory of Protein Chemistry, Institute of Microbial Genetics (GNIIGenetika), 1 Dorozhny pr., 1, Moscow 113545, Russia
b Department of Molecular Biophysics, Moscow Institute of Physics and Technology, Moscow, Russia
c Research Group of Prokaryotic Cell Biology, Institute for Molecular Biotechnology, Jena, Germany
d Centre for Pharmaceutical Biotechnology-m/c 870, University of Illinois, Chicago, IL 60607, USA

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Abstract

A 15-bp mini-gene was introduced into *Bacillus subtilis* and into stable protoplast-like L-forms of *Proteus mirabilis*. This mini-gene encoded the peptide MVLFV and modeled a fragment of *Escherichia coli* 23S rRNA responsible for *E. coli* erythromycin (Ery) resistance. Expression of the introduced mini-gene conferred permanent Ery resistance on *B. subtilis*. In L-forms of *P. mirabilis*, the Ery-protective effect was maintained in the course of several generations. Herewith, the mechanism of Ery resistance mediated by expression of specific short peptides was shown to exist in evolutionary distant bacteria. Three new plasmids were constructed containing the gene under study transcriptionally fused with the genes encoding glutamylendopeptidase of *Bacillus licheniformis* or N-endotoxin of *Bacillus thuringiensis*. The Ery resistance pentapeptide (E-peptide) mini-gene served as an efficient direct transcriptional reporter and allowed to select bacillar glutamylendopeptidase with improved productivity. The mini-genes encoding E-peptides may be applied as selective markers to transform both Gram-positive and Gram-negative bacteria. The small size of the E-peptide mini-genes makes them attractive selective markers for vector construction. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

A new mechanism of resistance towards macrolide antibiotics was suggested recently when a five-codon open reading frame (ORF) from 23S rRNA of *Escherichia coli* was shown to render cell resistance to low concentrations of erythromycin (Ery) [1,2]. Further studies showed that other mini-genes encoding tetra-, penta- or hexapeptides could confer higher resistance to Ery and other macrolides [3]. A consensus sequence of Ery resistance pentapeptides (E-peptides) was derived from comparison of mini-genes selected from a random pentapeptide mini-gene plasmid library for their ability to confer the Ery-resistant (Ery r) phenotype to the transformed *E. coli* cells [3]. A ‘bottle brush’ model of the E-peptide action was proposed, where translation of E-peptides ‘cleans’ the ribosome from Ery [3].

In this work, we present an evidence that E-peptides efficiently functioning in *E. coli* confer permanent Ery resistance to Gram-positive *Bacillus subtilis*. This extends the range of the organisms where mini-genes may be efficient...
transcriptional selective markers. The mini-gene was used as transcriptional reporter and allowed to improve an activity of a naturally poor expressed gene of a glutamylendopeptidase from Bacillus licheniformis (gseBL). Furthermore, our results show that the mechanism of Ery resistance mediated by expression of specific mini-genes probably operates in evolutionary distant organisms.

2. Materials and methods

2.1. Bacterial strains and plasmids

The following strains were used: E. coli JM109 [4], B. subtilis AJ73 kindly supplied by Jurgis Jomantas, Proteus mirabilis LVI [5].

Plasmid pES7 containing a mini-gene encoding a pentapeptide Met-Val-Leu-Phi-Val (MVLVF) (Fig. 1A) was isolated from a random mini-gene plasmid library where mini-ORFs with optimized translation initiation signals were expressed under control of the lac-tac promoter [2,3].

Plasmid pLFgap14 was constructed by cloning MfeI-BamHI fragment containing the gseBL gene [6] into EcoRI/BamHI sites of the pLF14 vector ([7]; EMBL accession number X85430). It contained a promoter sequence of the gseBL and no own transcriptional terminator.

Plasmid pA5G12 containing the cry1Gal δ-endotoxin gene from Bacillus thuringiensis in vector pUK21 was described in [8].

Plasmid pLFgap14-Em was constructed by insertion of the MfeI-EcoRI fragment containing the E-peptide mini-gene from pES7 into EcoRI site of the pLFgap14 plasmid (the multi-copy pLFgap14 plasmid [7]) downstream from gseBL [6]. In the resulting plasmid, pLFgap14-Em, the E-peptide mini-gene was expressed in transcriptional fusion with the gseBL (Fig. 1B, 1 and 2). Transcription of the bicistronic mRNA containing GseBL and MVLVF ORFs was controlled by the gseBL promoter and the kanamycine (Km) phosphotransferase promoter from TN903 [12]. This construct was produced in B. subtilis and the plasmids isolated from Ery resistance clone were reintroduced into E. coli. The plasmid sequence was tested directly.

To construct plasmid pBA-G12, the cry1Gal endotoxin gene of B. thuringiensis ssp. galleriae with its own promoter was excised from the plasmid pA5G12 using AflII restriction enzyme and introduced into AflII site of pES7 upstream of MVLVF (Fig. 1B, 3). The minimal replicon from the pBA4, a natural plasmid of Bacillus amyloliquefaciens (kindly provided by J. Jomantas), was introduced as a 2.5-kb EcoRI fragment into the unique EcoRI site of the resulting construct. As in pLFgap14-Em, E-peptide ORF in pBA-G12 was expressed as a second cistron in a bicistronic mRNA whose transcription was regulated by own promoter of the cry1Gal gene and possibly by lac-tac promoter of the original pES7 plasmid. The pBA-G12 construct was assembled in E. coli JM109 and then transformed to B. subtilis.

Plasmid pFPS1-Em for P. mirabilis was constructed by introducing the mini-gene-containing MfeI-EcoRI fragment from pES7 into the unique EcoRI of pFPS1 containing a hybrid gseBL gene with an attached secretory leader of the alkaline phosphatase (PhoA) (Fig. 1D). In the resulting construct, the bi-cistronic mRNA containing E-peptide ORF and the gseBL cistron were expressed from the lac-tac isopropyl-thiogalactopyranoside (IPTG) inducible promoter.

2.2. Media and reagents

B. subtilis was cultivated on the Lennox broth (Difco). L-forms of P. mirabilis were cultivated in FB medium as described earlier [10]. Ery free base was purchased from Serva and its 100-mg ml⁻¹ work stock was prepared adjusting pH to 7.0 with 10 M acetic acid. A chromogeneous synthetic carbobenxy glutamyl-paranitroaniline (Z-Glu-PNA) substrate for measurement of the specific glutamylendopeptidase activity was synthesized by E. Milgotina.

2.3. Cell transformation and selection of the superresistant transformants

The cells of E. coli were transformed with the standard Ca-mediated procedure [4]. The cells of B. subtilis were transformed using a modified Spizizen protocol [9].

The B. subtilis cells transformed by pLF derivatives were selected on a standard Lennox medium [4] containing 10 mg l⁻¹ Ery. For further selection of the most resistant to Ery clones, 100 colonies of the transformants were placed into the wells of a replicator and transferred on the plates containing 10, 30, 50, 80, 150 or 300 mg l⁻¹ of Ery. A similar procedure was run with pBA4 derivatives with 10, 30, 50 mg l⁻¹ of Ery (Table 1). Scarce clones appeared on a highest Ery concentrations and are referred further as 'Ery preselected'.

The stable protoplast-like L-forms of P. mirabilis were maintained and transformed following protocols described previously [10] in order to overcome their inability to start growing in a low density and a necessity to be adapted after transfer from agarized to the liquid medium. Plasmids pES7 (ampicillin-resistant), pFPS1 (Km-resistant (Kmr')) and pFPS1-Em (Kmr') were transformed using a standard method [5,10]. Selection was carried out on FB medium containing either 30 mg l⁻¹ Km and 1% glucose or 10–30 mg l⁻¹ Ery and 2 mM IPTG at 37°C (5 μg of pure plasmid for each transformation).

Six individual clones of LVI (pFPS1) and a single clone of LVI (pFPS1-Em) were selected using the Km-containing medium. No clones appeared even at a minimal Ery concentration of 10 mg l⁻¹. The Km-selected clones were adapted using the agarized FB medium containing 30 mg l⁻¹ Km at 37°C for four passages. Then, the clones were
transferred to the liquid FB medium of the same composition, supplemented with 30 mg l\(^{-1}\) Km and passed five times at 30°C. Later, a conventional composition of the FB medium with no glucose was used (1% sucrose, 0.5% yeast extract and 30 mg l\(^{-1}\) Km) for seven more passages. Induction was performed by addition of IPTG up to 2 mM to a freshly inoculated culture after 6 h of incubation in the FB medium containing 30 mg l\(^{-1}\) Km at 30°C. The cultivation was stopped and the cells were harvested 24 h after induction.

In parallel, the LVI (pFPS1-Em) culture after passage #7 (five passages with 1% glucose in the medium and two passages without it) was used as an inoculum for fermentation under Ery selection: 10 ml of the inoculum was transferred to 30 ml of a fresh FB medium containing 2 mM IPTG and 5, 10 or 20 mg l\(^{-1}\) Ery. Further, the clones were passed under the standard growth conditions in the presence of 2 mM IPTG at growing Ery concentrations in the medium by five various schemes (Table 2).

2.5. Assay of the secreted GseBL activity in the cultural medium of the pLFgap14 and pLFgap14-Em transformants of B. subtilis

The individual clones preselected with Ery and the random control clones were placed to the sloped LB agar (3 ml without addition of antibiotics) and incubated at 30°C for 24–48 h. Then, the cells were washed by 75 ml LB medium to Erlenmeyer flasks and grown for 48 h at 30°C with vigorous agitation. The cells were harvested and a GseBL activity measured in the liquid cultural medium fraction towards the selective synthetic Z-Glu-pNA substrate as described in [11].

3. Results

3.1. Introduction of the E-peptide mini-gene into B. subtilis

Transformation of both pLFgap14-Em and pBA-G12 constructs into B. subtilis cells resulted in growth of Ery\(^{r}\) colonies (Table 1). No colonies were observed under a Ery concentration of 80 mg l\(^{-1}\) or higher. One colony appeared at 50 mg l\(^{-1}\) Ery, showing that cells transformed with pLFgap14-Em could grow in the presence of up to 50 mg l\(^{-1}\) of Ery. The cells transformed with pBA-G12 were growing at Ery concentrations not higher than 20 mg l\(^{-1}\).

3.2. Introduction of the E-peptide gene into L-forms of P. mirabilis

L-forms of Gram-negative bacteria were shown to be highly sensitive to Ery due to better permeability for this compound [5]. Therefore, it was interesting to study E-peptide gene action in these cell wall-less L-forms of P. mirabilis, where translational machinery is quite equal with E. coli but a protecting effect of the cell wall is absent.

The E-peptide gene taken from pES7 was transferred into pFPS1 plasmid containing a gseBL adapted for secretion of the protease in L-forms of P. mirabilis. The pFPS1
Ery concentration (mg l$^{-3}$) harbored a modified gseBL under control of the lac-tac promoter on the basis of the pSIS2 vector. The pFPS1 contained a Km resistance (Km$^r$) gene essential for selection of the L-form transformants. In pFPS1-Em, the E-peptide gene was located upstream of the gseBL gene under the control of the lac-tac promoter. We successfully used this construct for direct selection of the E. coli JM109 transformants on LB medium containing 3000 mg l$^{-1}$ Ery and 2 mM IPTG.

A drastic decay of the cell viability was observed after repetitive passages in an induction/selection medium (Table 2). Cell growth was considered poor, if the cell density exceeded 1 OU(D$_{680}$) after 48 h or more incubation. The growth was normal at the steps 1 and 2 and rather poor at step 3 (scheme A). Poor growth was observed at step 2 of the schemes B and C; no growth took place at step 3 of these schemes. Almost no growth was seen even at step 1 of the schemes D and E and obviously, there was no growth at following steps (Table 2).

The recombinant protein yield was assessed for 24, 48 and 72 h of the steps 1 and 2, schemes A and B. Other steps were skipped since scarce cells and strong lysis were observed there.

A large volume of the inoculum required for passaging of L-forms precluded an evaluation of the cell viability by a conventional OD$_{680}$ measurement in the culture. Besides the reason of the L-form death under subsequent passaging on Ery was not clear. Therefore, a production of GseBL was used as marker of transcription and translation in the cells under selection. No difference in protein yield was noted for the steps 1 and 2 of the schemes A and B. The protein synthesis stopped after 24 h incubation but the protein produced was quite stable in the cultural medium regardless to the cell lysis. No substantial difference was observed either for protein production of LVI(FPS1) or LVI(FPS1-Em) without Ery selection. On the other hand, addition of IPTG was only shown to cause no cell death. Therefore, death of the cells was induced by alteration of the Ery protection system in the course of fermentation, not due to IPTG treatment or overexpression of the gseBL.

The DNA of pFPS1-Em, pFPS1 and pES7 was used for transformation of L-forms of *P. mirabilis* LVI. The transformants were selected on either 20 mg l$^{-1}$ Km or 10, 20 and 30 mg l$^{-1}$ Ery supplemented with 2 mM IPTG as described earlier [10]. No transformants of pFPS1-Em were obtained on the Ery-containing medium though eight colonies with this construct appeared under the Km selection. The same efficiency of transformation was noted for the pFPS1 construct when Km selection was used. In contrast to pFPS1-Em, a certain efficiency of transformation was obtained in the case of the pES7 construct, containing the E-peptide mini-gene under the control of the lac-tac promoter separately from any additional genes. The number of colonies at pES7 DNA transformation decreased with increasing Ery concentration: 1000 colonies at 10 mg ml$^{-1}$, 100 colonies at 20 mg ml$^{-1}$ and 30 colonies at 30 mg ml$^{-1}$. The DNA was purified from the transformants to examine stability of the constructs. The isolated transformants were adapted to grow under the same conditions as used for selection. It could be noted that the growth rate increased for pFPS1 and pFPS1-Em at each passage on the solid medium under the Km selection, whereas it subsequently decreased for pES7 under Ery selection from 10 to 30 mg l$^{-1}$. The growth of all pES7 transformants completely stopped after three passages regardless of the presence of the intact plasmid. The control culture of untransformed LVI strain was not able to grow even under 10 mg l$^{-1}$ Ery in the medium. The cultures of pFPS1 and pFPS1-Em were subsequently transferred into the liquid medium containing Km. Each pFPS1-Em-containing clone was further kept under either Km or Ery selection. It was demonstrated that no growth occurred under Ery selection in the absence of IPTG. The addition of IPTG allowed to perform three passages only in liquid medium when 1/10 of the final cultural volume was used as inoculum. Then, since the third passage, the growth became scarce and completely stopped at the fourth passage (10–12 generations). However, the growth of the same transformants was quite normal under Km selection.

The production of the recombinant GseBL and the influence of IPTG was detected for pFPS1-Em clones. No difference in the recombinant protein production was observed for the cultures selected by Km and Ery, while the

### Table 1

<table>
<thead>
<tr>
<th>Ery concentration (mg l$^{-3}$)</th>
<th>pLFgap14-Em</th>
<th>pBA-G12</th>
<th>pLF14</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>ND</td>
<td>ND</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>1200</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>460</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>18</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>150</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, no data available.

### Table 2

Passaging L-forms of *P. mirabilis* harboring pFPS-Em construct on the Ery-containing liquid media

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Passages and growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ery 5 mg l$^{-1}$ (good) $\rightarrow$ Ery 5 mg l$^{-1}$ (good) $\rightarrow$ Ery 5 mg l$^{-1}$ (poor)</td>
</tr>
<tr>
<td>B</td>
<td>Ery 5 mg l$^{-1}$ (good) $\rightarrow$ Ery 10 mg l$^{-1}$ (average) $\rightarrow$ Ery 10 mg l$^{-1}$ (poor)</td>
</tr>
<tr>
<td>C</td>
<td>Ery 5 mg l$^{-1}$ (good) $\rightarrow$ Ery 15 mg l$^{-1}$ (poor) $\rightarrow$ Ery 15 mg l$^{-1}$ (no)</td>
</tr>
<tr>
<td>D</td>
<td>Ery 10 mg l$^{-1}$ (poor) $\rightarrow$ Ery 10 mg l$^{-1}$ (no)</td>
</tr>
<tr>
<td>E</td>
<td>Ery 20 mg l$^{-1}$ (poor) $\rightarrow$ Ery 20 mg l$^{-1}$ (no)</td>
</tr>
</tbody>
</table>

The rate of the growth after 28 h incubation is shown in brackets (good, OD$_{680}$ > 2.0; average, OD$_{680}$ 1.0–2.0; poor, OD$_{680}$ < 1.0.)
culture was viable under Ery selection. No mortality or alteration of the growth was induced by permanent presence of IPTG in the medium for the Km-selected clones.

Herewith, a temporary protection effect from Ery by the E-peptide gene expression in the L-form cells was demonstrated.

4. Discussion

We used one of the E-peptide mini-genes isolated earlier and known to confer Ery resistance on _E. coli_ [1–3]. The MVLFV ORF, together with its translation initiation signal, but with no promoter, was introduced into three plasmid constructs capable of replication in _B. subtilis_ as well as in _E. coli_. The gseBL rendering a low-level expression in both natural and recombinant strains [6] and cry1Ga1, which was completely silencing in the natural host [8] with their own promoters, were used as models for expression optimization with a selection method based on the mini-gene.

As minimal inhibitory concentration of Ery for untransformed _B. subtilis_ cells was only 0.3 mg l⁻¹ and transformation of _B. subtilis_ with similar constructs without MVLFV did not make cells Ery⁺. We conclude that expression of MVLFV confers Ery resistance not only upon the Gram-negative _E. coli_, but also upon Gram-positive _B. subtilis_. The observed difference in Ery resistance conferred by two constructs may be caused by several factors. Promoter strength and/or mRNA stability may affect translation of MVLFV cistron. Similarly, a difference in the plasmid copy number could cause a lower level of expression of MVLFV ORF in the cells transformed with pBA-G12 compared to pLFgap14-Em.

In L-forms of _P. mirabilis_, the Ery-protective effect was maintained in the course of several generations. Our results show that the mechanism of Ery resistance mediated by expression of specific short peptides exists in evolutionary distant bacteria.

Individual clones of both recombinant and natural producer strains, though genetically identical, exhibit variable production of the secreted proteins. Therefore, one should be able to directly select better producing clones to provide high productivity of a culture. Usually, this monitoring is carried out by an assay of the product of interest in series of isogenic clones. We demonstrated that the E-peptide gene might facilitate this procedure if transcriptionally fused with a structural gene of interest. A method of a rapid clone screening was based on a hypothesis that transcription of the GseBL and the E-peptide gene was mutually dependent, and the Ery protection effect was proportional to the E-peptide gene transcription. In this respect, we compared pLFgap14-Em clones, preselected under the highest possible Ery concentration, and control clones randomly chosen after transformation for production of the GseBL. The GseBL activity of the Ery-preselected clones corresponded to about 0.06 U per ml, whereas only 0.023 U per ml was obtained from a control clone (almost 3-fold less). Therefore, we suggest that E-peptide genes could serve as suitable selectors in fast screening procedures for better transcription of any DNA fragment and gene expression optimization. A prospective result was observed also with cry1Ga1 δ-endotoxin gene which was completely silencing in the natural _B. thuringiensis_ ssp. _galleriae_ strain. The Ery selection of cry1Ga1 transcriptional fusion with the E-peptide mini-gene allowed to obtain its expression in _B. subtilis_ without any modification of the promoter region. The transformed cells of _B. subtilis_ AJ73 (pBA-G12) were not only Ery⁺ but in contrast to the control AJ73 strain accumulated microscopically detected protein crystals (data not shown) putatively containing crystal protein cry1Ga1.

The small size of Ery resistance mini-genes makes them attractive as selective markers for transformation. The small size is particularly valuable in combination with rolling-circle plasmids whose stability essentially depends on the total size of the construct [13]. Being transcriptionally fused with other genes, the E-peptide mini-genes may efficiently serve as reporters for gene expression.

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