Acta Scientific MEDICAL SCIENCES (ISSN: 2582-0931)

Volume 6 Issue 8 August 2022

Research Article

Group B Streptococci Epsilon/Zeta Toxin-antitoxin System Stabilizes Plasmid pAT29 in *S. agalactiae*

Eugenia Kuleshevich[1], Dmitrii Linnik[1], Valentin Shevchenko[1], Tatiana V Gupalova[1], Natalia Grudinina[2], Anastasia Malchenkova[1], Svetlana Giliazova[1] and Alexander N Suvorov[1,3]*

[1]Department of Molecular Microbiology of Federal State Budgetary Scientific Institution "Institute of Experimental Medicine", Saint-Petersburg, Russia

[2]Department of Molecular Genetics of Federal State Budgetary Scientific Institution "Institute of Experimental Medicine", Saint-Petersburg, Russia

[3]Department of fundamental Medicine and Medical Technology of Saint-Petersburg State University, Saint-Petersburg, Russia

***Corresponding Author:** Alexander N Suvorov, Department of Molecular Microbiology of Federal State Budgetary Scientific Institution "Institute of Experimental Medicine", Saint-Petersburg, Russia.

DOI: 10.31080/ASMS.2022.06.1302

Received: May 04, 2022

Published: July 03, 2022

Abstract

Group B streptococci (GBS) or *Streptococcus agalactiae* is the major cause of various diseases in newborns and elderly people. The genome of GBS is highly heterogeneous and contains mobile genetic elements, which play a significant role in the rapid adaptation of GBS to different ecological niches. Some mobile genetic elements are stabilized in a cell by toxin-antitoxin systems. The present study focused on investigating the putative Epsilon/Zeta toxin-antitoxin system, which is encoded by the GBS pathogenicity island PAI-A. Genes of the putative Zeta toxin, Epsilon antitoxin, and the entire Epsilon/Zeta operon from the GBS strain 07y/08 were cloned and expressed in *Escherichia coli*. The overexpression of the Zeta toxin gene caused temporary inhibition of *E. coli* growth. PAI-A's Epsilon antitoxin could bind to the Zeta toxin and eliminate its toxicity. The Epsilon/Zeta toxin-antitoxin system inserted into plasmid pAT29 stabilized an otherwise unstable plasmid in GBS. Based on the experimental data, we concluded that the biological role of this genetic element in GBS was to prevent translocation of the pathogenicity island PAI-A from the genome of *S. agalactiae.* Thus, the Epsilon/Zeta toxin-antitoxin system of GBS might contribute to the adaptation of the microorganism to new ecological niches. The present research might contribute to the development of a new class of antimicrobial substances.

Keywords: Group B Streptococci; Pathogenicity Island; Epsilon/Zeta Toxin-Antitoxin System

Abbreviations

GBS: Group B Streptococci; MGEs: Mobile Genetic Elements; TA: Toxin-antitoxin; UNAG: Uridine Diphosphate-N-acetylglucosamine; THB: Todd Hewitt Broth; LB: Luria-Bertani Broth; OD: Optical Density; IPTG: Isopropyl β-D-1-thiogalactopyranoside; THBSpe: THB Agar Supplemented with 100 μg/ml Spectinomycin

Introduction

For a long time, *Streptococcus agalactiae* or group B streptococci (GBS) were considered as agents of bovine mastitis; however, it became recently evident that GBS can also cause various human diseases [17], for example, in newborns, including sepsis,

meningitis, and pneumonia [21], while the presence of GBS in breast milk may cause B-streptococcal infection in newborns [20]. GBS may also cause illnesses in elderly people [21]. Moreover, *S. agalactiae* infects livestock animals and fish, which leads to economic losses worldwide [3,12].

Numerous mobile genetic elements (MGEs) found in GBS genome may carry the genes encoding for antibiotic resistance, enzymes, or virulence factors, which might be important for rapid adaptation of the organism to different ecological niches [1,4,19]. Therefore, horizontal gene transfer is believed to contribute to the emergence of new, highly virulent strains. Some of these MGEs are named as pathogenicity islands [4].

The pathogenicity island PAI-A that contains several genes, including *sspB1* encoding a putative surface adhesin, is found in the genome of *S. agalactiae* [7]. Previously, it has been shown that the products of the *sspB1* gene and its homologues correlate with the emergence of urogenital infections in humans [23]. Furthermore, PAI-A contains an additional lactose operon Lac.2-1 [20] and the set of genes encoding the type IVC secretion system [7]. Li., *et al*. [11] showed that the type IVC secretion system is located on the pathogenicity island 89K of *Streptococcus suis* and is important for the conjugal transfer of this MGE. Other authors proposed that the type IVC secretion system enhances bacterial pathogenicity and can facilitate the outbreaks of human diseases [28].

The Zeta toxin gene (*zeta*) and the Epsilon antitoxin gene (*epsilon*) are localized on the GBS pathogenicity island PAI-A. Molecular structures of the proteins encoded by these genes are similar to those of the proteins belonging to the type II toxin-antitoxin (TA) system Epsilon/Zeta [7,8].

TA systems or bacterial programmed cell death systems were discovered in most gram-positive and gram-negative bacteria [10]. To date, eight types of TA systems have been discovered and are classified into different families according to the operon's structure and mode of action [10,22]. The first investigated TA systems are located on plasmid DNA, and their true function is believed to be ensuring stable inheritance of plasmids through many cell divisions [18]. In type II TA systems, the effect of the toxin protein is prevented by antitoxin binding and through the formation of a stable toxin-antitoxin complex [18]. In contrast to toxin, antitoxin is an unstable protein prone to hydrolysis involving Clp and Lon ATP-dependent serine proteases. Therefore, plasmid loss leads to postsegregational cell death caused by the stable toxin, which is released from the complex with antitoxin. Later, it was revealed that TA systems might also be encoded by a host bacterial chromosome [18]. Many authors assume that, along with stabilization of MGEs, TA systems possess several other potential functions, such as programmed cell death under conditions of starvation and other types of stresses, which may cause antitoxin inactivation (serine protease activation, negative operon's regulation) [13,15,18]. Some authors stated that such systems can be used as an effective target for search of new antibiotics [6,16]. Chromosomal TA systems, depending on their type, may also play a key role in global gene regulation, biofilm formation, and defense from alien gene invasion [25].

The Epsilon/Zeta TA system was originally discovered on the pSM19035 plasmid of *Streptococcus pyogenes.* However, apart from the Zeta toxin and Epsilon antitoxin genes, the plasmid carries an Omega transcriptional regulator gene [2,29]. The gene (encoding PezT) that is homologous to the *S. pyogenes zeta* gene is present in the pathogenicity island PPI-1 of *Streptococcus pneumoniae*, but in contrast to the plasmid's Epsilon/Zeta TA system, the PezA antitoxin itself plays the role of a transcriptional regulator with a DNA-binding helix-turn-helix motif [5,15]. A similar TA system (designated SezAT) was detected in *S. suis*. Genes of this system are localized on the pathogenicity island SsPI-1. Overexpression of the SezT toxin demonstrated a bactericidal effect. This effect could be terminated by co-expression of the SezA antitoxin. The authors described that SezAT promotes inheritance of SsPI-1 during cell division [26].

Recently, it was found that the TA system genes located on the *S. agalactiae* PAI-A pathogenicity island are homologues to those of the Epsilon/Zeta TA system of *S. pneumoniae*. The Zeta toxin phosphorylates the peptidoglycan precursor uridine diphosphate-*N*-acetylglucosamine (UNAG) that, in turn, disrupts normal cell wall synthesis [16]. Nevertheless, there are still no data regarding its functionality.

Thus, based on the previous data, the present study aimed to discover the function of Zeta and Epsilon proteins, the genes of which are carried by the GBS pathogenicity island PAI-A.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

In this research, the following strains and plasmids were used: GBS strains 07y/08 and 02/08 [8]; *E. coli* strain M15 from the collection of Molecular Microbiology Department of the Institute of Experimental Medicine (Russia, St. Petersburg); expression bacterial plasmid vector pQE-32 (QIAexpressionist, Qiagen, Germany); and shuttle vector pAT29 [24].

Growth conditions: GBS strains were cultivated in Todd Hewitt Broth (THB) (Pronadisa, Spain) overnight at 37ºC. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (Amresco, USA) at 37ºC with shaking.

Computational and statistical analysis

Primers for genes were designed using Primer 3 software (Table 1). Sequences of the TA system genes were found in the genome of NEM316 (NC_004368.1), that carrying the pathogenicity island PAI-A, in the Genome database (NCBI). References for the Epsilon antitoxin gene and the Zeta toxin gene were the genes gbs1344 (GBS_RS07090) and gbs1343 (GBS_RS07085), respectively, of strain NEM316. The PCR primers were designed to incorporate the endonuclease restriction sites for BamHI and HindIII for subsequent cloning in the expression vector pQE-32. Primers corresponding to the gene of resistance to spectinomycin were designed according to the gene encoding the spectinomycin adenyltransferase AAD(9) [14].

Primer name	Sequence, 5'-3'	PCR product size, bp	Gene
epsfor	gggatcccgaggaggaagcagtat	508	Epsilon antitoxin
epsrev	ccaagcttgggggccaccacctggtga		
zetafor	gggatcccgtcaccaggtggtggccta	762	Zeta toxin
zetarev	cccaagcttgggggttctcatacttaactc		
SpcF	gctatgatcctgatttggctattg	322	Gene of resistance to spectinomycin
SpcR	cactctccccgatagccttttc		

Table 1: Sequences of primers used in this study. Specific endonuclease restriction sites for BamHI and HindIII are indicated in bold.

The open reading frames of the *epsilon* and *zeta* genes were confirmed using the Translate tool. Theoretical molecular masses of the Zeta toxin and the Epsilon antitoxin were calculated using the Compute pI/Mw tool.

Statistical analysis was performed using Pearson's chi-squared test for two-by-two contingency tables ($p \leq 0.05$).

Gene cloning in *E. coli*

DNA from the strain GBS 07y/08, which carries the pathogenicity island PAI-A, was used as a template for PCR. PCR was performed using the DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. The PCR conditions were as follows: initial denaturation at 95°C for 3 min; 30 cycles: 95°C - 30 s, 55°C - 30 s, 72°C - 60 s; and final extension at 72 °C for 15 min. The Qiagen Plasmid Mini Kit (Qiagen, Germany) and the "DNA express" kit (Litex, Russia) were used for the isolation of plasmid and chromosomal DNA, respectively, according to the manufacturers' protocols. Restriction digestion was carried out using endonucleases BamHI FastDigest and HindIII FastDigest (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. T4 DNA ligase (Promega, USA) was used to ligate the digested DNA fragments according to the manufacturer's protocol. The method of calcium transformation in *E. coli* was used for GBS gene cloning into vectors. The transformed *E. coli* cells were inoculated on LB plates containing the appropriate antibiotic (100 μg/ml ampicillin and 25 μg/ml kanamycin for the pQE-32 expression vector; 100 μg/ml spectinomycin for pAT29).

E. coli growth curves based on logarithm of CFU over time

E. coli strains were cultivated in LB medium (Amresco, USA) containing 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37ºC with shaking (180 rpm). The logarithm of colony forming unit (CFU) over time was calculated from zero point, at which optical density (OD) at wavelength 600 nm was 0.22-0.23. The gene expression was induced at zero point by using 1 mM isopropyl

β-D-1-thiogalactopyranoside (IPTG) (Helicon, Russia). Samples were taken every hour (from 1h to 6h) to determine the number of viable cells. Three independent experiments were conducted.

Fluorescent staining with dsGreen and propidium iodide

The expression of Zeta toxin and Epsilon antitoxin genes, the Epsilon/Zeta operon, and the pQE32 vector without insert was induced using the same approach as described previously. After 1h, 1 ml of *E. coli* culture was centrifuged and then re-suspended in 1 ml of 0.85% NaCl. Cells of *E. coli* strains containing genes of Zeta toxin, Epsilon antitoxin, and the Epsilon/Zeta operon and the pQE32 vector were stained with 1 µl of 10000× dsGreen (an equivalent of SYBR® Green I) (Lumiprobe, Russia) and 1 µl of 3.4 mM propidium iodide (Invitrogen, USA) for 15 min at room temperature in dark. Images were captured using an inverted confocal laser scanning microscope "LSM 510 Meta" (Zeiss, Germany) and an immersion lens C-Apochromat 40X/1.20 W CORR (Zeiss, Germany). Laser with a wavelength of 488 nm was used to excite the fluorescence of both dyes; a 505-550 nm filter was used to detect dsGreen fluorescence, while a filter of more than 650 nm was used to detect the fluorescence of propidium iodide. In transmitted light, images were obtained using the Differential Interference Contrast method. For each experiment, 14 images were obtained. Image processing and analysis were carried out using the software supplied with the microscope and Fiji software (ImageJ). The cell counting was performed using the threshold method. All images were analyzed with the same threshold value.

Isolation and purification of recombinant proteins

Forty milliliters of overnight *E. coli* culture was inoculated in 800 ml of LB medium and incubated at 37°C with mild shaking until it reached OD of 0.9 at the wavelength of 600 nm. The operon expression was induced using 1 mM IPTG for 4h. The cells were disrupted by ultrasonic disintegration using a Soniprep 150 Plus Ultrasonic Disintegrator (MSE, UK) three times. The sonication duration was 20 s at 4°C with a break of 40 s at 23 kHz. Protein isolation from the *E. coli* cell lysate was conducted using Ni-sepharose (GE Healthcare, USA) according to the manufacturer's recommendations. After purification, recombinant proteins were dialyzed overnight against phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 8.2). The molecular weight of the obtained proteins was determined by 12% SDS-PAGE [9].

Electroporation

One colony of GBS strain was inoculated in 5 ml of THB and incubated overnight at 37ºC. On the next morning, 1 ml of the night culture was inoculated in 50 ml THB supplemented with 0.6% glycine, and the bacterial culture was grown until OD 0.2-0.4 at the wavelength of 600 nm. Cells were centrifuged at 3000 rpm for 30 min at 4°C. The supernatant was discarded. The cells were washed three times on ice by using 25 ml of 10% glycerol. The bacterial pellet was suspended in 0.3 ml of 10% glycerol on ice, and plasmid DNA (0.5-1 µg) was added to 50 µl of bacterial cells. Electroporation was performed in a cuvette with a 1 mm electrode spacing at 2100V. The pulse durations were 4.2-4.7 ms. Next, 1 ml warm THB was added to the cells. The bacterial cells were then incubated at 37°C for 1h. The cells were plated on THB agar supplemented with 100 µg/ml spectinomycin.

Plasmid stability assay

One colony of GBS strain was inoculated in 1 ml containing 100 µg/ml spectinomycin and incubated overnight at 37ºC. The overnight culture (10 µl) was added to 1 ml of THB without spectinomycin and incubated for 24h at 37ºC. On the next day, 10-fold overnight culture dilutions in phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) were prepared. Twenty microliters of each dilution was dropped onto THB agar and THB agar supplemented with 100 µg/ml spectinomycin (THBSpe) and incubated for 24h at 37ºC. The number of CFUs was determined in THB and THBSpe agar. Ten microliters of overnight culture was re-inoculated into 1 ml of THB and re-incubated for 24h at 37ºC. The above mentioned procedures were repeated for 7 days. Three independent experiments were conducted.

Results and Discussion

Effect of Zeta toxin and Epsilon antitoxin on the growth of *E. coli*

To assess the influence of the putative Epsilon/Zeta TA system on *E. coli* growth, cloning of the Zeta gene (*zeta*), the TA system operon (*epsilon/zeta*), and the Epsilon gene (*epsilon*) from GBS strain 07y/08 was carried out. For this purpose, a series of primers for the genes of *zeta, epsilon*, and *epsilon/zeta* were constructed (Table 1), and the open reading frame was chosen in compliance with the pQE-32 expression vector. For operon amplification, a forward primer was used for *epsilon* (epsfor) and a reverse primer

for *zeta* (zetarev). Consequently, *E. coli* M15 transformants (M15_zeta, M15_epsilon, and M15_epsilon/zeta, containing the pQE-32 expression vector with *zeta, epsilon*, and *epsilon/zeta* genes, respectively) were obtained.

After the beginning of the induction of *zeta* expression with IPTG, the number of viable cells was determined every hour. The results are presented in figure 1. After 1-h induction of *zeta* gene expression, a dramatic decrease in cell number was observed; this indicates a temporary inhibition of *E. coli* growth. In the next hour, there was a slight decrease in CFU. Three or more hours of *zeta* overexpression led to an increase in the number of cells and a visible heterogeneity in colony size.

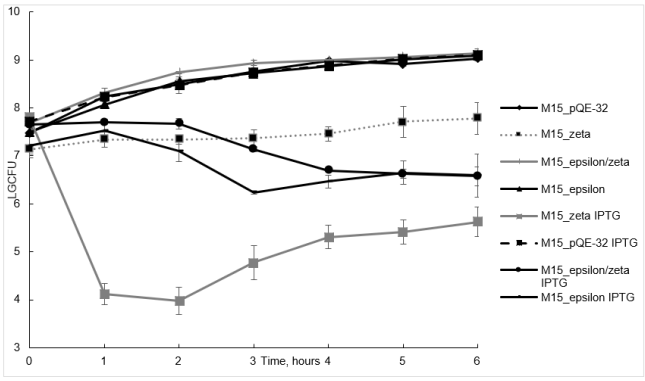

Figure 1: The influence of the overexpression of the Zeta toxin gene, Epsilon antitoxin gene, and Epsilon/Zeta operon on the growth of *E. coli*. The number of cells at zero point is statistically indistinguishable.

The induction of expression by using IPTG in *E. coli* strain M15 containing the pQE-32 expression vector with the GBS *zeta* (M15_zeta) led to temporary growth inhibition of *E. coli* culture as compared to *E. coli* strain M15 contained pQE-32 expression vector without insert (M15_pQE-32) (Figure 1). Restoration of the cell growth can be explained by plasmid elimination or mutation of the *zeta* gene in *E. coli*. In addition, it might be possible that the Zeta protein started to degrade during the period of cell growth inhibition, and the *E. coli* cells that survived the bacteriostatic influence of toxin or somehow eliminated the *Zeta* restored their viability. Furthermore, plasmid elimination was confirmed as randomly chosen colonies were re-inoculated on new agar plates with antibiotics, and some of the colonies failed to grow on new agar plates. The same effect was reported for the Zeta toxin of *S. pneumoniae* and *S. pyogenes* [5,29]. Zeta toxin is considered to be involved in arresting peptidoglycan layer synthesis; thus, it strongly affects gram-positive bacteria by causing cell wall damage and cell division disruption. In the present study, the Zeta toxin demonstrated mostly bacteriostatic properties against gram-negative *E. coli* cells, presumably at the early stages of the logarithmic growth phase. Perhaps, this protein demonstrates a bactericidal effect only against gram-positive bacteria. This hypothesis could also be confirmed by the fact that the Zeta/Epsilon TA system is abundant mostly among gram-positive bacteria [5,26].

After the beginning of induction of *epsilon/zeta* expression by IPTG in *E. coli*, the number of cells remained constant for 2h. A slight decrease in the number of cells over the next 2 h was observed (Figure 1). This growth retardation may be explained by exhaustion of resources for protein production. We therefore propose that in *E. coli*, the streptococcal Epsilon antitoxin bound to the Zeta toxin and blocked its toxic properties.

The induction of *epsilon* expression demonstrated the same effect as the *epsilon/zeta* overexpression at the beginning (Figure 1). However, after 3-h induction, a more significant decrease in the number of cells was observed than that during *epsilon/zeta* expression induction. Perhaps overproduction of the Epsilon antitoxin as a DNA-binding protein with unknown targets led to the delay in *E. coli* growth. It could also be possible that by-products of highly unstable Epsilon protein degradation may have a negative influence on bacterial cells. This may be a reason why this decrease started only after several hours of induction. No such effects were observed when *zeta* and *epsilon* were co-expressed; thus, we can state that Epsilon could be slightly toxic to the host. This effect of Epsilon in *E. coli* M15 strain remains unclear and is yet to be elucidated.

To exclude the influence of IPTG on *E. coli* growth, induction of expression of strain M15 contained pQE-32 expression vector without insert (M15_pQE-32) was performed. This strain was used as the control strain.

For counting live and dead cells, fluorescence staining with dsGreen and propidium iodide was performed with *E. coli* M15_

pQE-32 (control strain), M15_zeta, M15_epsilon, and M15_ epsilon/zeta. dsGreen was used as a vital staining agent, while propidium iodide was used to stain dead and damaged cells. Staining was carried out 1h after the start of *zeta, epsilon*, and *epsilon/zeta* overexpression. *E. coli* M15_pQE-32 cells (control strain) were diluted 4 times in saline solution before staining because of high bacterial cell culture density. The results of staining are presented in table 2. The most representative images of fluorescence staining with dsGreen and propidium iodide are shown in figure 2. As a control strain, M15_pQE-32 was diluted 4 times before staining, and the number of cells was multiplied by 4.

Cells	M15_zeta	M15_epsilon/zeta	M15_epsilon	M15_pQE-32 (control strain)
Live	625	925	950	3076
Dead	30	7	26	28
Percentage of dead cells	4.6	0.8	2.7	0.9

Table 2: The number of live and dead cells of *E. coli* M15_pQE-32 (control strain), M15_zeta, M15_epsilon, and M15_epsilon/zeta strains after 1-h induction of expression.

Figure 2: Fluorescence staining of *E. coli* M15_pQE-32 (control strain), M15_zeta, M15_epsilon, and M15_epsilon/zeta after 1-hour induction of expression. The induction of *zeta* expression led to a significant increase in the number of dead cells as compared to that after *epsilon/zeta* and pQE-32 overexpression.

The data obtained were analyzed by Pearson's chi-squared test for two-by-two contingency tables. The number of dead cells was statistically significantly different in the following pairs: M15_zeta - M15_epsilon ($p = 0.0372$), M15_zeta - M15_epsilon/zeta ($p < 0.0001$), M15_zeta - M15_pQE-32 (control strain) ($p < 0.0001$), M15_epsilon - M15_epsilon/zeta ($p = 0.0014$), and M15_epsilon - M15_pQE-32 (control strain) ($p < 0.0001$). In contrast, the number of M15_epsilon/zeta and M15_pQE-32 (control strain) dead cells did not meet the criterion ($p = 0.6628$). Percentage ratios of dead cells among the total number of cells for M15_zeta, M15_pQE-32 (control strain), M15_epsilon, and M15_epsilon/zeta strains were 4.6%, 0.9%, 2.7%, and 0.8%, respectively.

The induction of *zeta* expression led to a significant increase in the number of dead cells as compared to the overexpression of *epsilon/zeta* and pQE-32 (Table 2, Figure 2). Nevertheless, the induction of *epsilon* expression slightly influenced *E. coli* growth. The results obtained correlated with CFU count for strains M15_ zeta, M15_pQE-32, M15_epsilon, and M15_epsilon/zeta.

In this study, we observed that the Zeta toxin exhibited bacteriostatic properties against gram-negative *E. coli*. The same effect was reported for the Zeta toxin of *S. pneumoniae (*PezT*)* and *S. pyogenes* [5,29]. Yao., *et al*. [26] reported that the activity of Zeta toxin of *S. suis* (SezT) in *E. coli* was bactericidal rather than bacteriostatic. Perhaps GBS Zeta toxin expressed more bactericidal effect than bacteriostatic one, because in the CFU counts, all the colonies were considered, including those that lost the vector and consequently the *zeta* genes. Moreover, this could be proven by the fact that there was an increase in the number of dead cells after the induction of *zeta* expression.

It should be noted that in our experiments, the induction of epsilon expression slightly influenced *E. coli* growth. This was confirmed by fluorescence staining with dsGreen and propidium

iodide (Table 2, Figure 2). This decrease in *E. coli* growth during *epsilon/zeta* expression could be explained by exhaustion of resources for protein production. However, according to Khoo., *et al*. [5] and Yao., *et al*. [26] data, the overexpression of homologues TA system had no effect on *E. coli* growth, and the Epsilon antitoxin was nontoxic. This contradiction could be explained by the fact that different promoters were used in the studies. Accordingly, in the current study, the expression vector pQE-32 with T5 promoter, which is known to have a "leakage" effect, was used, while Khoo., *et al*. [5] and Yao., *et al*. [26] used expression vectors with T7 and BAD promoters.

Protein complex formation between Zeta and Epsilon

The number of living cells during 1-h induction of epsilon/zeta expression was not reduced as compared to that after the induction of z*eta* expression (Figure 1). We proposed that the antitoxin bound to the toxin and inhibited its toxic activity, similar to the TA system PezAT of *S. pneumoniae* [5]. To prove the antitoxin's ability to form a stable toxin/antitoxin complex, the overexpression of *epsilon/zeta* operon genes was conducted, followed by Ni-sepharose affinity chromatography. Because only the antitoxin contained His-tag, it was selectively absorbed on metal ions. In this case, the toxin protein would not have been purified unless it was strongly bound with the antitoxin.

Molecular weights of the two proteins after Ni-sepharose affinity chromatography and dialysis were determined by SDS-PAGE (Figure 3). The obtained molecular weights, namely 31 kDa and 20 kDa, corresponded to the theoretical molecular masses of Zeta toxin and Epsilon antitoxin, respectively, calculated by the Compute pI/Mw tool. Both proteins were purified by Ni-sepharose.

Figure 3: Electrophoregram of Zeta toxin and Epsilon antitoxin after Ni-sepharose affinity chromatography. Molecular weight marker PageRuler Plus Prestained (Bio-Rad, USA).

Thus, it can be concluded that GBS's Zeta and Epsilon could form a stable protein complex. The Epsilon antitoxin bound to the toxin and inhibited its toxic activity. Khoo., *et al*. [5] described that the same effect (inhibition of the toxic activity of the toxin by the antitoxin) was observed in the homologous TA system PezAT of *S. pneumoniae*. Therefore, we considered that the Epsilon antitoxin encoded by the pathogenicity island PAI-A could perform its function, which consisted of binding to the toxin and inhibiting its toxic activity.

Plasmid stability assay

To determine the possibility of the TA system's influence on plasmid stabilization in the genome of *S. agalactiae*, the *epsilon/zeta* operon was re-cloned (by using endonucleases EcoRI and HindIII) from pQE32 in shuttle vector pAT29 [24]. The shuttle vector pAT29 carrying the *epsilon/zeta* operon was designated as pAT29ez.

Genome of the GBS strain 02/08 does not contain the pathogenicity island PAI-A nor the genes of the Epsilon/Zeta TA system [8]. Therefore, this strain was transformed by electroporation with shuttle vectors pAT29 and pAT29ez. The presence of vectors pAT29 and pAT29ez in the 02/08 strain after electroporation was determined by PCR with primers corresponding to the gene of spectinomycin resistance and the *epsilon/zeta* operon (Table 1). Two strains of *S. agalactiae* were selected for further analyses: strain 3-1 containing pAT29*ez* and strain 7 containing pAT29.

To determine the ability of the TA system to stabilize MGE, the GBS strains 3-1 and 7 were cultivated for 7 days without spectinomycin selective pressure. Every day, 10 µl of overnight culture was re-inoculated in 1 ml of THB, and CFUs were counted in plates with and without spectinomycin. The results are presented in figure 4. Strain 7 containing the vector pAT29 without insert started eliminating the plasmid at day 2 and eliminated it almost completely at day 7. In contrast, no detectable loss of plasmid was observed over the same period for strain 3-1. The data obtained demonstrated that the functional system Epsilon/Zeta stabilized an otherwise unstable plasmid in *S. agalactiae.* Therefore, we can conclude that this system can stabilize the pathogenicity island PAI-A in the genome of GBS.

Thus, it was shown that the pathogenicity island PAI-A contained genes of the functional TA system Epsilon/Zeta and could be

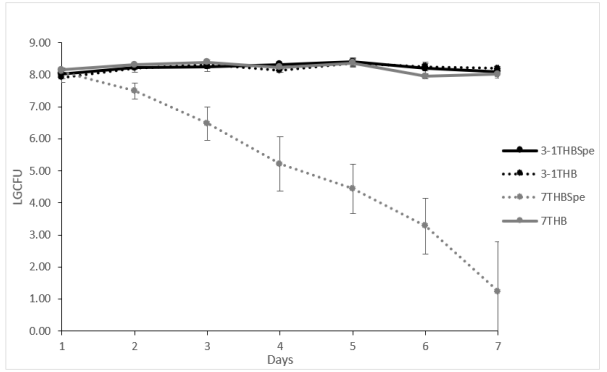

Figure 4: Plasmid stability assay in streptococci. The number of cells of strain 7 on THB supplemented with spectinomycin began to decrease on the second day because of the elimination of the plasmid. The number of cells of strain 3-1 on THB supplemented with spectinomycin remained constant as the number of cells of strains 3-1 and 7 on THB without spectinomycin.

transferred to other bacteria through the type IVC secretion system. Yao., *et al*. [26] reported that attempts to eliminate the pathogenicity island SsPI-1 encoding the TA system SezAT by using mitomycin C were not successful. The authors also showed that this system stabilizes plasmid in *E. coli* (after cloning of the genes encoding for the SezAT system) [26]. Therefore, we propose that after acquisition of the pathogenicity island PAI-A, this MGE would be stably presented in the genome of recipient bacteria due to the functional TA system Epsilon/Zeta. This ability of the Epsilon/Zeta TA system to stabilize a plasmid was thus proven in *S. agalactiae*.

Genetic analysis revealed that the pathogenicity island PAI-A contained additional lactose operon Lac.2-1 [7,20]. The presence of this operon demonstrated the ability of GBS strains with the PAI-A to utilize lactose more efficiently. Cases of neonatal group B streptococcal disease associated with infected breast milk have been reported [27]. Therefore, GBS containing the additional lactose operon Lac.2-1, localized on the stable pathogenicity island PAI-A, might have additional advantages while colonizing breast and bovine milk, which may be the cause of newborn diseases.

Previously, some authors suggested that the TA systems can be used as an effective target for new antibiotics [6,16]. Therefore, the investigation of functionality of the Epsilon-Zeta TA system of GBS would contribute to the development of a new class of antimicrobial substances.

Conclusion

In the present study, it was shown that the Zeta toxin of the type II TA system, encoded by the GBS pathogenicity island PAI-A, demonstrated toxicity in *E. coli*. For the first time, the ability of the Epsilon antitoxin to bind and block the Zeta toxin was proven. On the basis of the results, we affirmed that the investigated TA system belongs to the Epsilon/Zeta type II TA system. This TA system stabilizes an otherwise unstable plasmid in *S. agalactiae.* After acquisition, the pathogenicity island PAI-A containing Epsilon/Zeta genes would be stably presented in the genome of recipient bacteria. Thus, the Epsilon/Zeta TA system of *S. agalactiae* can contribute to the adaptation of microorganisms to new ecological niches. Additionally, the results of the present research might contribute to the development of a new class of antimicrobial substances.

Acknowledgements

- We would like to thank Olga Goupalova for her valuable assistance in the editing of this manuscript and Marina Karpenko for her valuable assistance in statistical analysis.
- This research was supported by Budgetary funds of the Institute of Experimental Medicine.

Conflict of Interest

We have no conflicts of interest to disclose.