BioNanoSci. (2017) 7:623–626
https://doi.org/10.1007/s12668-017-0460-9

Pleiotropic Effect of Salt Stress on Motility and Synthesis of Secreted Ribonucleases by *Bacillus pumilus*

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Published online: 19 October 2017

Abstract Members of the genus *Bacillus* can successfully counteract a sudden increase in salinity. In addition to the accumulation of osmolytes, saline stress also affects other aspects of bacterial physiology such as exoenzymes synthesis and motility. Here, we have shown that increase of salinity in growth medium leads to elevated biosynthesis level of low-molecular weight ribonuclease (RNase) binase I from *Bacillus pumilus*. The same effect was established previously for high-molecular weight binase II. Transmission electron microscopy revealed the absence of flagella and some other changes in salt-stressed cells of *B. pumilis*. We also detected the gene sequences homologous to the recognition sites of response regulator DegU in the binase I and binase II promoters. Using the *B. subtilis* strains with various mutations in DegU gene, we found that the two-component signal transduction system DegS-DegU which regulates the motility under salt stress participates in the control of biosynthesis for both secreted RNase of *B. pumilis* (binase I and binase II).

Keywords Ribonuclease · Binase I · Binase II · *Bacillus pumilus* · Salt stress · Motility · Flagella

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1 Introduction

Members of the genus *Bacillus* are often exposed to significant fluctuations in osmotic pressure as a result of drying or flooding of habitats. Like other bacteria, they resist the negative influences of salt stress primarily by regulating the concentration of soluble ions and organic compounds to support the turgor. It is also known that high salinity exerts pleiotropic effect on the physiology of the bacilli. In particular, the salt stress is an environmental signal affecting exoenzymes (alkaline protease, levansucrase) synthesis *in Bacillus subtilis* [1]. At the same time, salt stress causes severe impairment of the motility of *B. subtilis* [2]. We have shown that increase of salinity in growth medium leads to elevated biosynthesis level of secreted ribonuclease (RNase) of *B. pumilus* 7P binase II [3]. Binase II is the second secreted RNase, found in *B. pumilus* 7P. It differs from binase I by the absence of substrate specificity, the mode of RNA cleavage, and more than twice the molecular weight [4, 5]. Binase I is widely known as a promising antitumor therapeutic agent [6, 7] and despite the fact that biosynthesis of binase I has been thoroughly studied [8], there is no information on synthesis under salt stress. It is also interesting to find a correlation between the level of synthesis of both enzymes and the behavior of *B. pumilus* associated with motility.

2 Materials and Methods

Bacterial strains and plasmid are *B.subtilis* 168 trpC2, (*Bacillus* Genetic Stock Center), *B. pumilus* 7P (All-Russian Collection of Microorganisms—VKM), *B.subtilis* 8G5 (*trpC2*; *tyr*; *his*; *nic*; *ura*; *rib*; *met*; *ade*; lacks the *sipP* genes),

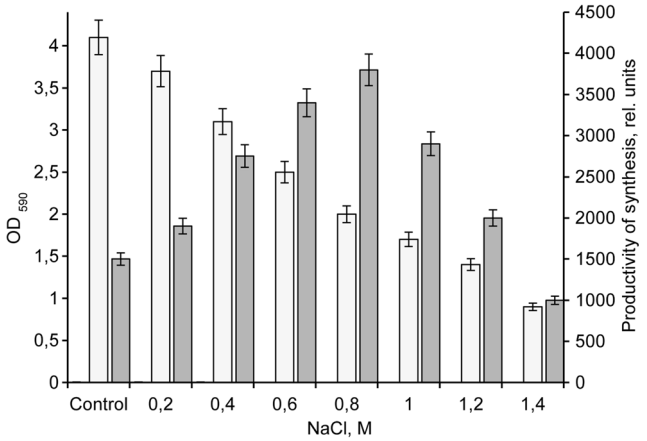

Fig. 1 The effect of high NaCl concentrations on the biosynthesis of binase I by the strain *B. subtilis* 168 (pMZ55): light columns—biomass, OD_{590}, dark columns—productivity of synthesis, relative units. Control—medium without added NaCl

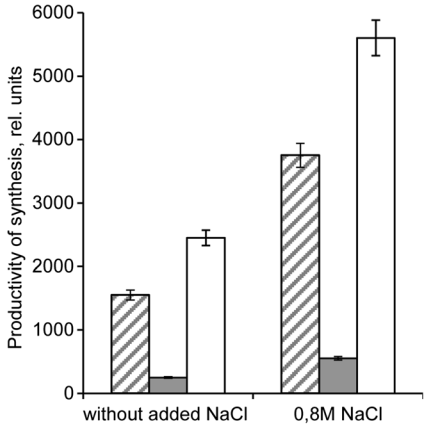

Fig. 3 Biosynthesis of binase I by the recombinant *B. subtilis* strains with mutations in genes encoding proteins of the DegS-DegU system: striped columns—*B. subtilis* 8G5 (pMZ55), dark columns—*B. subtilis* 8G5 ΔdegSΔdegU (pMZ55), light columns—*B. subtilis* 8G5 degU32(Hy) (pMZ55)

B.subtilis 8G5 *ΔdegSΔdegU Km*^{r}, *B.subtilis* 8G5 *degU32*(Hy), and *Km*^{r} (Jan Maarten van Dijl, University of Groningen, The Netherlands), plasmid pMZ55 bearing the kanamycin resistance gene, ampicillin resistance gene, and the *birA* gene of binase I [8]. *B. subtilis* cells were transformed with plasmid DNA as described by Chang and Cohen [9]. Strains were grown at 30 °C in a complex phosphate deficient medium containing: low phosphate peptone 2.0%, glucose 1.0%, $CaCl_2$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.03%, NaCl 0.3%, $MnSO_4$ 0.01%, and pH 8.5. Sterile solution of NaCl (0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.2 M, 1.4 M) was added to the culture medium together with the 1% inoculum. Biomass was determined from the culture density measured at 590 nm. The activity of RNases was determined as described previously [8]. The productivity of RNase synthesis was estimated as the ratio of the RNase activity of the culture liquid to the culture biomass. A transmission electron microscope (TEM) imaging was carried out using TEM Hitachi HT7700 Exalens (100 kV in TEM mode). Analysis of the binase I and binase II gene promoter and genomes of *B. pumilus* was performed using the BLAST algorithm at the National Center for Biotechnology Information NCBI (http://www.ncbi.nlm.nih.gov/). Experiments were performed in triplicate. The results were computed for a 95% confidence interval.

3 Results and Discussion

Biosynthesis of binase II and binase I occurs at the growth retardation phase [8, 10]. At this stage of growth, bacteria are exposed to stresses, mainly because of the depletion of easy available nutrients. It was previously shown that under conditions of phosphate deficiency, binase II and binase I are synthesized intensively [8, 10]. Besides, other stresses can affect biosynthesis level. We have compared effect of salt stress on binase I biosynthesis (Fig. 1) with the data for binase II [3]. To estimate the effect of salt stress on the synthesis of binase I, we transformed the strain *B. subtilis* 168 which has no guanyl-preferring RNases, with plasmid pMZ55 carrying the binase I gene. The productivity of binase I synthesis was measured in media with a gradually increasing salt concentration. The maximal biosynthesis level was detected at 0.8 M NaCl. It was 2.5-fold higher than in the medium without additional salt. As for the binase II, a maximum of productivity was observed at higher salt concentrations (2.0 M NaCl). Thus, high osmolality causes an increase in the level of biosynthesis of both secreted RNAses.

Control of binase II biosynthesis under the salt stress conditions is carried out with the participation of the two-component signal transduction system DegS-DegU [3]. The

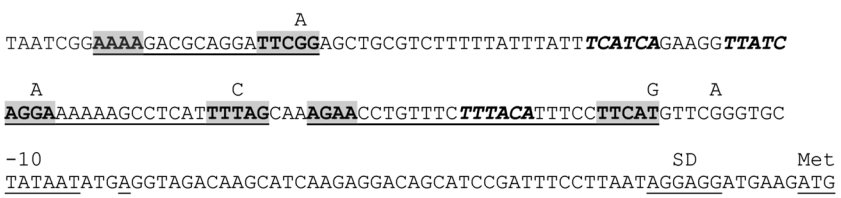

Fig. 2 Promoter region of the binase I gene of *B. pumilus* 7P. Potential binding sites for the DegU are underlined and their conserved motifs are shown in gray boxes. Pho-boxes are given in italics. The initiating codon, − 10 box, transcription start site and Shine-Dalgarno box are underlined

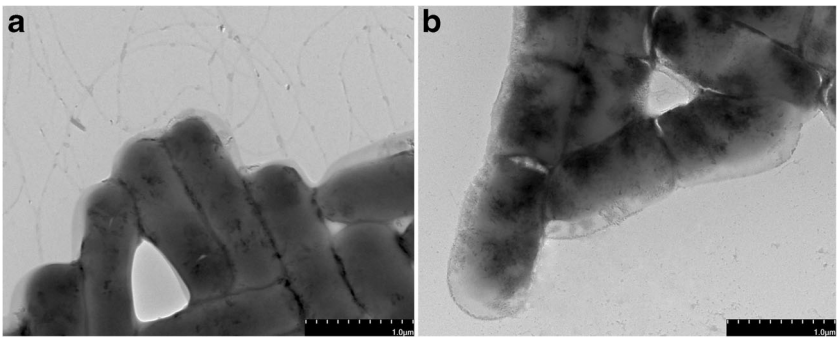

Fig. 4 TEM image of *B. pumilus* 7P cells growing in optimal (**a**) and salt stress (**b**) conditions. The bar corresponds to1 μm

binase II gene promoter contains a consensus sequence for DegU regulator protein and, in contrast to binase I gene promoter, has no pho-boxes for PhoR regulator protein that activates gene expression during phosphate-deficiency stress. In the promoter of binase I gene, in addition to pho-boxes, we have detected potential binding sites for the regulatory DegU protein (Fig. 2). DegU interacts with the respective consensus sequence AG (A) $AAN_{11\text{-}13}$TTCAG (DegU box). Sequence identity of two conservative motifs of DegU box separated by 9 and 12 variable nucleotides was 89 and 78%, respectively. The putative third site probably has 89% identity of conservative motifs, but contains an excessive number of variable nucleotides (N_{25}). Expression of binase I gene was studied in the mutant strain with deletions in the *degS* and *degU* genes and in the mutant strain with an enhanced production of the phosphorylated form (*degU32*(Hy)) of the regulatory DegU protein (Fig. 3). The productivity of RNase synthesis by *B. subtilis* 8G5 ΔdegSΔdegU (pMZ55) was about 15% of the productivity of RNase synthesis by *B. subtilis* 8G5 (pMZ55). At the same time, the level of biosynthesis of binase I by *B. subtilis* 8G5 *degU32*(Hy) (pMZ55) was 1.5–1.6 times higher than in the control. The data obtained are comparable with the results obtained earlier for binase II and confirm the participation of the DegS-DegU system in the regulation of binase I gene expression under conditions of salt stress.

It is known that *B. subtilis* strains with degU (Hy) mutations are characterized not only by the overexpression of secreted enzymes, but also by the loss of flagella [11]. To investigate whether *B. pumilis* 7P undergoes similar morphological changes, we compared cells that were grown in optimal medium with cells that were grown in high salinity (1 M NaCl) at the growth retardation phase. Transmission electron micrographs of the non-stressed cells showed apparently intact envelops with evenly distributed electron-dense cytoplasmic contents and numerous flagella (Fig. 4). The cells exposed to salt stress exhibited a number of differences. The most noticeable difference is the absence of flagella in cells under hyperosmotic conditions. Other differences included local condensation of cytoplasmic contents and a thicker and heterogeneous capsule. These changes may be associated with the activation of the transcription factor DegU that under conditions of salt stress inhibit flagellar-based motility and activates production of extracellular polymers consisting of poly-γ-glutamate and levan [1, 12].

4 Conclusions

Thus, it is established that under salt stress the two-component signal transduction DegS-DegU system is involved in the biosynthesis control of both secreted RNase of *B. pumilis* 7P (binase I and binase II). Activation of RNase gene expression is also accompanied by a DegU-dependent stopping of motility, which is probably associated with the onset of biofilm formation. An increase in the level of RNase biosynthesis under salt stress is part of a protection strategy that facilitates the use of hard-to-reach nutrients in the absence of the ability to move to readily available nutrients.

Acknowledgements The study was performed within the Russian Government Program of Competitive Growth of Kazan Federal University and was supported by the Russian Science Foundation (project no. 14-14-00522).

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