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Phosphate regulation of biosynthesis of extracellular RNases of endospore-forming bacteria

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Abstract The gene for the extracellular ribonuclease of *B. pumilus* KMM62 (RNase Bp) has been cloned and sequenced. The structural gene for this enzyme is similar to those of the extracellular ribonucleases of *B. intermedius* 7P (binase) and *B. amyloliquefaciens* H2 (barnase), as are the regulatory regions of binase and RNase Bp. The regulatory region of the barnase gene, however, is quite different from the other two. In the promoter of the genes for binase and RNase Bp, but not in that for barnase, is a region similar to the Pho box of *E. coli*. We have established that inorganic phosphate suppresses the synthesis of the binase and RNase Bp, but does not effect the synthesis of barnase.

Key words: Barnase; Binase; Ribonuclease Bp; Gene cloning; Pho box; Inorganic phosphate

1. Introduction

Extracellular low-molecular guanylspecific RNases (EC 3.1.27) of different species of *Bacillus* are similar in their physico-chemical and catalytic properties and have high homology in primary structure [1–4], indicating the evolutionary conservatism of these RNases. At the same time one can expect essential differences in the regulation of biosynthesis of these enzymes taking into account deep differences between species of the genus *Bacillus*. Nucleotide sequences of genes for extracellular guanylspecific RNases of *B. amiloliquefaciens* H2 (barnase) [1] and *B. intermedius* 7P (binase) [5,6] have been cloned earlier. Here we report the cloning of the gene for the extracellular ribonuclease of *B. pumilus* KMM62 (RNase Bp) and describe a comparative investigation of the structure of whole Bacilli RNases genes and the regulation of biosynthesis of these enzymes.

2. Materials and methods

B. intermedius 7P (B3073), *B. pumilus* KMM62, *B. amyloliquefaciens* H2 strains were used. *E. coli* SURE (e14-(mcrA), Δ (mcrCB-hsdSMR-mrr) 171, supE44, thi-1, gyrA96, endA1, relA1, lac, recB, recJ, sbcC, umuC::Tn5(kan^r), uvrC,[F′,proAB,lac1^{q}ZΔ M15,Tn10,(tet^r)] was used as a plasmid host for expression studies.

Plasmid isolation was performed as described by Birnboim and Doly [7]. Transformation of competent cells with plasmid DNA was carried out as described in Maniatis et al. [8]. The structural gene for RNase Bp was cloned on its own promoter and signal peptide in *E. coli* SURE on the basis of its homology with binase gene by RC-PCR [9]. Clones containing gene for RNase Bp were selected by hybridization with the radio labeled 25-mer EC59, 5′-TGTATTCAAGTGACTGGCTC, complementary to the coding sequence for binase. The resulting plasmid, pML86, contained a 5kb fragment inserted upstream of the barstar (barnase inhibitor) gene in pMT316. Subcloning was carried out and pML61 was obtained. This plasmid contains barstar gene, since gene expression of bacilli RNases is lethal for *E. coli* cells [1]. The following oligonucleotides were used for the PCR:

EC25 5′-CTTACTCCCCATCCCCCTGTTGACA

EC26 5′-TCTGATGAAATAAATAACTGGCCGTCGTT

EC27 5′-TTACAACGTCGTGACTGGGAAAACCCTG

EC28 5′-AAACAGCCCTACGTCAAATGGGGCTG

EC30 5′-AACGACGGCCAGTTATTTATTTCATCAGA

EC62 5′-AGAATATCAGCAGAAAAGCCTC

EC63 5′-AGCTCGAATTCAAAAAACAGC

EC64 5′-GCTGTTTTTTGAATTCGAGCT

Plasmid pML61 was prepared using oligonucleotides EC30 + EC28 and EC63 + EC62 for RNase Bp gene amplification, and oligonucleotides EC26 + EC25 and EC64 + EC27 for pMT316 amplification. The conditions for Taq polymerase PCR were: 10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl, pH 8.3; 0.2 mM dNTPs; 10 ng of plasmid template; 200 ng of each primer; 0.5–1 units of Taq DNA polymerase, in a final volume of 25 μl. 25 PCR-cycles were performed, each consisted of 1 min at 94°C, 1 min at 50–65°C (depends on primer used) and 3 min at 72°C. PCR products were isolated from 1% agarose gel, precipitated with ethanol and annealed in 10 μl of 1½ SSC buffer. The RNase Bp gene was sequenced by dideoxy chain-termination method of Sanger et al. [10], using Sequenase Version 2.0 (USB) and the primers to binase structural gene.

B. intermedius was cultivated in a medium containing (%): peptone, 2.0; glucose, 1.0; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; $MnSO_4$, 0.01, pH 8,5. For *B. amyloliquefaciens* and *B. pumilus* culture media optimal for RNases biosynthesis on the base of phosphate-deficient peptone were selected. Medium for cultivation of *B. pumilus* contained (%): peptone, 5; glucose, 0.55; other ingredients as in the medium for *B. intermedius*. Medium for cultivation of *B. amyloliquefaciens* contained (%): peptone, 3,75; glucose, 0.65; Na_2HPO_4, 0.04; other ingredients as in the medium for *B. intermedius*. RNase activity was indicated by modified method of Anfinsen et al. [11].

3. Results and discussion

The gene coding for RNase Bp of *B. pumilus* KMM62 was cloned and sequenced (Fig. 1). The nucleotide sequence of the cloned gene has been submitted to GenBank and has been assigned the accession number U06867. The primary structure of RNase Bp and binase are identical [2]. At the same time the structural gene for RNase Bp shares 98% identity with that of binase (10 nucleotide substitutions) and its identity with

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