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,lacI^{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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                          G T        A
1   TTATTTATTT CATCAGAAGA ATATCAGGAG AAAAGCCTCA TTTTAGCAAA

                  -35                          -10
    A[****      ** * *   *  * ]G
51  GATCCTGTTT CTTTACATTT CCTTCATATT CGGGTGCAT  AATATGAGGT

                                                SD
101 AGACAAGCAT CAAGAGGACA GCATCCGATT TCCTTAATAG GAGGATGAAG

                  -50                                    -40
    Met Lys Lys Ile Ser Ser Val Phe Thr Met Phe Ala Leu Ile
151 ATG AAA AAA ATC AGT TCG GTT TTT ACT ATG TTT GCT CTG ATC

                                          -30
    Ala Ala Ile Leu Phe Ser Gly Phe Ile Pro Gln Gln Ala Tyr
192 GCT GCT ATT CTG TTT TCT GGT TTT ATT CCG CAG CAA GCC TAT

                Pro     -20 Gln                Glu
    Ala Glu Thr Thr Leu Thr Pro Thr Ala Thr Asn Lys Thr Ala
234 GCC GAA ACA ACA CTT ACA CCG ACC GCC ACA AAT AAA ACA GCT
    Thr -10     C T          A                 G1
    Ser Ile Gln Leu Thr Ser Asp Val His Thr Leu Ala Val Ile
276 TCT ATT CAA CTG ACA TCA GAT GTT CAT ACC CTT GCC GTC ATT
    A           T           10
    Asn Thr Phe Asp Gly Val Ala Asp Tyr Leu Ile Arg Tyr Lys
318 AAT ACG TTT GAT GGT GTA GCA GAT TAT TTA ATT CGC TAC AAA

            20                                      30
    Arg Leu Pro Asp Asn Tyr Ile Thr Lys Ser Gln Ala Ser Ala
360 CGA CTG CCT GAT AAC TAC ATC ACA AAA TCA CAA GCA AGT GCT
      T                             40
    Leu Glu Trp Val Ala Ser Lys Gly Asn Leu Ala Glu Val Ala
402 CTT GGA TGG GTG GCA TCG AAG GGA AAT CTA GCA GAG GTT GCC
                  50   A
    Pro Gly Lys Ser Ile Gly Gly Asp Val Phe Ser Asn Arg Glu
444 CCA GGC AAA AGC ATC GGT GGA GAT GTT TTC TCT AAC CGG GAG
 60   T                                  70
    Gly Arg Leu Pro Ser Ala Ser Gly Arg Thr Trp Arg Glu Ala
486 GGA CGT CTT CCT TCA GCA AGC GGC CGC ACA TGG CGT GAA GCA
                      80                A               G
    Asp Ile Asn Tyr Val Ser Gly Phe Arg Asn Ala Asp Arg Leu
528 GAT ATC AAC TAC GTC TCA GGC TTC CGC AAT GCT GAC CGC CTC
            90            T               A           100
    Val Tyr Ser Ser Asp Trp Leu Ile Tyr Lys Thr Thr Asp His
570 GTG TAT TCA AGT GAC TGG CTC ATT TAT AAA ACA ACA GAC CAT
                                      C
    Tyr Ala Thr Phe Thr Arg Ile Arg ---
612 TAT GCG ACA TTC ACA CGT ATT CGA TAA
        A   T
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Fig. 1. Nucleotide sequence of the whole gene for the extracellular ribonuclease of *B. pumilus* KMM62 (RNase Bp) and the amino acid sequence of the signal peptide and mature part of RNase Bp. Binase nucleotides in the promoter region and amino acids in the signal peptide different from those of RNase Bp are shown above. Different nucleotides in the leader sequence and in structural gene of binase gene are shown below. The putative ribosome binding site and the −35 and −10 regions are overlined. The sequence with similarity to the Pho box (CTGTCATAATATACTGTCAACT) of *E. coli* is enclosed by square brackets, with asterisks denoting identical bases.

barnase structural gene is 72% (92 nucleotides changes, 3 deletions) [1]. In the regulatory region of gene for RNase Bp there are slight differences from that of binase gene. The signal peptide of RNase Bp differs from that of binase in four amino acids. Three of them do not change the polarity of peptide and the fourth changes polarity (Glu→Lys); the gene for the leader sequence of RNase Bp has six nucleotide substitutions. Promoter region of RNase Bp has 5 nucleotide substitutions and one deletion. In the −35 promoter region of the genes for binase and RNase Bp the nucleotide sequence is found similar to Pho box of *E. coli*, which is known to determine the regulation of genes of the phosphate regulon in phosphate deficient conditions (Fig.1) [12]. The nucleotide sequence of the regulatory region of the gene for barnase differs essentially from those of the genes for RNase Bp and binase. The promoter region of the barnase gene has no sequence homologous to Pho box of *E. coli* (Fig. 2). These comparisons suggested there would be differences in phosphate regulation of the biosynthesis of binase, RNase Bp and barnase.

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1   CAATAAGAAG AAAAATCCCG GTTGGTTCAG CCGGGGTTTA TTTTTCGCTA

51  GATAAAAAGT ACTATTTTTA AATTCTTTCT ATTCCTTTCT TTCGTTGCTG
                                                SD
101 ATACAATGAA AAGGAATCAG CTTCACATGA TGAAAATGGG AGGTATTGCT

                                          -30
    Met Lys Lys Arg Leu Ser Trp Ile Ser Val Cys Leu Leu Val
151 TTG AAA AAA CGA TTA TCG TGG ATT TCC GTT TGT TTA CTG GTG

                      -20
    Leu Val Ser Ala Ala Gly Met Leu Phe Ser Thr Ala Ala Lys
193 CTT GTC TCC GCG GCG GGG ATG CTG TTT TCA ACA GCT GCC AAA

    -10
    Thr Glu Thr Ser Ser His Lys Ala His Thr Glu
235 ACG GAA ACA TCT TCT CAC AAG GCA CAC ACA GAA
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Fig. 2. Nucleotide sequence of the regulatory region of the gene for barnase.

Earlier we determined that biosynthesis of binase is suppressed by inorganic phosphate [13] and the decrease of enzyme activity in culture liquid in the presence of phosphate is not due to inhibition of the enzyme. A comparative analysis of effect of inorganic phosphate on the biosynthesis of ribonucleases by bacilli was, therefor, carried out. Inorganic phosphate was added as Na_2HPO_4 calculated on P. Adding of inorganic phosphate to the culture medium in concentrations of 20 to 800 mg/l

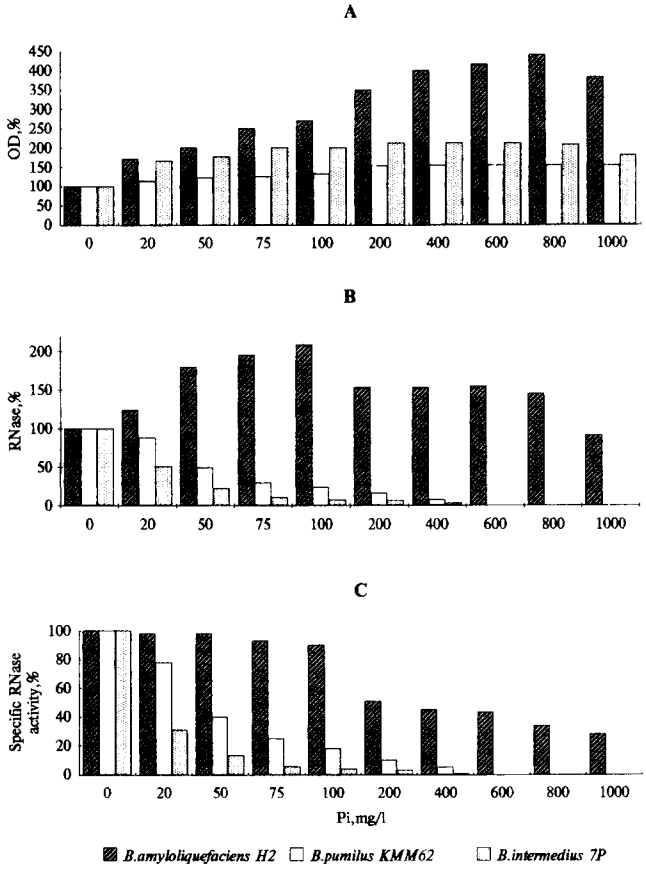

Fig. 3. Effect of inorganic phosphate on growth (A), RNase biosynthesis (B) and specific RNase activity (C) of Bacilli strains. Phosphate deficient culture medium was used was as described in Methods as medium for cultivating of *B. intermedius* 7P. Inorganic phosphate was added as Na_2HPO_4 calculated on content of P.

caused an increase in cell harvest of all three Bacilli strains (Fig. 3a). An increasing P_i concentration caused decrease in binase and RNase Bp activity. Barnase activity grew with increasing of concentration of P_i in medium till 100 mg/l, while binase and RNase Bp activity decreased to 7% and 16% accordingly. Barnase activity then decreased a little but even at P_i concentration 1000 mg/l it was 28% of its maximum (Fig. 3b). Changes of specific RNase activity were analogous (Fig. 3c).

Thus three cloned genes of Bacilli RNases have high degree of homology in the structural parts and essential differences in signal and promoter regions between binase and RNase Bp on the one hand and barnase on the other, including the presence of a Pho box like sequence in −35 promoter regions of the genes for binase and RNase Bp and the absence of such a sequence in the promoter of the barnase gene. Differences in nucleotide sequences of the promoter regions of bacilli RNase genes correlate with results of experiments investigating the effect of inorganic phosphate on biosynthesis of these enzymes: expression of binase and RNase Bp genes is suppressed by inorganic phosphate while barnase synthesis is not.

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