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Barnase and binase: twins with distinct fates

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RNases are enzymes that cleave RNAs, resulting in remarkably diverse biological consequences. Many RNases are cytotoxic. In some cases, they attack selectively malignant cells triggering an apoptotic response. A number of eukaryotic and bacterial RNase-based strategies are being developed for use in anticancer and antiviral therapy. However, the physiological functions of these RNases are often poorly understood. This review focuses on the properties of the extracellular RNases from *Bacillus amyloliquefaciens* (barnase) and *Bacillus intermedius* (binase), the characteristics of their biosynthesis regulation and their physiological role, with an emphasis on the similarities and differences. Barnase and binase can be regarded as molecular twins according to their highly similar structure, physical–chemical and catalytic properties. Nevertheless, the 'life paths' of these enzymes are not the same, as their expression in bacteria is controlled by diverse signals. Binase is predominantly synthesized under phosphate starvation, whereas barnase production is strictly dependent on the multifunctional Spo0A regulator controlling sporulation, biofilm formation and cannibalism. Barnase and binase also have some distinctions in practical applications. Barnase was initially suggested to be useful in research and biotechnology as a tool for studying protein–protein interactions, for RNA elimination from biological samples, for affinity purification of RNase fusion proteins, for the development of cloning vectors and for sterility acquisition by transgenic plants. Binase, as later barnase, was tested for antiviral, antitumour and immunogenic effects. Both RNases have found their own niche in cancer research as a result of success in targeted delivery and selectivity towards tumour cells.

Introduction

In addition to their basic functions in RNA metabolism, RNases are involved in the control of gene expression, cell growth and differentiation, host defence and physiological cell death pathways [1–4]. Many RNases show potent antineoplastic activity, but do not exhibit appreciable immunogenicity or nonspecific toxicity [5–7]. RNases are potential antitumour drugs because of their selective cytotoxicity. Their therapeutic value has been demonstrated by an RNase derived from the frog *Rana pipiens*, onconase (Alfacell, Inc., Monmouth Junction, NJ, USA), which has advanced to phase IIIb confirmatory clinical trials for the treatment of unresectable malignant mesothelioma [8]. The inhibition of tumour cell growth has been established for close analogues of onconase from *Rana catesbiana* and *Rana japonica* oocytes [9], bull semen RNase [10], precursor of human eosinophil-derived neurotoxin [11] and human eosinophil cationic protein [12]. Although the first paper demonstrating the antitumour activity of bacterial nuclease from *Serratia marcescens* appeared in 1964 [13], the development of bacterial RNases into antitumour drugs has remained almost uncharted territory for many years. Recently, microbial RNases have stepped out of the shadow of their well-known animal relatives, demonstrating different biological activities suitable for practical applications.

The superfamily of microbial N1/T1 RNases (EC 3.1.27.3) consists of about 25 members of fungal/bacterial α + β proteins that share sequence and tertiary structural similarities [14]. They are small extracellular enzymes that catalyse the overall hydrolysis of single-stranded RNA preferentially at guanylyl residues, yielding new guanosine 3′-phosphate and 5′-OH ends. This occurs in a two-step process with cleavage of the RNA chain by transesterification of a 5′-phosphoester bond to form a guanosine 2′,3′-cyclic phosphate terminus in the first step, followed by its hydrolysis to a 3′-phosphate product in the second independent step. The best known member of this family is RNase T1, an extremely well-characterized fungal RNase from *Aspergillus oryzae*. Here, we introduce its bacterial relatives, 'molecular twins' barnase (*Bacillus amyloliquefaciens* RNase) and binase (*Bacillus intermedius* RNase) (Figs 1A and S1A). According to recent genotypic identification, the strain known as *B. intermedius* belongs to the species of *B. pumilus* and is renamed appropriately (GenBank Accession No. HQ650161.1). However, given the historical preference, we address the guanyl-preferring RNase as binase. Binase and barnase are unrelated to any mammalian RNases, and therefore are not susceptible to omnipresent eukaryotic RNase inhibitor [16]. As a consequence, they are more toxic towards mammalian tumour cells than are RNases belonging to the pancreatic RNase A superfamily. This makes bacterial RNases favourable for the development of new antitumour drugs.

Here, we summarize our current knowledge on the properties of binase and barnase, as well as their biosynthesis regulation, with an emphasis on the similarities and differences. We also discuss possible physiological reasons for their production by bacteria, and recent advances in the application of these toxic RNases.

Two peas in a pod: phenotypic similarity

Both barnase and binase are small, single-domain proteins consisting of 110 and 109 amino acid residues with molecular masses of 12 382 and 12 213 Da, respectively [17,18]. Their primary structures share 85% identity. Their secondary and tertiary NMR [19,20] and X-ray [21,22] structures are almost superimposable (Fig. 1A). All functionally important amino acids incorporated into the active centre, the substrate binding site and the hydrophobic core are conserved among these two enzymes; substitutions are confined to the surface residues only. The number of charged amino acids differs by just one additional Asp in barnase. Therefore, the physical–chemical and catalytic properties of binase and barnase should be very similar.

Indeed, both barnase and binase are cationic proteins with isoelectric points of 9.2 and 9.5, respectively. The enzymes are highly thermostable over a broad pH range (pH 3–9). They cleave RNA molecules predominantly after purine (guanine) residues with the formation of 3′-mononucleotides and 3′-dinucleotides as the final products. The optimum temperature for hydrolysis is 37 °C and the optimum pH value is pH 8.5. The enzymes do not require any cofactors for activity.

Inside the cell, barnase is specifically inhibited by barstar, a small protein of 89 amino acids [23,24]. Barnase and barstar form a noncovalent one-to-one complex (Fig. 1B), which is one of the tightest known and fastest forming [25]. The shape and charge of the interacting surfaces are highly complementary [26]. Barstar inhibits barnase activity by sterically blocking its active site with an α-helix and adjacent loop. The association is stabilized by charge interaction involving the positively charged amino acid residues Lys27, Arg59, Arg83 and Arg87 of barnase and the negatively charged Asp35, Asp39 and Glu76 of barstar [25,27]. Binase has also been shown to be blocked by barstar *in vitro*, but with much lower affinity [28,29]. Nevertheless, all attempts to purify a physiological inhibitor of binase have failed.

Thus, binase and barnase can be regarded as molecular twins sharing the same phenotypic traits. The question is whether the 'life paths' of the RNases are similar. To obtain a better understanding of their physiological roles, one has to explore their origins.

Two branches of the genealogical tree: the basis of similarity

The *Bacillus* genus is a large group including 244 validly described species to date (http://www.bacterio.cict.fr/b/bacillus.html). The genus is phenotypically and genotypically heterogeneous, and researchers continue to separate new genera from it. Although *B. amyloliquefaciens* and *B. pumilus* do not belong to the same phylogenetic cluster generally referred to as the '*Bacillus subtilis* group', their 'branches' on the phylogenetic tree of *Bacillus* species are adjacent (Fig. S1B [15]). The physiology of the two species is quite similar. The main habitat is soil, from which bacteria are spread to other areas. Both *B. amyloliquefaciens* and *B. pumilus* are often found in the rhizosphere. They are able to promote plant growth and suppress plant pathogens [30]. Thus, *B. amyloliquefaciens* and *B. pumilus* constitute closely related species that occupy the same ecological niches.

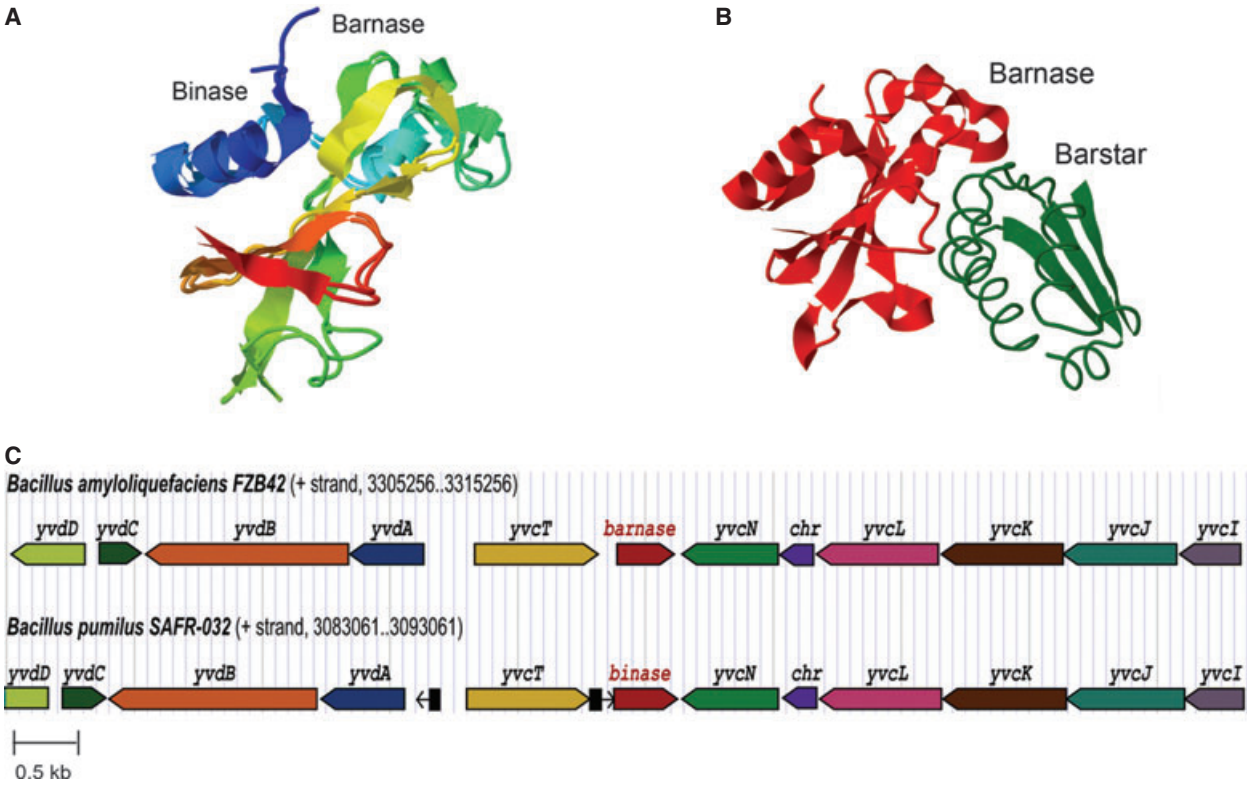

Fig. 1. Protein and gene structures of barnase and binase. (A) Superimposed three-dimensional NMR structures of barnase (PDB ID 1bnr) and binase (PDB ID 1buj) in solution. The alignment was performed using the FATCAT web server (http://fatcat.burnham.org). (B) The 2.0-Å resolution crystal structure of a complex between barnase and its intracellular inhibitor barstar (PDB ID 1brs). Structures were viewed by Jmol (http://www.jmol.org). (C) Gene neighbourhoods of barnase and binase genes extracted from the MicrobesOnline Database (http://www.microbesonline.org). (D) Promoter regions of barnase and binase genes. Potential Spo0A binding sites in the barnase promoter are boxed in a coding strand and highlighted in a noncoding strand; potential PhoP binding sites in the binase promoter are double-boxed.

The capability to secrete guanyl-preferring RNases, which can be advantageous in certain environments, is another common feature of these species. The organization of chromosomal loci containing binase and barnase genes is almost the same and differs from that of other representatives of the *B. subtilis* group. In particular, barnase and binase genes are preceded from the 5′-end by the *yvcT* gene encoding gluconate 2-dehydrogenase, a probable bicistronic operon *yvdA-yvdB* encoding carbonate dehydrogenase and permease of the SulP family, the *yvdC* gene encoding nucleotide pyrophosphohydrolase and the *yvdD* gene encoding a conserved protein with a probable function in DNA recombination or processing. Barnase and binase genes are followed at the 3′-end by a putative *yvcI-yvcN* operon encoding triphosphate pyrophosphate hydrolase,

ATPase/kinase, gluconeogenesis factor, regulatory protein, phosphocarrier protein and *N*-hydroxyarylamine *O*-acetyltransferase in consecutive order (Fig. 1C). Such a genetic neighbourhood assumes that the guanyl-preferring RNases are involved in carbon, nitrogen and phosphorus metabolism. By cleaving RNA molecules to mono- and dinucleotides, these extracellular RNases make the polymer readily available for phosphatases that supply the cell with phosphorus. The remaining nucleosides are further taken by the cell and metabolized to ribose and nitrogen bases, which can be reutilized in the biosynthesis of nucleotides or catabolized as carbon and nitrogen sources.

The above backgrounds indicate the complete similarity of binase and barnase from both molecular and genomic points of view. Is there still any chance of identifying distinguishing traits between the twins?

Mystery of birth: the main difference

In spite of being synthesized during growth impairment phases and accumulated to maximum levels at the beginning of the stationary phase, barnase and binase are 'born' under diverse conditions and through different mechanisms. Dissimilarities in both the signal peptides and regulatory regions of RNase genes demonstrate this fact.

The promoter of the barnase gene is several times stronger than that of binase. However, binase is secreted more effectively. About 30–40% of barnase can be stored inside the cells for up to 90 min in complex with barstar [31]. In contrast, 99% of binase is present outside of the cell and only < 1% is localized intracellularly [32]. Features of binase synthesis and secretion could be the cause of the lack of the RNase inhibitor inside the cells of *B. pumilus*. According to our BLAST searches, barstar is present only in *B. amyloliquefaciens* strains. They also produce the paralogous RNase inhibitor, YrdF, which is 49% identical to barstar. Taking into account positive amino acid substitutions, the similarity between the two proteins increases to 72%. YrdF homologues are widespread among *Bacillus* species and are found in *B. subtilis*, *B. pumilus*, *B. licheniformis* and *B. cereus*. Despite the overall similarity in the topology of barstar and YrdF (Fig. 2A, B), there are subtle differences in the secondary structures of these proteins, strengthened by the two substitutions in the barnase binding site (Fig. 2C). Barnase and barstar interacting surfaces are known to have a high degree of charge complementarity [26]. The

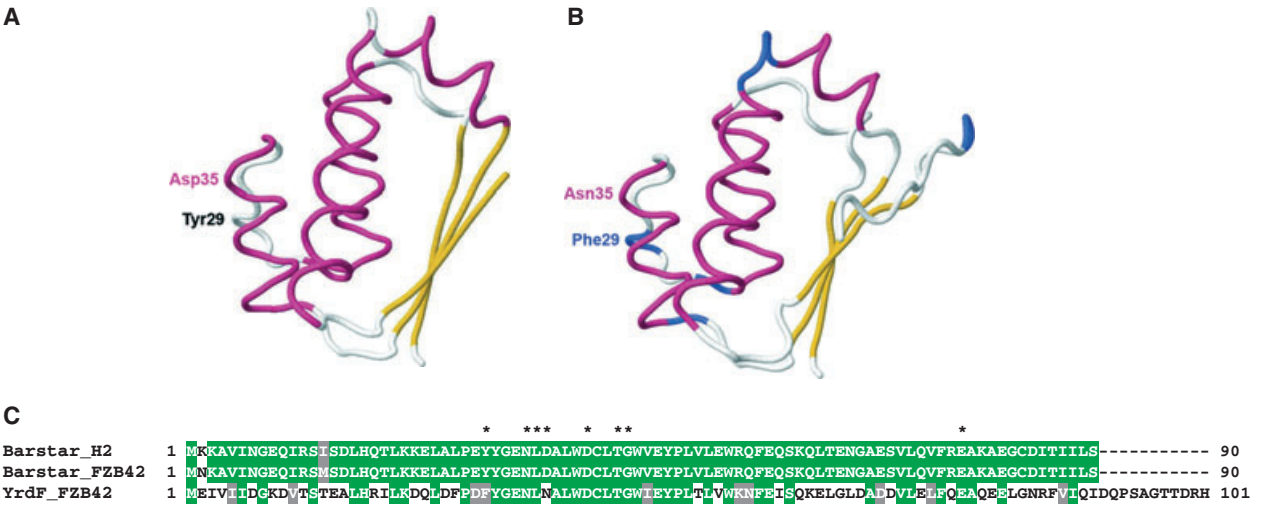

Fig. 2. RNase inhibitors from *Bacillus amyloliquefaciens*. (A) The three-dimensional structural prediction of the YrdF protein (UniProtKB Accession No **A7Z8U3**) obtained with I-TASSER without specifying any template [33]. The confidence score of the model is 0.22. The confidence score normally ranges from −5 to +2; higher scores are attributed to models with higher confidence. The Cα rmsd value is 3.5 ± 2.4 Å, indicating the medium resolution of the model obtained. The TM score is 0.74 ± 0.11, indicating the correct topology of the model. The amino acid residues that are involved in barnase binding and differ between the barstar and YrdF proteins are shown. The pictures were generated using JMOL software (http://www.jmol.org). (B) The 2.25-Å resolution crystal structure of barstar (PDB ID code **3da7C**). (C) Multiple alignment of amino acid sequences of *Bacillus amyloliquefaciens* RNase inhibitors. The proteins with the following UniProtKB accession numbers were used for comparison (strain names are given after protein numbers): **P11540**, Barstar_H2; **A7Z2K7**, Barstar_FZB42; **A7Z8U3**, YrdF_FZB42. Alignment was performed using the CLUSTALW 2.1 algorithm at EMBL-EBI Web Services with default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The amino acids of barstar that are involved in the interaction with barnase are marked by asterisks.

mutations of charged residues alter the rate of complex association [34]. Therefore, two substitutions of the key amino acid residues (Tyr29 and Asp35of barnase/Phe29 and Asn35 of YrdF) could account for the inability of YrdF to effectively block barnase. Lethal expression of the gene for barnase alone without barstar in *B. subtilis* host cells [23] supports this assumption. The YrdF protein is likely to inhibit another type of secreted RNase in *Bacillus*, namely high-molecular-weight RNase Bsn or binase II [35,36]. These nonspecific RNases cleave RNA molecules with the formation of 5′-terminal phosphate products, compared with the hydrolysis of RNA to produce ribonucleotide-3′-phosphates by guanyl-preferring RNAses.

Moreover, the biosynthesis of binase and barnase depends on diverse factors. Unlike the production of barnase by *B. amyloliquefaciens*, binase is not produced by *B. pumilus* when bacteria are grown on medium containing an inorganic source of nitrogen, perhaps suggesting that certain growth factors inherent in peptone or yeast extract are essential. One of these factors could be thiamine, as its addition to the culture medium leads to a considerable increase in RNase activity [37].

Binase, but not barnase, is involved in phosphate metabolism. Low concentrations of extracellular inorganic phosphate (0.12–0.14 mM) induce the expression of phosphate regulon (Pho regulon) genes, as well as the binase gene [38,39]. Transcription of the Pho regulon genes under phosphate starvation is controlled in *Bacillus*, mainly by a two-component signal transduction system PhoP–PhoR [40]. We have shown that only the binase promoter contains four TTAACA-like sequences, known as 'Pho boxes', for binding the transcription factor PhoP (Fig. 1D). The ability of the PhoP protein to specifically interact with these proposed binding sites has been demonstrated by us in an electrophoretic mobility shift assay (V. Ulyanova *et al.*, unpublished results). The promoter of the barnase gene was unable to form a complex with the PhoP protein. We have proven that binase expression is strongly dependent on a functional PhoP–PhoR two-component system [41]. The production of binase by recombinant *B. subtilis* strains bearing *phoPR*-null mutations is impossible, whereas barnase is synthesized on a basic level.

It is known that the Pho regulon is controlled by a regulatory network comprising, in addition to the PhoP–PhoR system, two other systems [38]: a ResD–ResE two-component system regulating respiration processes and activating the PhoP–PhoR system, and a Spo0A phosphorelay leading to the initiation of sporulation and repression of the Pho regulon. We studied the effect of ResD and Spo0A regulators on the production of RNases. Mutation in *resD* led to a significant decrease in binase production, whereas mutation in *spo0A* caused its hyperproduction [42]. These data are consistent with the expression profiles of other Pho regulon genes, in particular the alkaline phosphatase gene [38].

With regard to barnase, its expression is strictly dependent on Spo0A. In the *spo0A*-null mutant, barnase activity was not detected, indicating a positive role of the Spo0A regulator. Potential Spo0A binding sites are present in the barnase promoter (Fig. 1D), and direct regulation of barnase expression by Spo0A is proposed. The Spo0A protein is a multifunctional regulator that controls stress-related processes, such as sporulation, biofilm formation and cannibalism [43–46]. Taking this into account, we speculate that extracellular guanyl-preferring RNases could have extra activities under stress conditions in addition to their main digestive function.

Hidden threat from inside and outside: RNases serving the bacterial population

Nutrient deficiency induces the production of certain hydrolytic enzymes by bacteria in order to break up hard-to-reach macromolecules and thus liberate nutrients. As RNA is a potential source of phosphorus, nitrogen and carbon that are lacking in the early stationary phase, barnase and binase are usually considered as digestive enzymes. The above-mentioned experimental data and genetic neighbourhoods of the RNase genes demonstrate this function. However, the higher catalytic activity and guanyl preference on RNA hydrolysis enable us to assume that barnase and binase, in contrast with the nonspecific RNase Bsn (or binase II), might have certain particular functions. There has been a substantial increase in the number of reports concerning the involvement of RNases secreted or sequestrated inside the cell in a conserved cellular response to stress (for a review, see ref. [47]). This response includes the degradation of certain cellular RNAs which leads to translation disturbance or cell death. Fragments of the cleaved RNAs have been proposed to serve as a marker of cell damage [47]. Barnase and binase could help the population to win the competition with other bacteria for ecological niches, acting as toxins. Such a competition could also take place within the RNase-producing population; this process is called 'cannibalism' in *B. subtilis* [44].

Cannibalism is a type of bacterial programmed cell death, which is triggered by nutrient starvation and governed by the Spo0A protein [45]. The bacterial

population is divided into two subpopulations. In the first subpopulation, Spo0A regulator activates the synthesis of SkfA and SdpC killing factors, as well as a number of immunity proteins that confer resistance to SkfA and SdpC (Spo0A-ON population). In the second subpopulation, the Spo0A regulator is not functionally active, and the cells lack the protection mechanism against the bacteriocins (Spo0A-OFF population). As a result, the second subpopulation is lysed and the released nutrients are used by the first subpopulation for growth prolongation. This type of stress adaptation is described for *B. subtilis* only [44,45]. Our BLAST searches did not find similar killing factors and immunity proteins in *B. amyloliquefaciens* and *B. pumilus*. Thus, we suspect that toxic extracellular RNases and antitoxic barstar could build an analogous system. The ability of barnase to act as a bacteriocin supports this hypothesis [48].

The expression of barnase is strongly activated by the Spo0A regulator. Therefore, barnase is secreted only by Spo0A-ON cells, which are protected by barstar from the toxic action of the RNase. Spo0A-OFF cells are not able to produce barnase. Moreover, we suppose that they also do not express barstar at a level sufficient to inhibit extracellular barnase from Spo0A-ON cells because: (a) genes for barnase and barstar in the *B. amyloliquefaciens* genome are located at different loci, indicating distinct regulation of their expression; (b) the promoter of barstar contains possible binding sites for a SigW transcription factor that controls cell resistance to antimicrobial peptides and other compounds; and (c) the SigW-mediated protective mechanism is blocked in Spo0A-OFF cells [49]. Thus, Spo0A-OFF cells are not protected from the cytotoxic action of barnase and could be lysed under starvation to provide other sibling cells with nutrients (Fig. 3A).

In contrast, our data indicate that the expression of binase is possible only in Spo0A-OFF cells. Its biosynthesis rate in the *spo0A*-null strain was five to six times higher than in the wild-type strain. *Bacillus pumilus* does not possess barstar, but secretes binase very effectively, which protects Spo0A-OFF cells from the toxic action of RNase. Spo0A-ON cells are lysed because of the absence of protective mechanisms, which supports the growth of the RNase-producing cells (Fig. 3B).

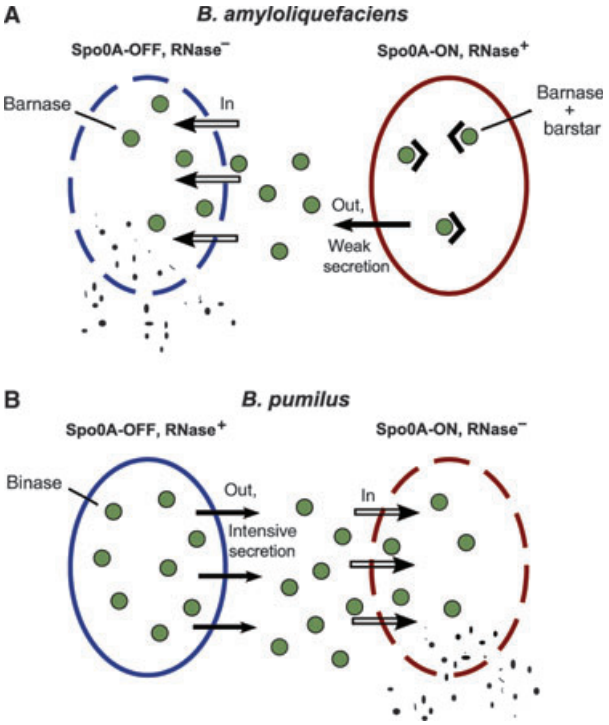

Fig. 3. Scheme illustrating the physiological roles of barnase (A) and binase (B) in starving *Bacillus* populations. RNase⁻, cells that do not produce RNase; RNase⁺, cells producing RNase; Spo0A-ON, cells in which the Spo0A regulator is functionally active (phosphorylated); Spo0A-OFF, cells with inactive Spo0A regulator. Filled arrows indicate the secretion of RNases outside the cells; open arrows show the penetration of RNases into the cells. (A) *Bacillus amyloliquefaciens* cells that do not contain activated Spo0A regulator are unable to synthesize barnase or its intracellular inhibitor barstar, and therefore are susceptible to the toxic action of extracellular barnase produced by Spo0A-ON cells. (B) *Bacillus pumilus* Spo0A-inactive cells hyperproduce binase and actively secrete it outside, affecting the Spo0A-active cells in which the production of binase is repressed. In both cases, nutrients released from lysed cells support the growth of the RNase-producing population.

Practical applications: RNases serving humanity

The fates of barnase and binase have also diverged in terms of their practical applications. In the very beginning, barnase was regarded as a potentially useful tool in biotechnology and research methodology [50–52], and only recently have a number of barnase-based strategies been suggested for use in medicine [53–55]. In contrast with barnase, binase was initially tested for therapeutic traits, such as antiviral and antitumour activities [56,57].

Barnase in research methodology and biotechnology

The ability of barnase to form a high-affinity complex with barstar has been exploited by biochemists for the study of protein–protein and protein–nucleotide interactions [50,58]. Either barnase or barstar can also act as an affinity tag when fused to proteins, such as antitumour antibodies and peptides, during their

quantification, immunodetection and purification [59,60]. It has been proposed that recombinant barnase may be applied as a reagent for the elimination of RNA from samples of plasmid DNA before their purification for genetic vaccination and gene therapy [61]. The cytotoxicity of barnase is used in the development of vectors suitable for direct cloning of PCR products [62]. The strategy involves cloning the desired DNA fragment into an open reading frame of the barnase gene, which leads to its dysfunction and therefore to the survival of the recombinant strains. If the cloning attempt is unsuccessful, the cells are lysed under the toxic action of barnase. A similar approach has been applied for the improvement of baculovirus expression vectors [63].

Numerous scientific publications have been devoted to the acquisition of male, female or bisexual sterility by transgenic plants with the help of barnase [51,64]. To this end, the barnase gene is placed under the control of a tissue-specific promoter, which enables it to be expressed in certain parts of the plant anther and/or pistil. Barnase expression leads to the destruction of the reproductive organs and therefore to sterility of the plant. The engineered sterility of plants is helpful for impeding the invasion of genetically modified plants into natural ecosystems and for the removal of allergenic pollen. It enhances biomass production in landscape species and bioenergy crops, facilitates hybrid seed production and extends the flowering period in ornamental species. In addition, the toxic potential of barnase can be useful for the self-destruction of cover crops [65], the development of disease insensitivity in plants [52] and the reduction in the antinutritional properties of rapeseed meal [66].

Binase and barnase in medical research

Binase and barnase demonstrate biological effects that make them promising tools in the development of specific agents attacking pathogens or malignant cells. Barnase and binase are cytotoxic proteins that exert certain mutagenic [56] and nephrotoxic [57] properties at high concentrations. At the same time, low doses of binase have been shown to promote the growth and stimulate the physiology of plants and microorganisms [67]. Binase possesses antiviral activity against murrain, influenza and rabies viruses [68]. However, the most intriguing feature of these RNases is their antitumour activity. They are able to selectively destroy malignant cells of certain types, inducing apoptotic reactions [69,70]. Importantly, binase lacks the properties of a superantigen, as it does not induce the expression of CD69 and IFN-γ activation markers in CD4+ and CD8+ T lymphocytes [69].

The biological effects of barnase and binase are mainly a result of the membrane-acting properties of these small and positively charged proteins and their catalytic activity. Cationicity of the protein is a crucial factor for membrane binding, cell internalization and cytosol entrance [70,71]. It is well known that tumour cells express an increased number of negatively charged carbohydrates and lipids on their surface in comparison with normal cells, and the enhancement of basicity using site-directed mutagenesis [72] or chemical modification [73] increases cytotoxicity. Barnase and binase manifest their catalytic activity inside the cells towards available RNA molecules as a result of their high stability and lack of susceptibility to the ubiquitous cytoplasmic eukaryotic RNase inhibitor.

To control the hydrolytic activity of RNases, it has been suggested that they should be converted into artificial zymogens [54,74]. This approach assumes the linkage of the enzyme's termini through a short peptide, with the subsequent recovery of its functional activity by protease cleavage. The design of the linking peptide so that it can be recognizable, for example, by HIV protease, will allow the construction of novel HIV/AIDS-targeted therapeutic agents [74].

Here, we turn our attention to two bacterial RNases as an alternative platform for novel anticancer therapy. Targeted delivery and selectivity towards tumour cells are crucial factors that determine the efficiency of the medical application of a potential drug. In this regard, barnase and binase have found their own niche in the field of cancer research.

Barnase has been shown to inhibit cell proliferation in human carcinoma and leukaemia cell lines [53]. To increase its targeted delivery to tumour cells, two barnase molecules are fused to the single-chain variable fragment (scFv) of humanized 4D5 antibody directed against the extracellular domain of human epidermal growth factor receptor 2 (HER2), a cancer marker that is overexpressed in many human carcinomas [53,55]. Thus, the cytotoxic effect of scFv 4D5-dibarnase on HER2-positive human ovarian carcinoma is increased 1000-fold compared with barnase alone [53]. The antibody fragments of immunoRNases do not only deliver the cytotoxic agent to the surface of the tumour cells, but also enable it to penetrate the cells via receptor-mediated endocytosis (for a review, see ref. [75]).

Nanoscale carriers (quantum dots, nanoparticles, carbon nanotubes) are also regarded as promising vehicles for the intracellular delivery of proteins for therapeutic purposes. Using RNase A as a model, the efficiency of self-assembled heparin-Pluronic nanogels and silica nanoparticles for the intracellular delivery of anticancer therapeutics has been demonstrated [76,77].

These nano-sized carriers use lipid rafts or involve clathrin-coated pits and actin microfilaments for their internalization. This allows them to escape from endosomal compartments, facilitating the retention of the biological activity of the delivered proteins. Fusion of nanoparticles with antibody fragments increases the targeting specificity of such conjugates. This is why the creation of multifunctional supramolecular structures built on barnase and barstar molecules fused to diverse antibody variable fragments and various nanoparticles has been proposed for the complex treatment of malignant cells [78].

The most important practical aspect of the cytotoxicity of bacillary RNases is their selectivity against tumour cells. Such selectivity has been clearly demonstrated for binase, although the basis for this effect is poorly understood. In the last few years, we have demonstrated that fibroblasts transformed with *ras* [79] and *kit* [69,80] oncogenes are more susceptible than nontransformed cells to the toxic effect of binase. However, binase has no antiproliferative effect on v-*src*- or v-*fms*-transformed fibroblasts [79]. We assume that the expression of certain oncogenes determines the susceptibility of tumour cells to binase. Recently, this assumption has been demonstrated for the *kit* oncogene: the inhibition of the tyrosine kinase Kit by imatinib led to a sharp decrease in the sensitivity of *kit*-transformed myeloid progenitor cells towards binase [80]. The synthesis of *kit* mRNA decreases significantly in binase-treated cells, supporting the direct or mediated effect of the RNase on the *kit* oncogene [81].

Many studies have reviewed the possible mechanisms of RNase cytotoxicity, including bacterial RNases [5–7]. Catalytic activity is agreed to be one of the key factors determining the regulation of intracellular processes by exogenous RNases. RNase-controlled RNA degradation plays an essential role in the control of gene expression, maturation and turnover, which are further associated with cancer progression [7]. Although binase is responsible for the reduction in the total amount of intracellular RNA in cancer cells, the decrease in the RNA content does not correlate with cell death [81]. As binase retains its catalytic activity after internalization in tumour cells for 48 h [81], its ability to induce regulatory alterations is obvious. Thus, it has been shown that the quantity of proapoptotic *p53* and *hSK4* mRNAs increases and the amount of antiapoptotic *bcl-2* mRNA decreases during binase treatment. Such effects may be a result of the degradation of miRNAs which silence the expression of certain genes and the generation of siRNAs on RNA cleavage [82,83]. These results may lead to novel research aimed at the effective elimination of cancer cells expressing certain oncogenes.

Conclusions

Recent data on the role of RNA interference in tumorigenesis have inspired new studies of enzymes cleaving RNAs as potential antitumour and antimetastatic agents. Many processes associated with the anticancer effect of RNases, namely the degradation of encoding RNAs followed by the arrest of protein synthesis, changes in the gene expression profile via RNA cleavage, degradation of noncoding RNAs and processes mediated by RNA interference, are known. The mechanism behind the RNase affinity with tumour cells, which is the most important question to be addressed, is currently under intensive study. Firstly, electrostatic interactions may provide an explanation for the preferential sensitivity of tumour cells to the strongly cationic binase and barnase, whereas the surfaces of cancer cells have a distinctly higher negative charge compared with their normal counterparts [84]. Therefore, the artificial cationization of RNases could be a promising basis for antitumour drug development. Secondly, targets for the selective action of cytotoxic bacterial RNases in malignant cells are to be found among the structures and/or processes which differ from those in normal cells. In particular, expression of the c-*kit* oncogene is one of these processes targeted by binase [80]. This suggests that bacterial RNases could participate in the blocking of other signalling pathways in tumour cells. Thirdly, the construction of bacterial RNase-based immunotoxins directed against tumour surface antigens is a very attractive tool for the targeted delivery and selective killing of cancer cells. Therefore, we assume that bacterial RNases have potential therapeutic utility. Emerging knowledge on the regulatory pathways guided by these enzymes in their own cells and in RNase-treated pro- and eukaryotic cells could aid in the development of new anticancer agents.

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Supporting information

The following supplementary material is available:
Fig. S1. Phylogenetic trees of guanyl-preferring RNases and their bacterial producers derived from amino acid sequences of the enzymes (A) and nucleotide sequences of 16S rRNA genes (B), respectively.

This supplementary material can be found in the online version of this article.

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