J. Mol. Biol. (1988) **202**, 913–915

Barnase and Barstar

Expression of Its Cloned Inhibitor Permits Expression of a Cloned Ribonuclease

Barnase is the extracellular ribonuclease of *Bacillus amyloliquefaciens* and barstar its specific intracellular inhibitor. The gene for barstar has now been cloned and sequenced. When the wild-type gene for barnase is reconstructed from its previously cloned parts on the same plasmid as the barstar gene, the lethal effect of its expression is suppressed. A plasmid has been devised which directs the secretion of 100 mg per active barnase liter by *Escherichia coli* and another which provides large (500 to 1000 mg/l) yields of barstar. The structure of these plasmids and the derived 89 amino acid sequence of barstar are reported.

The gene for barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens*, has heretofore been cloned only in inactive form, either with an insertion derived from the transposon Tn*917* (Paddon & Hartley, 1985; Hartley & Paddon, 1986), or with point mutations which allow expression of inactive barnase mutants (Paddon & Hartley, 1987). I report here the cloning of the gene for barstar, an intracellular protein of *B. amyloliquefaciens* which specifically inhibits barnase by combining with it in a one-to-one complex (Smeaton & Elliott, 1967; Hartley & Smeaton, 1973). Assembly of the wild-type barnase gene on a plasmid which also carries the barstar gene allows the synthesis of active barnase. Both barnase and barstar are small proteins (110 and 89 residues), with neither non-peptide components nor disulfide bonds, and both may be unfolded reversibly in solution. The folded structure of barnase is known (Maugen *et al.*, 1982), and it clearly resembles, in both sequence and folding, several other bacterial and fungal ribonucleases (Hill *et al.*, 1983). Each protein alone is an excellent subject for the use of random or site-directed mutagenesis in the study of sequence-directed folding. Together, they provide the added interest of a specific protein–protein interaction. The plasmids described below were designed with these points in mind.

We have reported the sequence of the barnase gene (Paddon & Hartley, 1985) and, under control of the promoter and signal sequence of the *Escherichia coli phoA* gene, expression and secretion of correctly processed barnases inactivated by point mutation (Paddon & Hartley, 1987). I have now cloned the gene for barstar by screening a library of *B. amyloliquefaciens* DNA in *E. coli* with a second-antibody immunostain procedure (Huynh *et al.*, 1985), using rabbit anti-barstar serum. The antigen-producing clone obtained carried a 7000 base-pair fragment of *B. amyloliquefaciens* DNA (in the plasmid vector pUC19). Barely detectable levels of barstar activity could be extracted from cells carrying this plasmid (pMT302). On the basis of a restriction map of this DNA, smaller fragments were cloned and tested for barstar production. This process provided a *Sma*I–*Cla*I fragment about 800 base-pairs long, which directed the synthesis of some 40 mg barstar/l. (Barstar yields given here and subsequently are based on activity; that is to say, inhibition of barnase.) Further subcloning, after digesting this fragment from either end with exonuclease III (Henikoff, 1984), yielded a fragment with the 436-base sequence included in Figure 1 (bases 640 to 1075). pMT311, with this fragment alone in pUC19, provided a yield of 50 mg barstar/l. Also shown in Figure 1 is the derived amino acid sequence of barstar. Its composition and 89 amino acid residue length agree with those reported for barstar isolated from *B. amyloliquefaciens* (Hartley *et al.*, 1972). The previous analysis reported one more leucine and one more proline and missed the two cysteine residues (b*40 and b*82). The gene has a good ribosome binding site (overlined in Fig. 1, bases 748 to 758) and the TAGAAT sequence starting at base 733 is presumed to be the "−10" portion of the promoter sequence (Pribnow, 1975).

When the gene for wild-type barnase, which is lethal alone, was assembled on the same plasmid as the barstar gene, extracellular ribonuclease activity was produced. With this combination on a pUC19-pC194 shuttle vector (Paddon & Hartley, 1987), barnase is correctly processed and secreted by *Bacillus subtilis* but remains cell-bound in *E. coli*. With barstar and the *phoA* promoter–signal sequence construct (Paddon & Hartley, 1987), which has an *E. coli* signal peptidase site at the N terminus of mature barnase, and induction by low phosphate, authentic barnase is secreted by *E. coli* at a level of 20 mg/l. A variable amount of this barnase, generally about half, appears in the culture medium, the rest remaining in the periplasmic space. On the addition of acetic acid to 5%, however, all of the barnase is released to the medium, from which it may be directly adsorbed onto phosphocellulose (Hartley & Rogerson, 1972). More than 90% of the protein eluting from the phosphocellulose in high salt is barnase. Essentially pure barnase may then be obtained by salt-gradient chromatography on a strong anion exchanger such as SP-Trisacryl (Pharmacia).

0022-2836/88/160913-03 $03.00/0

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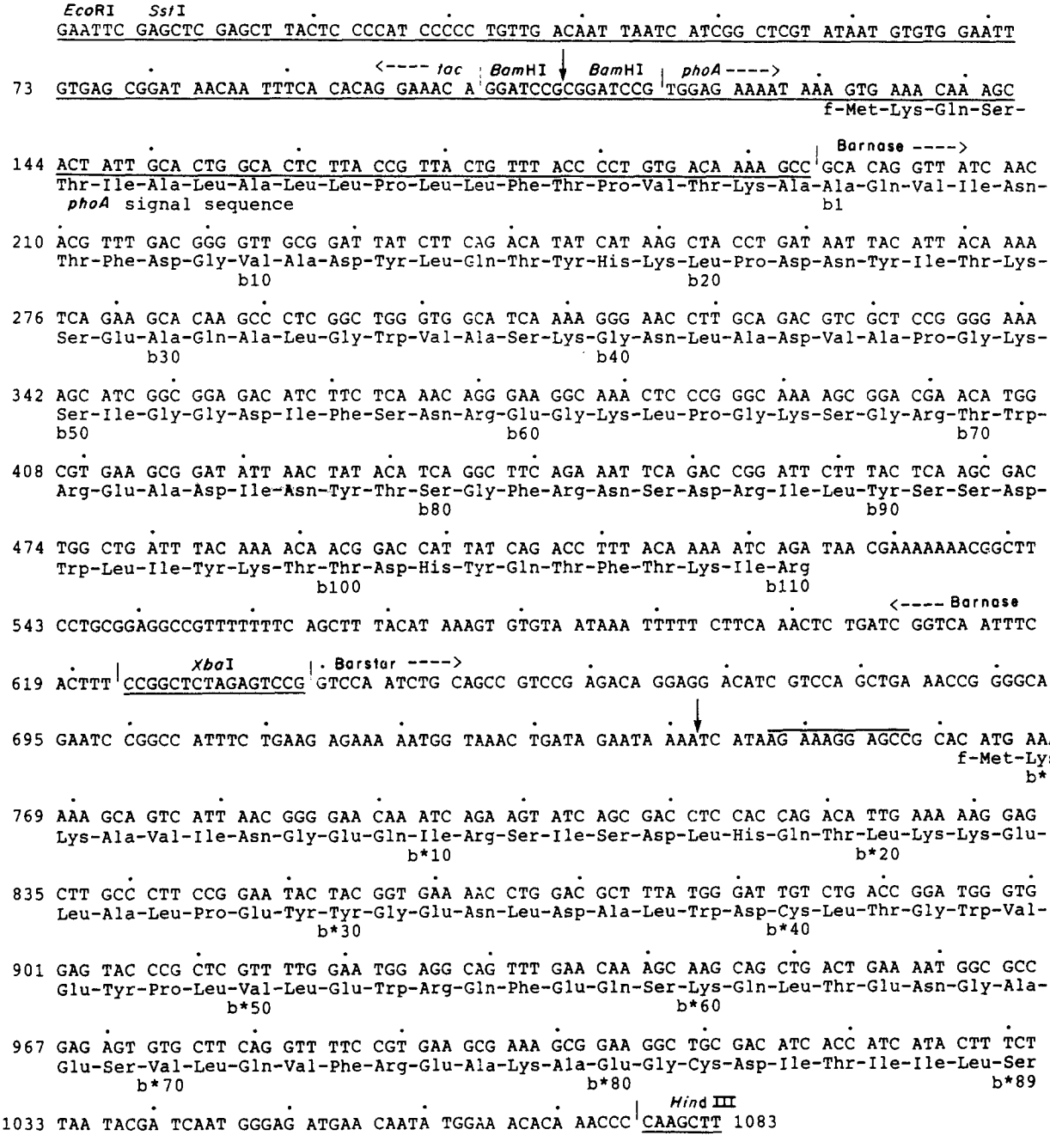

Figure 1. Sequence of the barnase secretion fragment of plasmid pMT416. The derived amino acid sequences of barnase (b) and barstar (b*) are also shown. Sequences not derived from *B. amyloliquefaciens* are underlined. Arrows mark the ends of the DNA (111 and 742) deleted to produce the barstar production plasmid, pMT316. The 3′ end of the previously published barnase sequence (Paddon & Hartley, 1985) has been corrected and extended in this Figure without affecting the amino acid sequence.

In order to increase barnase production, the *phoA* promoter was replaced by a synthetic *tac (trp-lac)* promoter (de Boer *et al.*, 1983), while keeping the *phoA* signal sequence. The entire sequence of this construction is shown in Figure 1. pMT416 is composed of this fragment linked to a plasmid vector comprising the 1831 nucleotides of pUC19 (Yanisch-Perron *et al.*, 1985) between the *Hae*II (680) site and the *Ssp*I (2501) site. In *lac*I^{Q} (high repressor) *E. coli* strain JM107, grown in a rich medium, pMT416 yields as much as 100 mg barnase per liter. Full induction of the *tac* promoter with IPTG (isopropyl-β-D-thiogalactopyranoside), however, is lethal, suggesting that the barstar available is not sufficient to provide protection from the increased barnase synthesis. The extra *Bam*HI linker seen at the *tac*/signal sequence junction apparently provides a better ribosome binding site. Removal of the linker reduces yield.

A barstar production plasmid, pMT316, with the barstar structural gene on the *tac* promoter, has been derived by deletion from pMT416. The deletion (arrows, Fig. 1) is from the *Bam*HI site at the 3′ end of *tac* through nucleotide 742 just to the

left of the barstar ribosome binding site. This was obtained by exonuclease III digestion (Henikoff, 1984) from the *Xba*I linker, followed by insertion of a *Bam*HI linker and deletion of the new *Bam*HI fragment thus formed. This construct yields as much as 1 g barstar/l on induction by IPTG.

Recombinant barnase from *E. coli* is indistinguishable from the authentic protein from *B. amyloliquefaciens* in size, specific activity, crystal morphology, N-terminal sequence, chromatographic behavior (on SP-Trisacryl) and thermal unfolding. Recombinant barstar has not yet been studied in such detail, but clearly resembles authentic barstar in size, activity and chromatographic behavior. Several active-site mutants of barnase, as well as mutants altered at each of its three proline residues, have been investigated. These studies will be reported elsewhere.

It is now clear, as suspected all along, that the function of barstar is to protect *B. amyloliquefaciens* from the damaging effects of barnase within its own cells. This in turn implies that, in *B. amyloliquefaciens*, *B. subtilis* and *E. coli*, some fraction of the barnase produced is folded into active form before it can be secreted. That barstar is not involved in some special mechanism for barnase secretion is indicated by the fact that inactive barnase mutants can be secreted perfectly well without it (Paddon & Hartley, 1987).

The existence of enzymes similar to barnase in eukaryotes suggests an ancient lineage for this enzyme. In view of the growing sense that the number of basic protein folds is limited, it is interesting to speculate on where one might look to find further homologs. While the bacterial colicins exert their killing effects in a variety of ways (for a review, see Konisky, 1982), several are ribonucleases and most or all appear to be synthesized in concert with specific inhibitors called immunity proteins (see, for example; Masaki & Ohta, 1985; Mankovich *et al.*, 1986). These inhibitors act, as does barstar, by forming one-to-one, non-covalent complexes with their respective targets. There is no obvious similarity between the sequence of barnase and that of any of the colicins, or between those of barstar and the immunity proteins. The question of homology, or descent from a common ancestor, therefore, must wait on comparisons of their three-dimensional folds, alone and in complex, which, when the requisite structures become available, will provide a more sensitive test.

The barnase–barstar pair is now available as a model system for the use of random or directed mutgenesis in studies of: (1) sequence-directed folding; (2) the enzymatic mechanisms of barnase; and (3) the protein–protein reaction between the two. While it is hoped that the availability of barstar in quantity will lead to its crystallization for X-ray diffraction studies, its structure may also be within the range of modern nuclear magnetic resonance techniques.

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Received 19 April 1988

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Edited by S. Brenner