Isolation and Purification of Two Novel Streptomyces RNase Inhibitors, SaI14 and SaI20, and Cloning, Sequencing, and Expression in Escherichia coli of the Gene Coding for SaI14

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Two new RNase inhibitors, SaI14 (Mr, ~14,000) and SaI20 (Mr, ~20,000), were isolated and purified from a Streptomyces aureofaciens strain. The gene saI14, coding for SaI14 protein, was cloned and expressed in Escherichia coli. The alignment of the deduced amino acid sequence of SaI14 with that of barstar, the RNase inhibitor from Bacillus amyloliquefaciens, showed significant similarity between them, especially in the region which contains most of the residues involved in barnase-barstar complex formation.

A number of investigations have been focused on understanding the nature of the interaction between protein molecules. Natural complexes, such as enzyme-inhibitor and antibody-antigen, have been used in many laboratories to study aspects of protein-protein recognition. One of these is the complex of barnase, an extracellular RNase, and barstar, its intracellular protein inhibitor, both produced by the spore-forming bacterium Bacillus amyloliquefaciens. These proteins form a very tight one-to-one complex with a dissociation constant (Kd) of 10^-14 M (4, 5, 16).

It was found recently that barstar also inhibits the streptomyces RNases Sa, Sa2, and Sa3 (6). The dissociation constants of the complexes of barstar with RNases Sa and Sa2 have been estimated to be on the order of 10^-12 M. The barstar-RNase Sa3 complex is even tighter, with a Kd of 10^-12 M. A practical consequence of inhibition of the streptomyces RNases by barstar is to allow high-level production of the recombinant enzymes when each of their genes is assembled with that of barstar on the same expression plasmid in Escherichia coli. All experiments with the expression of the RNase Sa, Sa2, and Sa3 genes alone failed due to the high toxicity of these enzymes for the host cells. Though the sequence identities of RNases Sa, Sa2, and Sa3 with barnase are rather low (from 23 to 27%), the amino acid residues of barnase which are involved in barnase binding (Lys27, Arg59, Glu60, Arg83, Arg87, His102, and Tyr103) have equivalent residues in RNase Sa, except for Lys27, the structural counterpart of which in barnase is Glu60.

S. aureofaciens was maintained on Bennet sporulation medium (0.1% yeast extract, 0.1% meat extract, 0.2% tryptone, 0.2% (NH4)2SO4, 0.2% NaCl, 0.25% corn steep (pH 5.8)]. Cells for chromosomal DNA isolation were grown in GPY medium (0.3% glucose, 0.25% corn steep, 0.2% (NH4)2SO4, 0.2% NaCl, 0.4% CaCO3, 0.2% (NH4)2SO4, 0.2% molasses, 0.25% corn steep (pH 5.8)]. Cells for chromosomal DNA isolation were grown in GPY medium (0.3% glucose, 0.3% peptone, 0.4% yeast extract, 1.0% glycine [pH 7.0 to 7.2]). Streptomyces was grown at 30°C.

DNA methods. Chromosomal DNA from S. aureofaciens was isolated according to the method of Hopwood et al. (7). Plasmid DNA was purified with a Wizard purification system (Promega). DNA fragments and PCR products separated on agarose gels were purified from the gel with the Wizard purification system. Restriction digestions, ligations, and transformations were done as described by Sambrook et al. (15).

Protein analysis and assays. Protein concentrations were determined by the method of Bradford (3). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (11) or that of Swank and Munkres (17). Gels were stained for protein with Coomassie brilliant blue R-250 or with silver (2).

The activity of the RNase inhibitor is given as the amount of
inhibitor which decreases the activity of 30 ng of RNase Sa to 50% as described previously (10).

**Purification of the RNase inhibitor from S. aureofaciens.** The existence of intracellular protein which inhibits RNase secreted by *S. aureofaciens* was discovered in 1982 (9). Surprisingly, we purified two inhibitors from soluble extracts of *S. aureofaciens* mycelium by a combination of chromatographic procedures. The isolation and purification of these proteins was hampered by their very low levels in the cells (less than 0.05 mg from 1 liter of culture) and their instability during purification. The results of a typical purification are summarized in Table 1. Less than 20% of the RNase-inhibitory activity was recovered after DEAE-Sephadex chromatography. The specific activity was enhanced more than 400-fold compared with the specific activity of the crude extract, but the inhibitors in the sample were still only a small portion (about 2%) of the total protein. A crucial advance in purification was the use of affinity chromatography involving immobilized RNase Sa. SDS-polyacrylamide gel electrophoresis of the final preparation revealed three components whose estimated molecular masses were 20 (SaI20), 14 (SaI14), and 9 kDa (SaI9). After the bands were cut out and the inhibitory activity was recovered, we found that all three proteins inhibited RNase Sa. Microsequencing of these proteins yielded the sequences TVTYVIDGEFDIFLEFNVGQAIGVDRGFHGHNLDADA for SaI14 and TDNELVLDRGRQIETLNDFFDADVPEF for SaI20. The sequence alignment of the N termini of SaI14, SaI20, and barstar revealed significant similarities, especially between SaI14 and barstar. Sequencing of the third protein showed that it was a truncated form of SaI14 lacking about 5 kDa of the C terminus. Whether SaI9 was a product of proteolytic degradation in vivo is not known, but it is interesting to note that inhibition of RNase Sa by this protein was observed.

**Cloning of the SaI14 RNase Sa inhibitor gene.** As the first step towards cloning, a DNA probe was prepared by PCR with the degenerate oligonucleotides DK1 (5'-ACNGTNACNTAY GTNATHGAYGG-3') and DK2 (5'-RAANGRCRNART TRTGRCCRAA-3') (R, A or G; Y, C or T; H, A, C, or T; N, A, C, G, T), corresponding to the segments TVTYVIDG (residues 1 to 8) and FGHNLDAF (residues 31 to 38) of the N-terminal sequence of SaI14, respectively, as primers and chromosomal DNA as a template. As expected, a single PCR product of approximately 120 bp was amplified. Southern blot analysis with the DK4 oligonucleotide probe, 5'-GARGAYTT YAACGACGTNGTNGG-3', which corresponds to an internal segment of the *sai14* N-terminal coding region, confirmed that a part of the *sai14* gene was amplified.

The PCR fragment was subsequently used as the probe for Southern hybridization of *S. aureofaciens* chromosomal DNA digested with a variety of restriction endonucleases. A *Kpn*I fragment of about 2.6 kb was considered to be most suitable for construction of the enriched genomic library. Two- to 3-kb fragments of *S. aureofaciens* DNA digested with *Kpn*I were ligated into *Kpn*I-digested plasmid pUC18. The recombinant DNA was transformed into *E. coli* XL1-Blue. Out of the 1,800 transformants screened, six clones which hybridized with the PCR product were selected, all of them carrying identical DNA fragments. Clone 2.4, named pSaI14, was chosen for further analysis.

The nucleic acid sequence of the segment of pSaI14 containing the inhibitor gene was determined by primer walking, beginning with the degenerate oligonucleotide primer DK1. As shown in Fig. 1, the open reading frame started with GTG, ended with TGA, and encoded a polypeptide of 126 amino acids with a calculated molecular mass of 14,034 Da. The coding region exhibited an overall G+C content of 71.4%, with an average G+C content at the third codon of 96.8%, which is typical of *Streptomyces* genes. The deduced amino acid sequence was in agreement with the N-terminal sequence determined experimentally by Edman degradation. Alignment of the deduced amino acid sequences of SaI14 and barstar (Fig. 2) revealed only 29.2% identity, which is about the same as that for barnase and RNase Sa. There are 13 amino acid residues in the region between residues 29 and 46 of barstar which form contacts with barnase in the barnase-barstar complex. In the corresponding region of SaI14 there are 10 residues identical to those of barstar. Among them are Tyr30, Asp36, and Asp40 (equivalent to Tyr29, Asp35, and Asp39 in barstar), whose contributions to the binding energy in the barnase-barstar complex are most important.

**Overexpression of the *sai14* RNase inhibitor gene.** In order to facilitate the cloning of the RNase inhibitor gene into the expression vector pTrc 99A, two PCR primers, SaI20 (5'-GA CCACACACAGCTTTTCAGCCGCGGAGG-3') and SaI13 (5'-GCATATCTCAGCCCCATGGCTGTGAATTAT GTG-3'), were designed to allow the introduction of an *Nco*I site on the upstream side of the gene and a *Hind*III site on the downstream side. The PCR fragment containing the inhibitor gene was then subcloned into the expression vector pTrc 99A, which has both the strong Trc promoter and a strong transcription termination signal. The recombinant plasmid, designated pSaI14.7, was introduced into *E. coli* DH5α cells. Upon induction by IPTG of expression of the cloned gene, the transformed cells expressed inhibitory activity. We found that decreasing the cultivation temperature from 37°C to 28 to 30°C after induction increased the level of expression fivefold. The inhibitor produced by overexpression of the *sai14* gene was purified to homogeneity by a slight modification of the procedure used for the native inhibitor (data not shown). The recombinant *sai14* protein inhibits all three streptomyte RNases, Sa, Sa2, and Sa3, as well as barnase.

The dissociation constant of the RNase Sa-Sai14 inhibitor complex has not yet been determined, but we assume that it will be comparable to the barnase-barstar dissociation constant, because SaI14 was bound to the RNase Sa affinity column very tightly and could only be eluted under strong denaturing conditions, i.e., in the presence of 6 M guanidine-HCl, 6 M urea, or 1% SDS. The comparison of the barstar and streptomycte inhibitors' dissociation constants could also be interesting from an evolutionary point of view. The interaction of barnase and barstar may be as close as it is because of the high intracellular toxicity of barnase, which has no disulfide bond and can readily fold to its active conformation inside the cells. In contrast, streptomycte RNases have one disulfide bond and may not fold properly in the reducing milieu of the cells. This might explain the lower toxicity of these enzymes. This idea is in agreement with the observation that RNase T1, which has two disulfide bonds, can be produced at a high level by *e. coli*
without any inhibitor (6). As a result of evolutionary development, the differences in toxicity of RNases may be reflected in differences in the dissociation constants of the RNase-inhibitor complexes and/or in the levels of inhibitors synthesized. Presumably, the role of inhibitors is to prevent RNase activity prior to secretion, which would be extremely harmful to the cell.

Our next approach will be structural work, which, combined with modern physicochemical techniques and protein engineering, will contribute to an understanding of the interactions between these RNases and their inhibitors. Employment of the three streptomycete RNases, Sa, Sa2, and Sa3, and their two inhibitors, SaI14 and SaI20, as well as others as they become available, together with barnase and barstar, will expand the study of enzyme-inhibitor complexes and should help clarify the details of protein-protein recognition.

**Nucleotide sequence accession number.** The GenBank accession number for the sequence shown in Fig. 1 is AF 020428.

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**REFERENCES**


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**FIG. 1.** DNA sequence of the gene encoding the SaI14 RNase inhibitor and its deduced primary structure. The initiation and termination codons are in boldface.


16a. **Sevcik, J.** Unpublished data.
