

## 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

DANIELA KRAJCIKOVA,<sup>1\*</sup> ROBERT W. HARTLEY,<sup>2</sup> AND JOZEF SEVCIK<sup>1</sup>

*Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia,<sup>1</sup> and Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892<sup>2</sup>*

Received 2 October 1997/Accepted 15 January 1998

**Two new RNase inhibitors, SaI14 ( $M_r$ , ~14,000) and SaI20 ( $M_r$ , ~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 sporegenic bacterium *Bacillus amyloliquefaciens*. These proteins form a very tight one-to-one complex with a dissociation constant ( $K_d$ ) of  $10^{-14}$  M (4, 5, 16).

It was found recently that barstar also inhibits the streptomycete 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  $10^{-10}$  M. The barstar-RNase Sa3 complex is even tighter, with a  $K_d$  of  $10^{-12}$  M. A practical consequence of inhibition of the streptomycete 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 barstar binding (Lys27, Arg59, Glu60, Arg83, Arg87, His102, and Tyr103) have equivalent residues in RNase Sa, except for Lys27, the structural counterpart of which in RNase Sa is Gln32. A superposition of C $\alpha$  atoms of the conserved structural cores of RNase Sa and barnase shows a very accurate match of the structure which is close to the active site and to the enzyme-barstar interface (6). The structures of RNases Sa2 and Sa3 were determined, and they have a high degree of similarity with that of RNase Sa. The structure of the RNase Sa-barstar complex has also been determined (16a).

We report here the isolation and purification of two novel RNase inhibitors from *Streptomyces aureofaciens* R8/26, the source of RNase Sa2. *sai14*, the SaI14 inhibitor gene, was cloned, sequenced, and overexpressed in *E. coli*, and the in-

hibitory activity of the recombinant protein was confirmed. The cloning and sequencing of the SaI20 inhibitor gene is under way.

**Bacterial strains and vectors.** *E. coli* XL1-Blue MRF' (Stratagene) and DH5 $\alpha$ F'IQ (Gibco BRL) were used as the hosts for DNA cloning and protein overexpression. *S. aureofaciens* R8/26, the industrial wild-type strain (14), was kindly provided by Biotika, Slovenská L'upča, Slovakia. The pUC18 vector (18) was used for genomic library construction, and the pTrc 99A expression vector (1) was used for overexpression of the protein.

**Media and growth conditions.** *E. coli* strains were routinely grown in Luria broth. Selection was made with 100  $\mu$ g of ampicillin per ml in agar or liquid medium at 37°C. For protein production, superbroth medium was used at 28 to 30°C, and protein expression was induced with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG).

*S. aureofaciens* was maintained on Bennet sporulation medium (0.1% yeast extract, 0.1% meat extract, 0.2% tryptone, 1.0% maltose, 1.5% agar) (8). An overnight culture was grown in Niedercorn medium [3.0% saccharose, 2.0% corn steep, 0.2% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.7% CaCO<sub>3</sub> (pH 7.0 to 7.2)] (13). Protein production was in 8/8 medium [3.0% saccharose, 2.0% soy bean flour, 0.25% NaCl, 0.4% CaCO<sub>3</sub>, 0.2% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 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

\* Corresponding author. Mailing address: Institute of Molecular Biology, Slovak Academy of Sciences, Dubravská cesta 21, 842 51 Bratislava, Slovakia. Phone: 4217-378-2426. Fax: 4217-372316. E-mail: umbidana@savba.savba.sk.

TABLE 1. Purification scheme of RNase Sa inhibitors SaI9, SaI14, and SaI20

| Purification step    | Total protein (mg) | Sp act (U · mg <sup>-1</sup> ) | Purification (fold) |
|----------------------|--------------------|--------------------------------|---------------------|
| Crude extract        | 12,000             | 2                              | 1                   |
| Streptomycin sulfate | 12,000             | 2                              | 1                   |
| DEAE-Spheron         | 1,300              | 8                              | 4                   |
| Sephadex G-50        | 42                 | 180                            | 90                  |
| DEAE-Sephadex        | 6                  | 700                            | 350                 |
| Affinity column      | 0.05 <sup>a</sup>  | 34,000 <sup>a</sup>            | 17,000 <sup>a</sup> |

<sup>a</sup> Estimated value.

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* R8/26.** 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 TVTYVIDGFIEDTLEDFNDVVGQAIGVDGRFGHNLDAFA for SaI14 and TDNELIVDLRGRQIETLNDFFDAVVEP 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'-ACNGTNACNTAYGTNATHGAYGG-3') and DK2 (5'-RAANGCRTCNRRTTRTGRCCRAA-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'-GARGAYTTYAACGACGTNGTNGG-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, SaI20.2 (5'-CA CCACCACCAAGCTTTCAGGCGCAGGCGGAGGC-3') and SaI13 (5'-GCATATCCTCGACCCCATGGCTGTGACTTATGTG-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* DH5 $\alpha$  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 streptomyces 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 streptomyces 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, streptomyces 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*

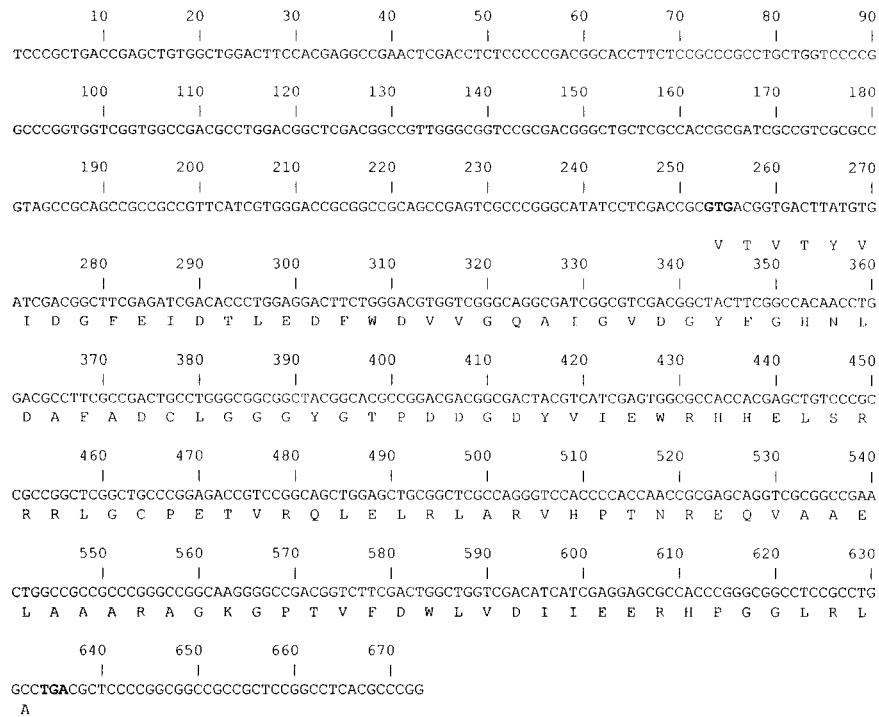


FIG. 1. DNA sequence of the gene encoding the SaI14 RNase inhibitor and its deduced primary structure. The initiation and termination codons are in boldface.

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, SaI1 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.

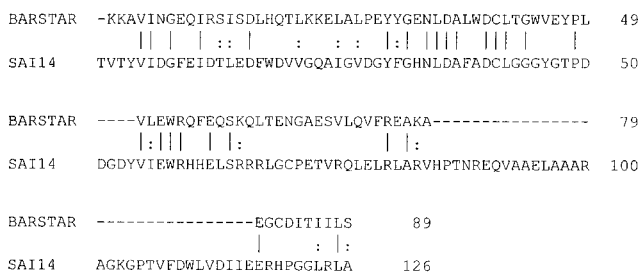


FIG. 2. Alignment of amino acid sequence of barstar and deduced amino acid sequence of SaI14. Alignment was made with PALIGN (12). The identical amino acids are denoted by vertical bars, and similar amino acids are indicated by dots.

We thank Freie Universität Berlin for sequencing of RNase inhibitors and Vladimir Kery for helpful advice on affinity column preparation.

This work was financed by Slovak Academy of Sciences grant 2/1070 and Howard Hughes Medical Institute grant 75195-547601. D.K. thanks NIH Bethesda for a fellowship, which facilitated this work.

REFERENCES

- Amann, E., B. Ochs, and K. J. Abel. 1988. Tightly regulated *tac* promoter vectors useful for expression of unfused and fused proteins in *Escherichia coli*. *Gene* **69**:301-303.
- Blum, H., H. Beier, and H. J. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**:93-99.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Hartley, R. W. 1989. Barnase and barstar: two small proteins to fold and fit together. *Trends Biochem. Sci.* **14**:450-454.
- Hartley, R. W. 1997. Barnase and barstar, p. 51-100. *In* G. Dalessio and J. F. Riordan (ed.), *Ribonucleases: structures and functions*. Academic Press, Inc., San Diego, Calif.
- Hartley, R. W., V. Both, E. J. Hebert, D. Homerova, M. Jucovic, V. Nazarov, I. Rybajlak, and J. Sevcik. 1996. Expression of extracellular ribonucleases from recombinant genes of four *Streptomyces* strains with the aid of the barstar gene. *Protein Pept. Lett.* **3**:225-231.
- Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf. 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation, Norwich, United Kingdom.
- Horinouchi, S., and T. Beppu. 1985. Construction and application of a promoter-probe plasmid that allows chromogenic identification in *Streptomyces lividans*. *J. Bacteriol.* **162**:406-412.
- Jeloková, J., E. Zelinková, and J. Zelinka. 1982. Relatívna molekulová hmotnosť ribonukleázy viazanej na ribozómy *Streptomyces aureofaciens* a dôkaz jej inhibítora v mycéliu. *Biologia (Bratislava)* **37**:357-362.
- Krajčíková, D., E. Kutejová, and J. Ševčík. 1990. Ribonuclease inhibitor from *Streptomyces aureofaciens*. *Biologia (Bratislava)* **45**:977-985.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.
- Myers, E. W., and W. Miller. 1988. Description of the alignment method used in program PALIGN. *CABIOS* **4**:11-17.

13. **Niedercorn, J. G.** 1952. U.S. patent 2,609,329.
14. **Pristaš, P., A. Godány, B. Ševčíková, B. Oktavcová, and J. Farkašovská.** 1992. Characterization of restriction endonuclease activities in tetracycline producing strains of *Streptomyces aureofaciens*. *Nucleic Acids Res.* **20**: 4364.
15. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
16. **Schreiber, G., and A. R. Fersht.** 1993. The interaction of barnase with its polypeptide inhibitor barstar studied by protein engineering. *Biochemistry* **32**:5145–5150.
- 16a. **Sevcik, J.** Unpublished data.
17. **Swank, R. T., and K. D. Munkres.** 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. *Anal. Biochem.* **39**:462–477.
18. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **17**:259–268.