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Streptomyces Subtilisin Inhibitor-Like Proteins Are Distributed Widely in Streptomycetes

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***Streptomyces* subtilisin inhibitor-like proteins were found to be distributed widely in streptomycetes by using the combination of the convenient, newly developed plate assay system and an established liquid culture assay. Almost all the strains formerly categorized as *Streptoverticillium* species produced proteins that exhibited inhibitory activity against both subtilisin BPN′ and trypsin. N-terminal regions of three purified proteins showed high structural similarity to those of other previously reported SIL inhibitors.**

Proteinaceous inhibitors of proteolytic enzymes have been found to be produced by a wide variety of animals and plants (5), and they occur also in microorganisms (17). Some have proved useful for medical and agricultural purposes (1, 16). These inhibitors share several common physical properties: they are rather small proteins with low molecular weights, and they tend to be stable at low pHs and high temperatures (7, 8). Although they are thought to control proteases under physiological conditions, little is known about their biological significance. Holzer suggested that the complicated interaction between yeast proteases and their respective specific inhibitors plays an important role in the regulation of specific proteolytic activation, inactivation, and enzyme activity modification (3).

Most of the extracellular inhibitory proteins discovered so far have been isolated from *Streptomyces* spp. and classified as members of the *Streptomyces* subtilisin inhibitor (SSI) family on the basis of their similar structures and protease inhibitory specificities (4, 10–12). A typical serine protease inhibitor, SSI, produced in large amounts by *S. albogriseolus*, is a stable dimeric molecule, and its structure-function relationships have been studied extensively (2).

We have already established a highly sensitive method of using an acid precipitant (liquid culture assay system) for screening and assaying SSI-like proteins (SIL series) in culture supernatants of various *Streptomyces* strains (14). By applying this system, we found that production of SIL inhibitors is distributed widely among *Streptomyces* spp. (13) and investigated their structure and function relationships (13, 14; unpublished data). Further discovery of a wide variety of SIL inhibitors would enable us to investigate the physiological significance and evolutionary process of such proteins as well as to reach a unified understanding of the biological role of protease-protease inhibitor systems in nature. Furthermore, it would be of interest to ascertain whether bacteria other than *Streptomyces* spp. produce SIL proteins. In order to realize these aims, we have developed a convenient plate assay system, using *Streptomyces* strains, for primary screening of such inhibitors.

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The protease inhibitory activities of 12 *Streptomyces* strains, *S. albogriseolus* S-3253 and its mutant strain (M1), *S. antifibrinolyticus*, *S. griseoincarnatus*, *S. lividans*, *S. longisporus*, *S. cacaoi*, *S. parvulus*, *S. coelicolor*, *S. lavendulae*, *S. cellulosae*, and *S. diastaticus*, were determined.

The plate assay system for SIL activity we have developed involves the following steps. (i) Spores of each strain are spread onto 1.5% (wt/vol) agar-containing tryptone soya broth (3% [wt/vol]) (15). (ii) These agar plates are incubated at 30°C until distinct colonies appear. (iii) Fully grown colonies are transferred to plates of the same medium containing 2% (wt/vol) skim milk. (iv) After incubation of the plate at 30°C for 2 to 3 days, 3.5 ml of melted soft agar (0.7% [wt/vol] agarose) containing an appropriate concentration of a target protease (standard amount in 3.5 ml, 500 μg) is quickly overlaid onto each plate. (v) The plates are left to stand for at least 2 h, after which a turbid area around each colony is clearly visible if the colony has excreted compounds that inhibit proteolysis of skim milk.

Figures 1A and B show examples of the results when subtilisin BPN′ and trypsin, respectively, were used as the target proteases. Nine strains showed inhibitory activity against subtilisin BPN′, and six were positive against trypsin. In each protease assay, the positive bacteria coincided with the strains identified previously as SIL producers, which produced SSI, plasminostreptin (PSN) (4), alkaline protease inhibitor (API)-2c′ (12), *Streptomyces* trypsin inhibitor (STI) 1 and 2 (11), and SIL1 to SIL4. Our previous experimental results by protein engineering of SSI proved that the primary determinant of inhibitor specificity is the amino acid residue at the P1 site (reactive-site) amino acid, which matches the substrate specificity of the target enzyme (6). Therefore, the different results against subtilisin BPN′ and trypsin found in the present study may correspond to the different protease inhibitory specificities of their SIL inhibitors.

No SIL activity was detected with *S. cellulosae* and *S. diastaticus* under the assay conditions employed. The mutant M1 of *S. albogriseolus* is one of the SSI-non-producing strains obtained during transformation with the multicopy plasmid pIJ702 (15). In this mutant, the genetic analysis of which is now in progress, SSI appeared nonessential for cell

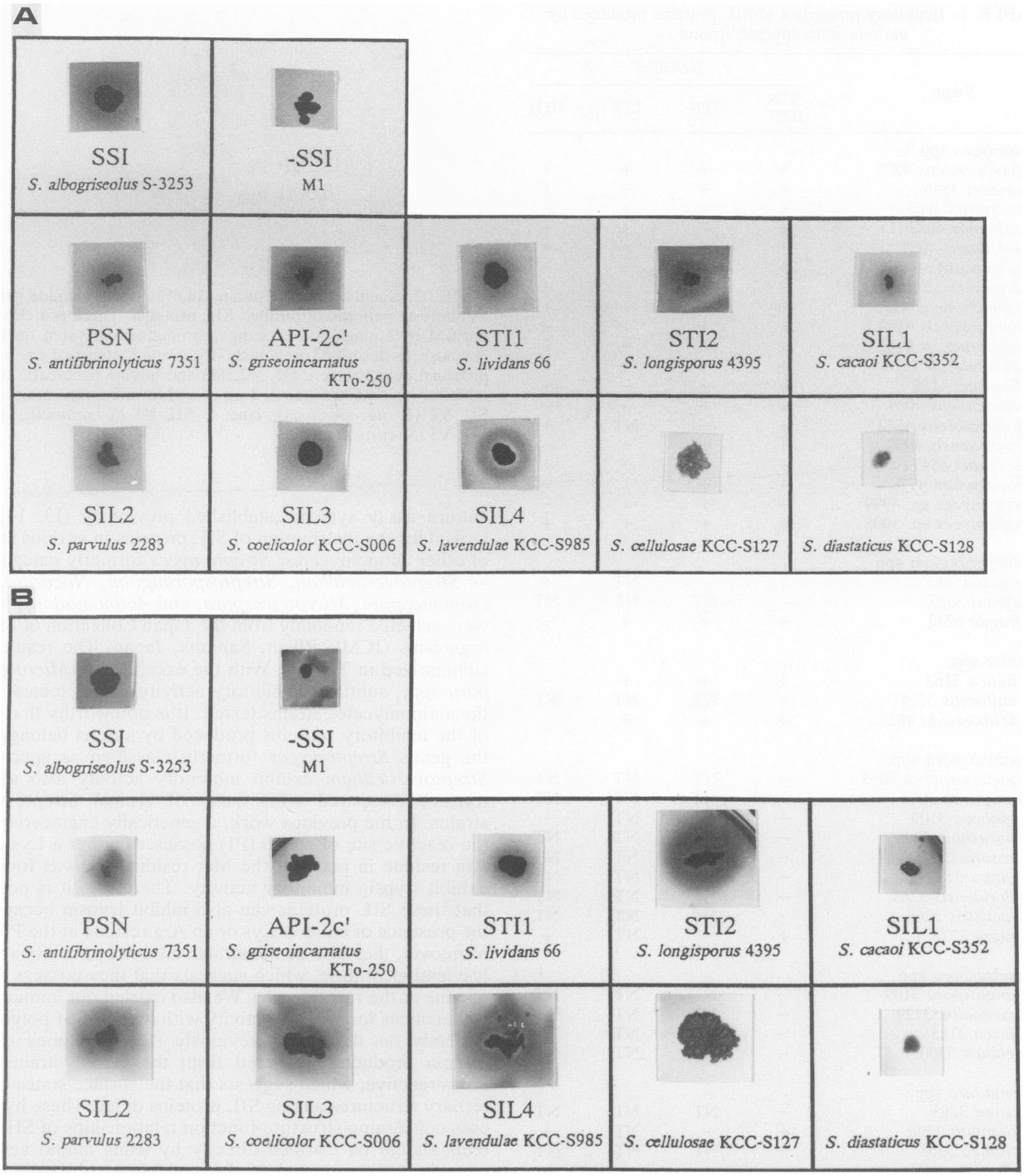

FIG. 1. Plate assays of protease inhibitors. The names of 12 *Streptomyces* strains and 9 SIL inhibitors identified are indicated below each colony; A and B denote the turbid areas forming patterns when subtilisin BPN' (A) and trypsin (B), respectively, were used as the target proteases. Inhibitors produced by nine positive strains against subtilisin (SSI, PSN, API-2c', STI1, STI2, and SIL1 to SIL4) and by six positive strains against trypsin (PSN, STI1, STI2, and SIL2 to SIL4).

growth when cultured in tryptone soya broth medium. The behavior of M1, which showed no inhibitory activity against subtilisin, indicates that SSI is actually responsible for the formation of the turbid area in *S. albogriseolus*. The same phenomenon was observed for the mutant of *S. griseoincarnatus* (data not shown).

On the basis of these results, we considered this simple, sensitive plate assay to be very valuable for the primary screening of SIL producers among *Streptomyces* spp. and other bacteria. This system may also be applicable to the detection of inhibitors of other types of proteases. In fact, by using this system, we have observed that some other types

TABLE 1. Inhibitory properties of SIL proteins produced by various actinomycete strains

Strain	Inhibition[a]			
	STN BPN'	TPN	LEP	CTN
Streptomyces spp.[b]				
S. flavopersicus 4307	+	+	+	+
S. orinoci 4546	+	+	+	+
S. netropsis 4063	+	+	+	−
S. salmonis 4083	+	+	+	+
S. thioluteus 4087	+	+	+	+
S. griseocarneus 4095	+	+	+	+
S. luteoverticillatus 4099	+	+	+	+
S. cinnamoneus 4152	+	+	+	+
S. kentuckensis 4153	+	+	+	+
S. mobaraensis 4168	+	+	+	+
S. olivoreticuli 4176	+	+	+	+
S. lilacinus 4188	+	+	−	+
S. eurocidicus 4029	+	+	+	−
S. luteosporeus 4542	+	−	NT	+
S. mashuensis 4059	+	+	+	−
S. septatus 4547	+	+	+	+
S. ladakanum 4778	+	+	+	+
Streptomyces sp. 4999	+	+	+	−
Streptomyces sp. 5008	+	+	+	+
Streptosporangium spp.				
S. roseum 3005	+	−	NT	+
S. album 3025	−	NT	NT	NT
S. fragile 6242	+	+	+	+
Nocardia spp.				
N. italica 3163	+	+	+	−
N. uniformis 3224	−	NT	NT	NT
N. salmonicida 4826	+	+	+	−
Micromonospora spp.				
M. melanosporea 3063	−	NT	NT	NT
M. purpurea 3074	−	NT	NT	NT
M. globosa 3109	+	−	NT	−
M. narashino 3129	−	NT	NT	NT
M. rosaria 3159	−	NT	NT	NT
M. citrea 3182	−	NT	NT	NT
M. floridensis 3265	−	NT	NT	NT
M. lacustris 3266	−	NT	NT	NT
M. nigra 3328	+	−	NT	+
Microtetraspora spp.				
M. angiospora 3109	−	NT	NT	NT
M. niveoalba 3129	−	NT	NT	NT
M. fusca 3183	−	NT	NT	NT
M. glauca 3300	−	NT	NT	NT
Actinomadura spp.				
A. citrea 3295	−	NT	NT	NT
A. kijaniata 3306	+	−	NT	+
A. cremea 3308	−	NT	NT	NT

[a] STN, subtilisin; TPN, trypsin; LEP, lysylendopeptidase; CTN, chymotrypsin; NT, not tested.
[b] All *Streptomyces* strains listed were formerly categorized as species of *Streptoverticillium*.

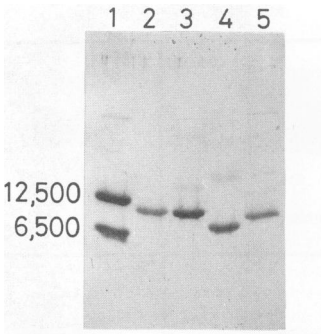

FIG. 2. Sodium dodecyl sulfate–18.8% polyacrylamide gel electrophoresis patterns of purified SIL proteins. Three proteins were purified by a single ion-exchange chromatography step on DE-32 cellulose, as described previously (15). Lane 1, standard size marker proteins: cytochrome *c* (M_r, 12,500) and bovine pancreatic trypsin inhibitor (M_r, 6,500); lane 2, 2 μg of SSI (*S. albogriseolus*); lane 3, SIL-V1 (*S. flavopersicus*); lane 4, SIL-V2 (*S. orinoci*); lane 5, SIL-V3 (*S. eurocidicus*).

of bacteria produce extracellular proteinaceous serine protease inhibitors (unpublished data). Therefore, this finding has encouraged us to investigate the distribution of inhibitory-protein-producing bacteria from physiological and ecological viewpoints.

We combined this plate assay system with the liquid culture assay system established previously (13, 14) and looked for the distribution of SIL proteins in various strains of other actinomycetes: *Streptomyces* formerly categorized as *Streptoverticillium*, *Streptosporangium*, *Nocardia*, *Micromonospora*, *Microtetraspora*, and *Actinomadura*, which were selected randomly from the Japan Collection of Microorganisms (JCM), Riken, Saitama, Japan. The results are summarized in Table 1. With the exception of *Microtetraspora* spp., subtilisin inhibitory activity was detected in all the actinomycetes strains tested. It is noteworthy that most of the inhibitory proteins produced by strains belonging to the genus *Streptomyces* formerly classified as species of *Streptoverticillium* exhibit inhibitory activity also against trypsin, compared with those of typical *Streptomyces* strains. In the previous work, a genetically engineered SSI, the reactive site of which (P1) possesses either a Lys or an Arg residue in place of the Met residue (6), was found to exhibit trypsin inhibitory activity. Therefore, it is possible that these SIL proteins can also inhibit trypsin because of the presence of either a Lys or an Arg residue at the P1 site. Moreover, they also demonstrated inhibitory activity against lysylendopeptidase, which suggests that they possess a Lys residue at the reactive site. We also carried out immunological analysis for cross-reactivity with an anti-SSI polyclonal antibody, as described previously (15), and none of the protein products recovered from the tested strains was cross-reactive, which suggests that the surface states of the tertiary structures among SIL proteins differ. These hypotheses concerning structure-function relationships of SIL proteins should be clarified directly by using amino acid sequencing, measurement of their inhibitory properties, and X-ray analyses of their complex samples with trypsin.

The structures of three novel protease inhibitors, SIL-V1 (*S. flavopersicus*), SIL-V2 (*S. orinoci*), and SIL-V3 (*S. eurocidicus*), isolated by using the purification procedures described previously (13, 14), were analyzed. The protease inhibitors resembled the SSI family in terms of their molecular weights estimated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns shown in Fig. 2, sequence homologies in both the β_1- and β_2-strands forming the part of 5-stranded β-sheet, which have been demonstrated to be required for the dimerization of SSI (9) (Fig. 3), and heterogeneities of signal peptide processing (unpub-

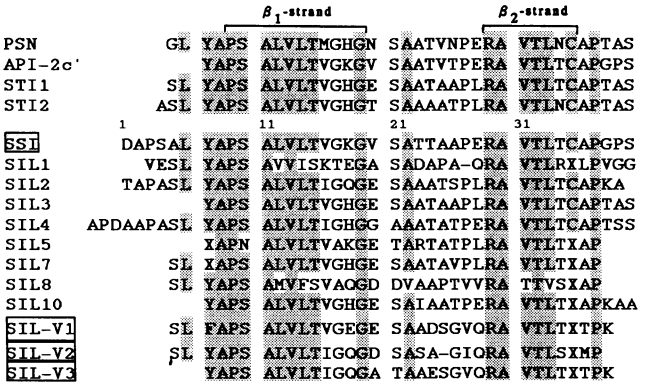

FIG. 3. Sequence comparisons of the N-terminal regions of the SIL proteins from various streptomycetes strains. Automated sequence analysis by Edman degradation was carried out by using an Applied Biosystems model 476A protein sequencer. The one-letter amino acid notation is used for alignment among the SIL proteins, and the residue numbering is that of SSI. Highly conserved residues are enclosed in shaded boxes. The β_1- and β_2-strands above the lines denote the secondary structure regions confirmed by X-ray crystallographic analysis of SSI (9). In the cases of SIL1 and SIL-V2, bars are used for optimum alignment. X, unidentified residue.

lished data). A deletion in the loop-out region between β_1- and β_2-strands of SIL1 and SIL-V2 indicates that *S. cacaoi* and *S. orinoci* are phylogenetically close to each other. Detailed characterization of the structures and functions of these SIL proteins and their genes is in progress.

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