MOLECULAR CLONING OF A XYLANASE GENE FROM *STREPTOMYCES* SP. NO. 36a AND ITS EXPRESSION IN STREPTOMYCETES

AKIO IWASAKI[a], HIROSHI KISHIDA[b] and MASANORI OKANISHI[c]

Department of Antibiotics, National Institute of Health
Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication December 26, 1985)

The gene for an extracellular xylanase from *Streptomyces* sp. No. 36a was cloned into *Streptomyces lividans* TK21 using pIJ702 as a vector plasmid. The smallest DNA fragment encoding the xylanase gene and its possible promotor was determined to be a 1.04 kb *Sph* I-*Sac* I fragment by sub-cloning studies. This xylanase gene fragment was transferred into the pSK2 series of plasmids and introduced into *Streptomyces kasugaensis* G3 protoplasts. The cloned xylanase gene was expressed in both *S. lividans* TK21 and *S. kasugaensis* G3, and these clones produced and secreted high yields of xylanase into the culture medium. The xylanase production was not detected when a foreign DNA fragment was inserted into the *Bcl* I site locating in the xylanase gene fragment.

The genus *Bacillus* produces many extracellular enzymes. Genes coding for a few of these enzymes such as α-amylase[1], β-lactamase[2], proteases[3] and xylanases[4,5] have been cloned and secretion vectors were developed from these genes[6,7].

Streptomycetes also produce many kinds of extracellular enzymes, including special enzymes for the utilization of insoluble materials existing in nature. The cloning of genes for α-amylase[8] and agarase[9] already have been studied in streptomycetes. Streptomycetes seem to be possible hosts for extracellular production of heterologous proteins by using secretion vectors constructed from their extracellular enzyme genes.

Xylanase which hydrolyzes xylan to xylose or xylooligosaccharides is a useful enzyme to degrade some industrial and agricultural wastes. This report deals with the cloning of an extracellular xylanase gene from *Streptomyces* sp. No. 36a into *Streptomyces lividans* TK21 using pIJ702[10] as a vector plasmid. The cloned xylanase gene was then sub-cloned into *Streptomyces kasugaensis* G3[11] using the pSK2 series of plasmids[12,13], and the smaller cloning vector allowing detection by insertional inactivation of the xylanase gene was constructed. Expression of the cloned xylanase gene was studied in both streptomycetes hosts.

Materials and Methods

Bacterial Strains and Plasmids

Plasmid-free *S. kasugaensis* G3[11] derived from *S. kasugaensis* MB273[11] and *S. lividans* TK21 provided by D. A. HOPWOOD were used as cloning hosts. Xylanase-producing *Streptomyces* sp.

Present address:
[a] Tokyo Rsearch Laboratories, Kowa Co., Ltd., Higashimurayama, Tokyo 189, Japan.
[b] College of Agriculture and Veterinary Medicine, Nihon University, Shimouma, Setagaya-ku, Tokyo 154, Japan.
[c] Exploratory Research Laboratories, Banyu Pharmaceutical Co., Ltd., Shimomeguro, Meguro-ku, Tokyo 153, Japan.

No. 36a was obtained from Hokko Chemical Industry Co., Ltd. Plasmid vectors pSK21-B4[12], pSK21-B5[12] and pSK21-TM6[13] were isolated from *S. kasugaensis* G3 harboring those plasmids. Plasmid pIJ702 was prepared from *S. lividans* 3131 which was provided by E. KATZ.

Media

GPY medium[12] and GPYG medium[11] in seed culture and growth culture for the preparation of protoplasts from *S. kasugaensis* have been described previously. YEME medium containing sucrose 34%, $MgCl_2$ 5 mM and glycine 0.5%[14] was used to prepare protoplasts from *S. lividans* TK21 cells. Protoplast regeneration medium R3X containing 5% of xylan to R3 lower layer medium[15] was used for the initial selection of the regenerated colonies producing xylanase. Melanin production agar consisted of NaCl 2.0%, $MgCl_2 \cdot 6H_2O$ 0.05%, $CuSO_4 \cdot 5H_2O$ 0.01%, trace elements solution (MTE) 0.5%, glucose 0.1%, L-tyrosine 0.1%, sodium glutamate 0.12%, Casamino Acids 0.5%, K_2HPO_4 0.3% and agar 2.0%. MTE contained $CuCl_2 \cdot 2H_2O$ 40 mg, $MnCl_2 \cdot 4H_2O$ 40 mg, $CoCl_2 \cdot 2H_2O$ 40 mg, $ZnSO_4 \cdot 7H_2O$ 80 mg, $Na_2B_4O_7 \cdot 10H_2O$ 80 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.24 g, $CaCl_2 \cdot 2H_2O$ 2 g and $FeSO_4 \cdot 7H_2O$ 0.2 g in 1,000 ml deionized water. XA medium for production of xylanase consisted of KCl 0.1%, $MgCl_2 \cdot 6H_2O$ 0.02%, K_2HPO_4 0.7%, KH_2PO_4 0.54%, $(NH_4)_2SO_4$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.001%, $FeSO_4 \cdot 7H_2O$ 0.0001%, Polypeptone 1.0% and xylan 1.0%. For detection of xylanase activity in a grown colony, 1.8% of agar was added to XA medium. Xylan was separately autoclaved.

Enzymes and Reagents

T4 DNA ligase, bacterial alkaline phosphatase (BAP), xylan and agarose LGT were purchased from Wako Chemical Co., Ltd., Toyobo Co., Ltd., Aldrich Chemical Company Inc. and Nakarai Chemicals Ltd., respectively. Thiopeptin capable of using instead of thiostrepton was obtained from Fujisawa Pharmaceutical Co., Ltd. Restriction endonucleases and other chemicals were obtained commercially.

Preparation of Total DNAs, Plasmid DNAs, Digestion with Restriction Endonucleases and Agarose gel Electrophoresis

Total DNAs were extracted from *Streptomyces* sp. No. 36a and *S. kasugaensis* G3 by the method described by OKANISHI and GREGORY[16]. All plasmids were prepared by the cleared lysate method as described previously[11]. The methods for restriction enzyme digestion and agarose gel electrophoresis (AGE) were virtually identical with those described previously[11]. In AGE, *Hin*d III fragments of λ-DNA and *Hae* III digests of $\phi \times 174$ phage DNA were used as molecular weight standards.

Transformation of Protoplasts

S. kasugaensis G3 cells were precultured in GPY medium containing 0.05% of glycine at 28°C for 2 days and then 1% volume of preculture was transferred into GPYG medium. Protoplasts were prepared from the mycelia grown in GPYG medium at 28°C for 52 hours by the method described previously[15]. In *S. lividans* TK21, protoplasts were prepared from a culture of YEME medium containing sucrose, $MgCl_2$ and glycine at 28°C for 72 hours. Transformation with DNA was carried out by the method described previously[12]. The treated protoplasts were spread on 20 ml of R3X medium plates, followed by embedding with 4 ml of the upper layer agar medium of R3[15]. For the selection of thiopeptin-resistant transformants, 4 ml of upper layer agar medium of R3 supplemented with 140 μg of thiopeptin was overlaid on the base plates after incubation at 28°C for 20 to 24 hours, and the plates were incubated further for 5 to 9 days to regenerate transformants.

Xylanase positive transformants had transparent halo-formation around the colonies on R3X medium. To reconfirm this, the halo-forming colonies were re-transferred to an XA agar plate and observed within 3 days after incubation at 28°C.

Detection of Plasmids in Transformants

Detection of plasmids in transformants was carried out by a modification of the alkaline lysis method reported by CHATER *et al.*[17]. The protoplasts of *S. kasugaensis* G3 were prepared using 5 ml of 42 hours culture in GPYG medium and the mycelia suspended in P3 medium[15] were treated with lysozyme. The protoplasts of *S. lividans* TK21 were also prepared from mycelia grown in YEME

medium containing sucrose, $MgCl_2$ and glycine for 72~96 hours. The resulting protoplast suspension (0.5 ml) was treated with 0.5 ml of a lytic mixture consisting of SDS 2.0%, trisodium EDTA 4.12% and NaOH 2.0% (0.5 M) at room temperature for 10 minutes. Then 0.125 ml of 2 M acetic acid and 1 ml of phenol - $CHCl_3$ solution were added and mixed vigorously. After centrifugation at 10,000 rpm for 15 minutes, 10 μl of the upper phase was submitted directly to AGE.

Cloning of Xylanase Gene

Bcl I-digested total DNA of *Streptomyces* sp. No. 36a was ligated to the *Bcl* I-digested and BAP-treated pSK21-TM6 using T4 DNA ligase at 4°C for 16 hours. Additionally, *Bgl* II digested total DNA of *Streptomyces* sp. No. 36a was ligated to the *Bgl* II-digested and BAP-treated pIJ702. These ligation mixtures were used to transform protoplasts of *S. kasugaensis* G3 or *S. lividans* TK21. After incubation on R3X medium, thiopeptin-resistant and halo-forming colonies were isolated.

Purified xylanase-positive plasmids were trimmed by *Sac* I or *Sph* I and then self-ligated to construct small plasmids. Sub-cloning was performed between xylanase-positive plasmids and the pSK2 series of plasmids such as pSK21-B4, pSK21-B5 and pSK21-TM6.

Measurement of Xylanase Activity

Streptomycetes to be assayed for xylanase activity were precultured with GPY medium supplemented with 10 μg/ml of thiopeptin at 28°C for 48 hours, and then 0.25 ml of seed culture was transferred into 25 ml of XA medium. After incubation for 72 hours, the culture broth was centrifuged. Xylanase activity of the supernatant was assayed by the method of PANBANGRED *et al.*[18] with the following modifications. Fifty mM of phosphate buffer (pH 6.4) was used, and the reaction mixture was incubated at 30°C for 15 minutes. One unit of enzyme was defined as the amount of enzyme capable of releasing 1 μmol/ml of reducing sugar in 1 minute.

Results

Cloning of Xylanase Gene

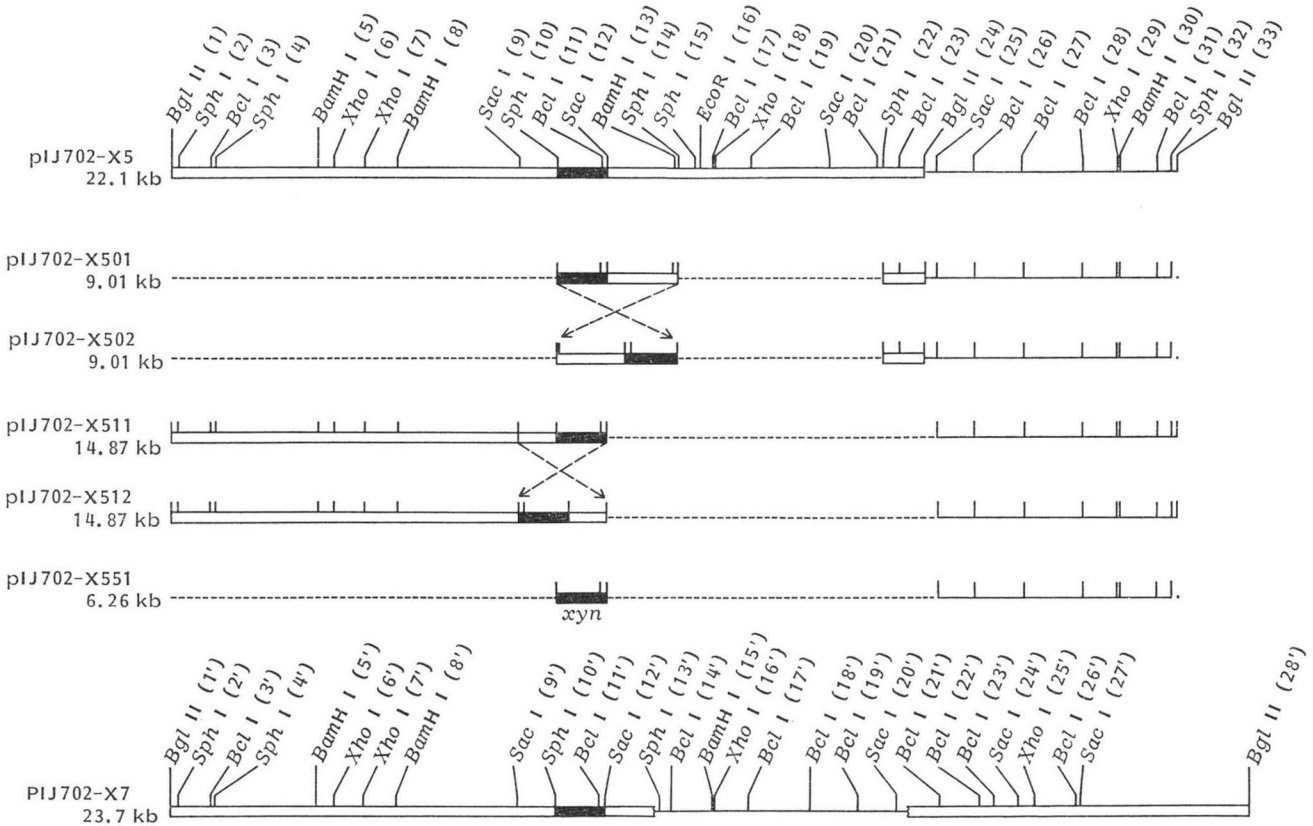
The ligation mixture of *Bgl* II digests of both pIJ702 and *Streptomyces* sp. No. 36a total DNA was introduced into *S. lividans* TK21 protoplasts. Many thiopeptin-resistant transformants appeared, and six transparent parts in which xylan was digested were observed in 10 plates. As the transparent parts were large, many resistant colonies were contained in there. Seventy-two colonies grown in these parts were transferred to XA agar and melanin production medium plates to check their characters acquired. Two halo-forming, melanin-negative strains due to insertional inactivation of *mel* gene at *Bgl* II site[10] were obtained. The plasmids contained in these strains were extracted, purified and designated pIJ702-X5 or pIJ702-X7, respectively. Re-transformation of *S. lividans* TK21 by pIJ702-X5 or X7 also gave colonies with halo-formation and thiopeptin-resistance.

Restriction Endonuclease Map and Trimming of the Inserted Region in the Plasmids

As shown in Table 1 and Fig. 1, the plasmid pIJ702-X5 contained a 16.45 kb insert into the *Bgl* II site of pIJ702. pIJ702-X5 had single site for *Eco*R I, 2 sites for *Bgl* II, 3 sites for *Sac* I, *Xho* I and *Bam*H I, and 6 sites for *Bcl* I and *Sph* I in the 16.45 kb insert fragment. The other plasmid pIJ702-X7 was 23.7 kb in size. A 9.50 kb fragment from the *Bgl* II site to *Sac* I site was identical to that of pIJ702-X5, but the *Bgl* II site derived from pIJ702 was missing in pIJ702-X7.

pIJ702-X5 was digested with *Sph* I or *Sac* I, and re-ligated. The ligated samples were introduced into *S. lividans* TK21 protoplasts, and the transformants showing thiopeptin-resistance and halo-formation were isolated. The smallest plasmids contained in these transformants were examined. From *Sph* I digests of pIJ702-X5, 2 plasmids which gave a different AGE-pattern by *Sac* I digestion

Fig. 1. Restriction endonuclease-cleavage maps of pIJ702-X5, X7 and trimmed plasmids derived from pIJ702-X5.



These closed circular plasmid DNAs are drawn in the linear form. Thin lines, open bars and solid bars indicate the pIJ702 portion, the fragment derived from *Streptomyces* sp. No. 36a genome and the xylanase gene fragment, respectively. Dotted lines indicate deleted fragments. Broken arrows indicate that the orientation of the fragments were reversed.

Table 1. Distance of each restriction sites in the plasmids pIJ702-X5 and X7.

pIJ702-X5						pIJ702-X7					
(1)	0.00	(13)	10.98	(25)	16.73	(1′)	0.00	(13′)	10.67	(25′)	18.89
(2)	0.19	(14)	11.07	(26)	17.56	(2′)	0.19	(14′)	10.95	(26′)	19.84
(3)	0.91	(15)	11.42	(27)	18.61	(3′)	0.91	(15′)	11.84	(27′)	19.93
(4)	1.02	(16)	11.55	(28)	19.95	(4′)	1.02	(16′)	11.87	(28′)	23.70
(5)	3.22	(17)	11.80	(29)	20.74	(5′)	3.22	(17′)	12.66		
(6)	3.60	(18)	11.81	(30)	20.77	(6′)	3.60	(18′)	14.00		
(7)	4.27	(19)	12.65	(31)	21.66	(7′)	4.27	(19′)	15.05		
(8)	4.97	(20)	14.38	(32)	21.94	(8′)	4.97	(20′)	15.89		
(9)	7.61	(21)	15.43	(33)	22.10	(9′)	7.61	(21′)	16.85		
(10)	8.46	(22)	15.54			(10′)	8.46	(22′)	17.71 (17.18)*		
(11)	9.39	(23)	15.92			(11′)	9.39	(23′)	18.04		
(12)	9.50	(24)	16.45			(12′)	9.50	(24′)	18.54		

Distance to each restriction sites in kilo-basepairs.
* The site 22′ in pIJ702-X7 cannot be determined.

were obtained and designated pIJ702-X501 and pIJ702-X502. As shown in Table 1 and Fig. 1, pIJ702-X501 contained a 2.61 kb *Sph* I-*Sph* I fragment and a 0.91 kb *Sph* I-*Bgl* II fragment derived from the inserted region in pIJ702-X5. The same 2.61 kb *Sph* I fragment was inserted in reverse orientation in pIJ702-X502. From *Sac* I digests of pIJ702-X5, two plasmids designated pIJ702-X511 and pIJ702-X512 were obtained. Their orientation in the inserted fragments was determined by digestion with *Sph* I. The plasmid pIJ702-X511 contained a 9.5 kb *Bgl* II-*Sac* I-*Sac* I fragment as shown in Table 1 and Fig. 1. The plasmid pIJ702-X512 had the opposite orientation of the 1.89 kb *Sac* I fragment.

Subsequently, pIJ702-X501 was digested with *Sac* I, re-ligated and introduced into *S. lividans* TK21. The smallest plasmid pIJ702-X551 was obtained from transformants forming halo and possessing resistance against thiopeptin. As shown in Table 1 and Fig. 1, pIJ702-X551 contained a 1.04 kb *Sph* I-*Sac* I fragment in which a single *Bcl* I site existed.

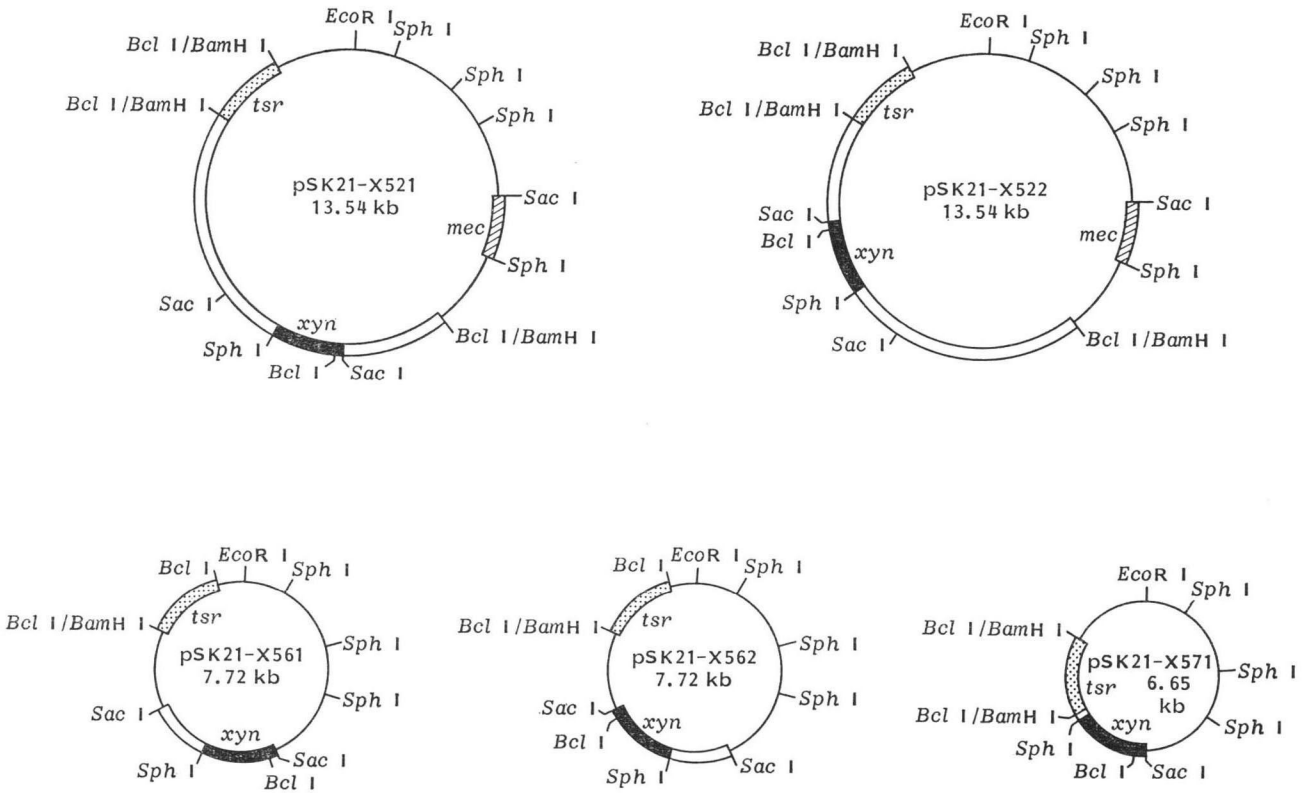
Sub-cloning of Xylanase Gene into the pSK2 Series of Plasmid Vectors

*Bam*H I digests of pIJ702-X5 and *Bcl* I digests of pSK21-TM6 were ligated and introduced into protoplasts of *S. kasugaensis* G3. Two plasmids, pSK21-X521 and pSK21-X522 containing a 6.01 kb *Bam*H I fragment in a different orientation were obtained (Fig. 2). *Sac* I digests of pIJ702-X511 and pSK21-B5 were ligated and the ligation mixture was introduced into protoplasts of *S. kasugaensis* G3. Two plasmids, pSK21-X561 and pSK21-X562 containing a 1.89 kb *Sac* I fragment in a different orientation were obtained (Fig. 2). Moreover, pIJ702-X501 was digested with *Sph* I, *Sac* I and *Bam*H I, and pSK21-B4 was digested with *Sac* I and *Bcl* I, then their digests were ligated and introduced into *S. lividans* TK21. As shown in Fig. 2, the desired plasmid pSK21-X571 containing the 1.13 kb *Bam*H I-*Sph* I-*Sac* I fragment derived from pIJ702-X501 was obtained.

The transformants carrying these 5 pSK2 series of plasmids showed halo-formation and thiopeptin-resistance.

To examine possible insertional inactivation at the *Bcl* I site of the cloned xylanase gene in pSK21-X521, *Bcl* I digested fragments of *S. kasugaensis* G3 total DNA were introduced into the unique *Bcl* I site, and the ligated sample was introduced into *S. kasugaensis* G3. The thiopeptin-resistant transformants obtained were transferred onto XA agar plates containing 10 μg/ml of thiopeptin. Twenty-four colonies out of 94 tested did not form a halo. All halo-minus transformants carried plasmids

Fig. 2. Restriction endonuclease-cleavage maps of the constructed pSK2 series plasmids.



Thin lines indicate the vector plasmid portion. Open regions indicate the fragment derived from *Streptomyces* sp. No. 36a genome. The selective markers described here were *tsr* for a thiostrepton-resistant gene, *xyn* for a xylanase gene and *mec* for a gene which restores methionine and cysteine requirement of *S. kasugaensis* G3 reported previously[13].

Table 2. Xylanase productivity in culture broth of the donor strain, the host strains and Xyn$^+$ transformants.

Host	Plasmid	Xylanase activity (units)	Ratio
Streptomyces sp. No. 36a	—	38.9	1.0
Streptomyces lividans TK21	—	3.1	0.08
	pIJ702	0	0
	pIJ702-X5	1,495	38.4
	pIJ702-X501	952	24.5
	pIJ702-X511	1,256	32.7
	pIJ702-X551	2,839	73.0
	pSK21-X571	1,777	45.7
Streptomyces kasugaensis G3	—	0	0
	pSK21-TM6	0	0
	pSK21-X521	454	11.7
	pSK21-X561	1,841	47.3

Units: μmol of reducing sugar/ml/minute.

larger than pSK21-X521. The vector pSK21-X521 was recovered by *Bcl* I digestion of the large plasmids.

The smallest plasmids pIJ702-X551 and pSK21-X571 obtained were digested with 26 restriction endonucleases. The following enzymes had no restriction cleavage site in the 1.04 kb fragment; *Aat* I, *Aat* II, *Apa* I, *Bam*H I, *Bgl* II, *Cla* I, *Eco*R I, *Hin*d III, *Hpa* I, *Kpn* I, *Mlu* I, *Nco* I, *Nde* I, *Pst* I, *Pvu* I, *Pvu* II, *Sma* I, *Xba* I and *Xho* I. Seven restriction endonucleases such as *Sph* I (0 kb), *Bal* I (0.15 kb), *Sst* II (0.37 kb), *Sal* I (0.61 kb), *Bss*H II (0.88 kb), *Bcl* I (0.93 kb) and *Sac* I (1.04 kb) had single restriction cleavage sites in the *Sph* I-*Sac* I fragment (size in parenthesis indicates distance from *Sph* I site).

Xylanase Productivity

The xylanase productivity of *S. lividans* TK21 and *S. kasugaensis* G3 carrying the newly constructed plasmids was examined together with their control strains and *Streptomyces* sp. No. 36a. As shown in Table 2, the strains carrying the plasmids containing xylanase gene had very high xylanase activity compared to *Streptomyces* sp. No. 36a from which the xylanase gene was obtained. *S. lividans* TK21/pIJ702-X551 showed a 73-fold increase in xylanase activity. Both hosts harboring the pSK2 series of plasmids, namely *S. kasugaensis* G3/pSK21-X561 and *S. lividans* TK21/pSK21-X571, had the same level of increase in xylanase activity.

Discussion

Recently, PANBANGRED *et al.* cloned the xylanase gene from *Bacillus pumilus* into *Escherichia coli* C600 using pBR322 as a vector[4]. BERNIER *et al.* also cloned the gene from *Bacillus subtilis* into *E. coli* WA802 using pBR325 as a vector[5]. Both *E. coli* transformants produced xylanases but the enzymes were not excreted into the medium.

An attempt to clone the xylanase gene of *Streptomyces* sp. No. 36a using a *Bcl* I digest of pSK21-TM6 as a vector was unsuccessful, while another attempt using a *Bgl* II digest of pIJ702 as a vector gave 2 plasmids pIJ702-X5 and X7 carrying the xylanase gene. The plasmid pIJ702-X7 had some DNA alterations in the vector portion, but the 9.50 kb *Bgl* II-*Sac* I fragment was identical to that of pIJ702-X5.

To elucidate the location of xylanase gene precisely, pIJ702-X5 was trimmed down by restriction

endonuclease treatment and by re-ligation. The plasmid pIJ702-X551 which includes a 1.04 kb insert fragment showed xylanase activity in *S. lividans* TK21. This 1.04 kb fragment was the shortest fragment coding for the xylanase gene. Plasmids pIJ702-X501 and X502 carrying the minimal *Sph* I fragment in opposite insert orientation expressed xylanase activity. Plasmids pIJ702-X511 and X512 also carrying the minimal *Sac* I fragment in opposite orientation expressed the activity. From these data, it is suggested that the *Sph* I-*Sac* I fragment of 1.04 kb contained the promotor as well as the xylanase structural gene. This is supported by the fact that the xylanase gene was expressed in the transformants harboring the pSK2 series of plasmids with inserts having the opposite orientation, such as pSK21-X521 or X522, and pSK21-X561 or X562.

Failure to clone the xylanase gene using a *Bcl* I digest of pSK21-TM6 suggested that the *Bcl* I site exists in the coding region of the xylanase gene. To confirm this possibility, *Bcl* I digests of *S. kasugaensis* G3 total DNA were introduced into the unique *Bcl* I site of pSK21-X521. All thiopeptin-resistant and xylanase-negative transformants tested showed the insertion of foreign DNA fragments. Therefore, the *Bcl* I site in the xylanase gene can be used as a insertional inactivation site for cloning of other genes. The smallest plasmid in the pSK2 series, pSK21-X571 which has a single *Bcl* I site in the xylanase gene, will be useful as a cloning vector to allow selection by insertional inactivation of xylanase activity. The *Sal* I and the *Bss*H II sites existing in the xylanase gene fragment should be also usable as cloning sites in pSK21-X571 and pIJ702-X551, respectively.

The cloned xylanase gene was expressed in *S. kasugaensis* G3 and *S. lividans* TK21. This indicates that its expression may occur in a wide host range. The transformants harboring the xylanase-positive recombinant plasmids produced about 10 to 70 times higher levels of extracellular xylanase than that of the donor strain *Streptomyces* sp. No. 36a. This increase in xylanase production may be due to a gene dosage effect by the multi-copy vector plasmids. The productivity of xylanase in *S. kasugaensis* G3 harboring pSK21-X521 was noticeably lower than that of other transformants. This might be caused by unstability of the vector pSK21-TM6 included in pSK21-X521. It is interesting that *S. lividans* TK21 possessed very low xylanase activity, and when it was transformed with pIJ702 did not show this activity.

Consequently, the plasmid pSK21-X571 is useful as a cloning vector owing to the apparent wide host range, and the insertional inactivation of the xylanase gene at the *Bcl* I site which was directly detected in the R3X regeneration medium. Moreover, it showed a high level of xylanase secretion into the medium, indicating its possible usefulness as secretion vector.

Acknowledgment

The authors acknowledge to Dr. D. A. HOPWOOD, John Innes Institute, England and Dr. E. KATZ, Georgetown University, U.S.A. for providing *S. lividans* TK21 and *S. lividans* 3131 harboring pIJ702, respectively, and also thank to Dr. M. KOIKE, Hokko Chemical Industry Co., Ltd. for providing *Streptomyces* sp. No. 36a and technical suggestions.

References

1) YAMAZAKI, H.; K. OHMURA, A. NAKAYAMA, Y. TAKEICHI, K. OTOZAI, M. YAMASAKI, G. TAMURA & K. YAMANE: α-Amylase gene (*amy*R2 and *amy*E$^+$) from an α-amylase-hyperproducing *Bacillus subtilis* strain: Molecular cloning and nucleotide sequence. J. Bacteriol. 156: 327~337, 1983
2) GRAY, O. & S. CHANG: Molecular cloning and expression of *Bacillus licheniformis* β-lactamase gene in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 145: 422~428, 1981
3) VASANTHA, N.; L. D. THOMPSON, C. RHODES, C. BANNER, J. NAGLE & D. FILPULA: Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. J. Bacteriol. 159: 811~819, 1984
4) PANBANGRED, W.; T. KONDO, S. NEGORO, A. SHINMYO & H. OKADA: Molecular cloning of the genes for xylan degradation of *Bacillus pumilus* and their expression in *Escherichia coli*. Mol. Gen. Genet. 192: 335~341, 1983
5) BERNIER, Jr., R.; H. DRIGUEZ & M. DESROCHERS: Molecular cloning of *Bacillus subtilis* xylanase gene in *Escherichia coli*. Gene 26: 59~65, 1983

6) OHMURA, K.; T. SHIROZA, K. NAKAMURA, A. NAKAYAMA, K. YAMANE, K. YODA, M. YAMASAKI & G. TAMURA: A *Bacillus subtilis* secretion vector system derived from the *B. subtilis* α-amylase promotor and signal sequence region, and secretion of *Escherichia coli* β-lactamase by the vector system. J. Biochem. 95: 87~93, 1984
7) PALVA, I.; M. SARVAS, P. LEHTOVAARA, M. SIBAKOV & L. KÄÄRIÄINEN: Secretion of *Escherichia coli* β-lactamase from *Bacillus subtilis* by the aid of α-amylase signal sequence. Proc. Natl. Acad. Sci. U.S.A. Biol. Sci. 79: 5582~5586, 1982
8) HOSHIKO, S.; O. MAKABE, C. NOJIRI, E. SATOH & K. NAGAOKA: Molecular cloning and expression of α-amylase gene from *Streptomyces hygroscopicus*. in preparation
9) KENDALL, K. & J. CULLUM: Cloning and expression of an extracellular-agarase gene from *Streptomyces coelicolor* A3(2) in *Streptomyces lividans* 66. Gene 29: 315~321, 1984
10) KATZ, E.; C. J. THOMPSON & D. A. HOPWOOD: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129: 2703~2714, 1983
11) OKANISHI, M.; K. KATAGIRI, T. FURUMAI, K. TAKEDA, K. KAWAGUCHI, M. SAITOH & S. NABESHIMA: Basic techniques for DNA cloning and conditions required for Streptomycetes as a host. J. Antibiotics 36: 99~108, 1983
12) NABESHIMA, S.; Y. HOTTA & M. OKANISHI: Construction of plasmid vectors from *Streptomyces kasugaensis* plasmids, pSK1 and pSK2. J. Antibiotics 37: 1026~1037, 1984
13) HIRASAWA, K.; M. ICHIHARA & M. OKANISHI: Nucleotide sequence of Mec$^+$ gene region of *Streptomyces kasugaensis*. J. Antibiotics 38: 1795~1798, 1985
14) THOMPSON, C. J.; J. M. WARD & D. A. HOPWOOD: Cloning of antibiotic resistance and nutritional genes in Streptomycetes. J. Bacteriol. 151: 668~677, 1982
15) SHIRAHAMA, T.; T. FURUMAI & M. OKANISHI: A modified regeneration method for streptomycete protoplasts. Agric. Biol Chem. 45: 1271~1273, 1981
16) OKANISHI, M. & K. F. GREGORY: Methods for the determination of deoxyribonucleic acid homologies in *Streptomyces*. J. Bacteriol 104: 1086~1094, 1970
17) CHATER, K. F.; D. A. HOPWOOD, T. KIESER & C. J. THOMPSON: Gene cloning in *Streptomyces*. Curr. Top. Microbiol. Immunol. 96: 69~95, 1982
18) PANBANGRED, W.; A. SHINMYO, S. KINOSHITA & H. OKADA: Purification and properties of endoxylanase produced by *Bacillus pumilus*. Agric. Biol. Chem. 47: 957~963, 1983