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Cloning and Expression of a *Paenibacillus* sp. Neopullulanase Gene in *Saccharomyces cerevisiae* Producing *Schwanniomyces occidentalis* Glucoamylase

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Abstract A gene, *npl*, encoding neopullulanase from *Paenibacillus* sp. KCTC 8848P was cloned and expressed in *Escherichia coli*. It consisted of an open reading frame of 1,530 bp for a protein that consisted of 510 amino acids with a molecular weight of 58,075 Da. The deduced amino acid sequence of the neopullulanase gene had 92% identity with the neopullulanase of *Bacillus polymyxa*. The *npl* gene was also expressed in *Saccharomyces cerevisiae* secreting *Schwanniomyces occidentalis* glucoamylase (*GAM1*) under the control of the yeast actin gene (*ACT1*) promoter. Secretion of the neopullulanase was directed by the yeast mating pheromone α-factor (*MFα1*) prepro region. Enzyme assays confirmed that co-expression of *npl* and *GAM1* enhanced starch and pullulan degradation by *S. cerevisiae*.

Key words: *Paenibacillus* sp. neopullulanase gene, *Saccharomyces cerevisiae*, *Schwanniomyces occidentalis* glucoamylase gene

The α-amylase family (family 13 glycosyl hydrolases) has been widely employed in starch-processing industries [4, 20]. Among these enzymes, neopullulanase (EC 3.2.1.135) hydrolyzes starch, including most of its subfamily members, and also degrades α-1,4-glycosidic bonds in pullulan, an α-glucan produced by *Aureobasidium pullulans*, with formation of trisaccharide panose (Glucose-α-1,6-Glucose-α1,4-Glucose). There are two other related types of enzymes that hydrolyze pullulan: pullulanase (EC 3.2.1.41) hydrolyzes α-1,6-glycosidic linkages to release maltotriose, and isopullulanase (EC 3.2.1.57) attacks α-1,4-glycosidic bonds and produces isopanose (Glucose-α1,4-Glucose-α-1,6-Glucose) [22]. Neopullulanase has been found in amylolytic bacteria such as *Bacillus* sp. KSM-1876, *B. stearothermophilus*, *B. polymyxa*, and *Bacteroides thetaiotaomicron*, and the genes encoding these enzymes have been cloned and characterized [6, 10, 18, 23]. In an attempt to supply *Saccharomyces cerevisiae* with amylolytic activity, several heterologous bacterial, fungal, and mammalian amylase genes have been cloned and expressed in *S. cerevisiae* [2, 7, 8, 9, 15]. Recently, the β-amylase gene from *Paenibacillus* sp. KCTC 8848P has been cloned and expressed in *S. cerevisiae* [7]. In this report, the cloning and characterization of a neopullulanase gene from this bacterium and its expression in *S. cerevisiae* secreting *Schwanniomyces occidentalis* glucoamylase (*GAM1*) with debranching activity [2] are described.

Paenibacillus sp. KCTC 8848P [7, 14] was used as the source of the neopullulanase gene, and *Sch. occidentalis* ATCC 26077 [13] as the source of the glucoamylase gene. *Escherichia coli* JM83 was used for all bacterial transformation and plasmid preparations. Plasmid pUC19 was used as the cloning vector and as the subcloning vector for the DNA sequencing. *S. cerevisiae* INVSC1 (*MATα*, *his3-Δ1*, *leu2*, *trp1-289*, *ura3-52*) was used as a host for yeast transformation. YEpACT-MFα1 [23] and pYES2ADC [8] were used for the construction of yeast recombinant plasmids. Manipulation and preparation of plasmid, and the transformation of *E. coli* were performed by the methods described by Sambrook and Russell [16]. *Paenibacillus* sp. KCTC 8848P and *E. coli* transformants were cultured on buffered Luria-Bertani (LB) media containing 0.1 M sodium phosphate buffer (pH 6.0), supplemented with 50 μg/ml of ampicillin and 1% soluble starch, when required [7]. The yeast cells were transformed according to the lithium acetate/DMSO method of Hill *et al.* [5]. To assay the amylase activity, the yeast transformants grown on the minimal selective media [17] were cultured on buffered YPD media containing 0.1 M sodium phosphate buffer (pH 6.0) [8]. Mitotic stability of the *GAM1* gene was determined by the method of Kim and Kim [9].

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Chromosomal DNA of *Paenibacillus* sp. was isolated according to the procedure of Murray and Thompson [12]. The DNA was partially digested with *Sau*3AI, and ligated to the *Bam*HI site of pUC19. The ligated mixture was then transformed in *E. coli*. The amylolytic clones were detected by the halos around the colonies on the LB plates supplemented with 3% soluble starch. The nucleotide sequence of the neopullulanase gene was determined by using an ABI PRISM 377 DNA sequencer. The PC/GENE and DNASIS software systems were employed to analyze the DNA sequence. For the amplification of the neopullulanase gene, two oligo primers (p5' and p3') were designed based on the sequence analysis. The sequences around the start codon and at the upstream of the TAG termination codon were used for the design of p5' and p3' oligo primers, respectively. In two oligo primers, *Sal*I site (GTCGAC) and *Sph*I site (GCATGC) were introduced to facilitate the cloning of the neopullulanase gene, respectively. The primer sequences were as follows: p5' (5'-GAAGAATTCGTCGAC-CCTATGCTCACGATGTTCGC-3') and p3' (5'-GAAGA-ATTCGCATGCCTATTTGGTGATTAGAGAGCTGTA-3'). On the other hand, the *GAM1* gene of *Sch. occidentalis* was amplified with oligonucleotides 5'-GGAGGATCCAC-CATGTTTTCTGAAGCTGAT-3' and 5'-GGAGGATCC-

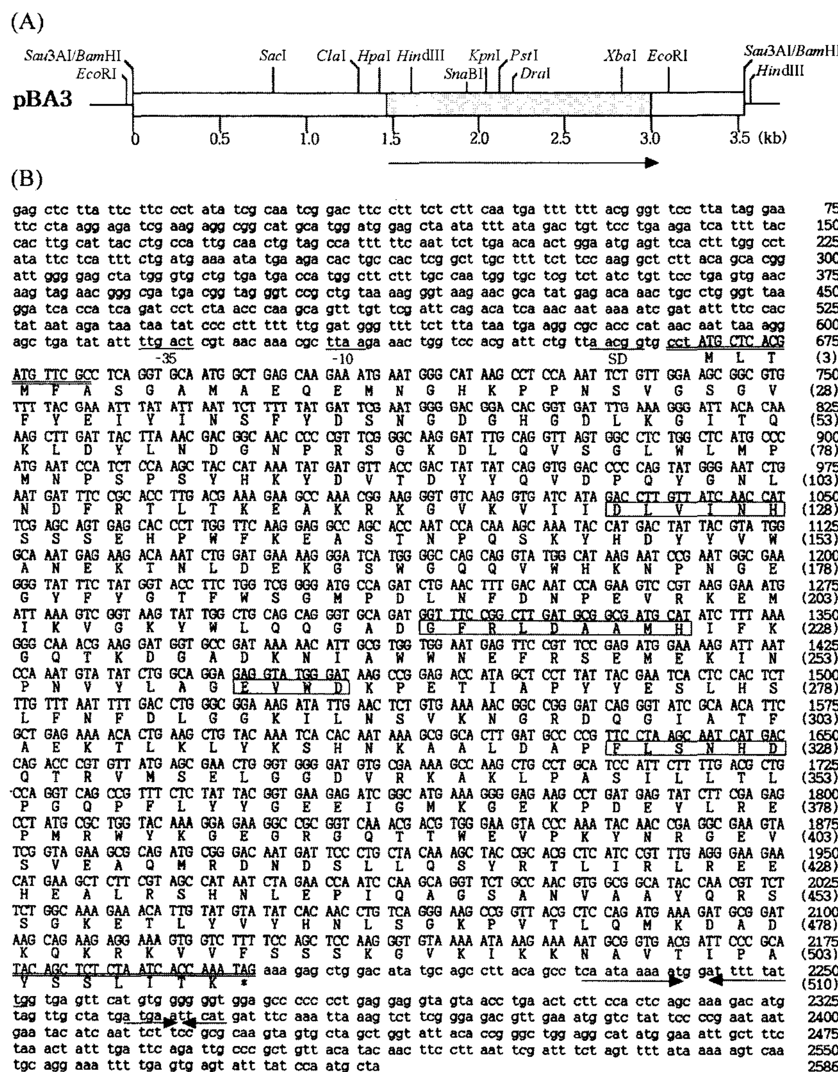

Fig. 1. Restriction map of the 3.6-kb insert DNA of pBA3 (A) and the complete nucleotide and deduced amino acid sequences of *Paenibacillus* sp. neopullulanase gene (*npl*) (B).

(A) The box represents the coding region of *npl* and the transcription direction is indicated by an arrow. The thick and thin lines represent the *Paenibacillus* sp. and pUC19 DNAs, respectively. (B) The putative promoter regions (–35 and –10 regions) and Shine-Dalgarno (SD) sequence are underlined. The primers used for the amplification of the neopullulanase gene are marked by double underlines. The four conserved sequence motifs in amylolytic enzymes are boxed. The arrows indicate inverted repeat sequences. The nucleotide sequence reported in this report was deposited in the GenBank database under the accession number of AF412311.

CGGGATGTTAAAATTACCAAGT-3'. These primers were synthesized from the published nucleotide sequence of the genomic copy of the *GAM1* gene from *Sch. occidentalis* ATCC 26076 (GenBank accession number M34666) [2]. Amylolytic activity (neopullulanase activity and/or glucoamylase activity) was determined by the method of Jeong *et al.* [7]. The pH of the enzyme assay mixture (0.5% soluble starch or pullulan as substrate and culture supernatant as a crude enzyme) and incubation temperature employed were 6.0 and 50°C, respectively. One unit of neopullulanase or glucoamylase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per ml per min. Reaction products were identified by thin-layer chromatography (TLC) according to the method of Bai *et al.* [1].

A genomic library of *Paenibacillus* sp. KCTC 8848P was constructed in *E. coli* by using pUC19, and approximately 3,000 transformants were screened for their amylase activity. Three amylase-positive clones were found to form halos on the LB plates supplemented with soluble starch. Two recombinant plasmids isolated from these clones had inserts exhibiting the same restriction map pattern of the β-amylase gene reported previously [7]. The other plasmid, pBA3, had an insert of about 3.6-kb, which exhibited quite different enzyme sites (Fig. 1A). The amylolytic activity of the amylase (0.11 U/ml) produced by the *E. coli* transformants harboring pBA3 was much lower than that reported for the β-amylase (1.12 U/ml), when soluble starch was used as a substrate [7]. However, the amylolytic enzyme degraded pullulan (0.06 U/ml), unlike the β-amylase which could not hydrolyze pullulan [21]. The analysis of the enzymatic reaction products by TLC indicated that the amylolytic enzyme from the *E. coli*/pBA3 broke down pullulan into trisaccharide (data not shown). Therefore, this enzyme seemed to be one of three types of pullulan-degrading enzymes. In order to analyze the location of the gene for amylolytic enzyme, the nucleotide sequencing of the downstream region from the *Sac*I site of the insert fragment was carried out. The 2,586-bp sequence contained the 1,530-bp open reading frame encoding a protein of 510 amino acids with a molecular mass of 58,075 Da (Fig. 1B). The deduced amino acid sequence analysis of the gene encoding *Paenibacillus* sp. amylolytic enzyme with BLAST programs showed 92% identity with *B. polymyxa* neopullulanase (*nplA*) (accession No. U89716). However, there was little similarities to other neopullulanases from *B. stearothermophilus* (AF233372) and alkalophilic *Bacillus* sp. KSM-1876 (D13255) belonging to family 13 glycosyl hydrolases [4, 6], with the identity being 30% with the former and 26% with the latter. Four conserved regions (I-IV), which are reported to be important for enzyme activities of the α-amylase family [20], were found in *Paenibacillus* sp. amylolytic enzyme. The amino acid residues of the catalytic site and the substrate-binding site in four conserved regions of the *Paenibacillus* sp. amylolytic enzyme coincided exactly with those from the *B. polymyxa* neopullulanase, but differed from those of *B. stearothermophilus* and *Bacillus* sp. KSM-1876 neopullulanases at two residues of region II corresponding to the substrate-binding site, which closely related to the production ratio of panose from pullulan [11] (Fig. 2). The neopullulanase from *B. stearothermophilus* is known to release panose simultaneously with maltose and glucose from pullulan, but the enzyme from *B. polymyxa* exclusively produces panose as the final product of pullulan hydrolysis [22].

	I	II	III	IV
Paeni	DLVINH	GFRLDAAMH	EVWD	FLSNHD
B.pol	DLVINH	GFRLDAAMH	EVWD	FLSNHD
B.ste	DAVFNH	GWRLDVANE	EIWH	LLGSHD
B.KSM	DAVFNH	GWRLDVANE	EIWH	LLGSHD
	* * **	* *** *	* *	* **

Fig. 2. Comparison of amino acid residues in the four conserved regions of the *Paenibacillus* sp. neopullulanase with those of other neopullulanases.

The amino acid sequences of the *Paenibacillus* sp. neopullulanase (Paeni) are shown compared with those of *Bacillus polymyxa* (B. pol), *B. stearothermophilus* (B. ste), and *Bacillus* sp. KSM-1876 (B.KSM). The identical amino acids are indicated by asterisks. Residues shown to be important for the catalytic site and substrate-binding site by Kuriki *et al.* [11] are underlined and boxed, respectively.

Since the *Paenibacillus* sp. gene promoter is not functional in *S. cerevisiae*, the amylolytic enzyme (neopullulanase) gene (*npl*) was expressed under the control of the promoter derived from yeast actin gene (*ACT1*). The secretion of neopullulanase in *S. cerevisiae* was found to be directed by the yeast mating pheromone α-factor (*MFα1*) prepro region, because *S. cerevisiae* was not capable of secreting the recombinant neopullulanase using the native neopullulanase signal peptide [23]. A 1.5-kb *Sal*I-*Sph*I amplified DNA fragment of the neopullulanase coding region was inserted into the same sites between the *MFα1* prepro region and 2-micron origin of YEpACT-MFα1, thereby generating YEpNPL (Fig. 3A). On the other hand, a 2.9-kb amplified DNA fragment of the *GAM1* gene (containing its own signal sequence) from *Sch. occidentalis* producing extracellular glucoamylase [2], after being digested with *Bam*HI, was inserted into pYES2 containing *ADC1* promoter [8]. For the stable expression of the *GAM1* gene in *S. cerevisiae*, a linearized integrating vector, YIpSG, was constructed by self-ligation after a 2-micron origin was excised by digesting the above recombinant plasmid with *Hpa*I and *Sna*BI (Fig. 3B). YIpSG exhibited a unique restriction site for *Nco*I within the *URA3* gene. Therefore, linearized YIpSG that was digested with *Nco*I could be integrated into a homologous sequence of the *URA3* or *ura3* loci on the chromosome

V of a recipient yeast cell by initiating homologous recombination [7, 14]. The mitotic stability of YIpSG in yeast transformants (grown in nonselective media) was calculated as 100% after 100 generations [9].

The recipient strain, *S. cerevisiae* INVSC1, was transformed to Trp^+ Npl^+ with YEpNPL, generating INVSC1/YEpNPL. *S. cerevisiae* was transformed to Ura^+ GAM^+ with YIpSG, generating INVSC1/YIpSG. All the transformants secreting neopullulanase or glucoamylase formed halos around their colonies on YPDS3 (YPD containing 3% soluble starch) agar plates. For the coexpression of the *npl* gene and *GAM1* gene, the *npl* gene was introduced into *GAM1*-integrated *S. cerevisiae* transformant (INVSC1/YIpSG) by using YEpNPL, thereby generating INVSC1/YIpSG/YEpNPL secreting both neopullulanase and glucoamylase. Neopullulanase and glucoamylase production by *S. cerevisiae* transformants harboring both YEpNPL and YIpSG grown in the media containing glucose were caused by the *ACT1* promoter and *ADC1* promoter without the regulatory site, respectively [9]. The culture supernatants of the transformants were examined for their amylolytic activities. As shown in Table 1, the amylolytic activity of INVSC1/YIpSG/YEpNPL capable of secreting both neopullulanase and glucoamylase was about 3 times higher than that of

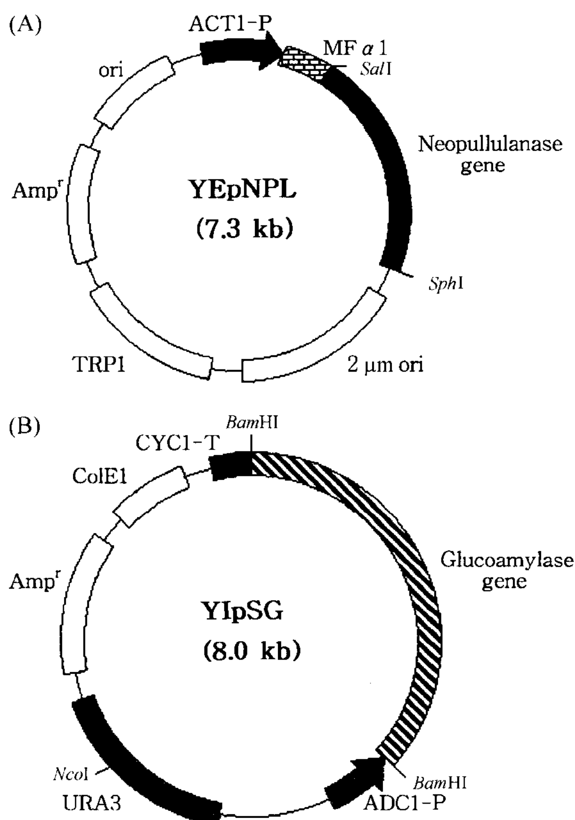

Fig. 3. Genetic maps of plasmids YEpNPL containing the neopullulanase gene (A) and YIpSG containing the glucoamylase gene (B).

Table 1. Amylolytic activities in cell-free culture supernatants of various yeast strains.

Yeast strains	Amylolytic activity (U/ml)	
	Starch	Pullulan[a]
S. cerevisiae INVSC1	0.00	0.00[b]
INVSC1/YEpNPL	0.13	0.08
INVSC1/YIpSG	0.23	0.12
INVSC1/YIpSG/YEpNPL	0.38	0.22

[a]Amylolytic activity was assayed by using 0.1 M sodium phosphate buffer (pH 6.0) containing 0.5% soluble starch or pullulan as a substrate.
[b]Values are the means of results from triplicate experiments, and express the amylolytic activity (neopullulanase activity and/or glucoamylase activity) present in the culture supernatants.

INVSC1/YEpNPL which secreted only neopullulanase. The effect of the *npl* and *GAM1* genes on the production of amylolytic activity appeared to be more or less additive when reducing sugar released from soluble starch or pullulan was assayed [19]. When the amylolytic activity was assayed by using starch as a substrate, the sum of neopullulanase activity (0.13 U/ml) produced by INVSC1/YEpNPL and the activity of the glucoamylase (0.23 U/ml) produced by INVSC1/YIpSG was 0.36 U/ml, similar to the value of 0.38 U/ml obtained with INVSC1/YIpSG/YEpNPL. The activity of the recombinant neopullulanase towards pullulan was lower than towards starch, in agreement with that obtained from *B. polymyxa* neopullulanase [23]. To produce useful food industrial products, such as oligosaccharides or branched oligosaccharides, from starch biomass [24], further attempts are now being made to develop industrial polyploid strains of *S. cerevisiae* capable of producing neopullulanase [3, 19].

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