An amylopullulanase from alkalophilic Bacillus sp. KSM-1378 hydrolyzes both α-1,6 linkages in pullulan and α-1,4 linkages in other polysaccharides, with maximum activity in each case at an alkaline pH, to generate oligosaccharides (Ara, K., Saeki, K., Igarashi, K., Takaïwa, M., Uemura, T., Hagihara, H., Kawai, S., and Ito, S. (1995) *Biochim. Biophys. Acta* 1243, 315–324). Here, we report the molecular cloning and sequencing of the gene for this enzyme and show that its dual hydrolytic activities are associated with two independent active sites. The structural gene contained a single, long open reading frame of 5,814 base pairs, corresponding to 1,938 amino acids that included a signal peptide of 32 amino acids. The molecular mass of the extracellular mature enzyme (Glu33 through Leu1938) was calculated to be 211,450 Da, a value close to the 210 kDa determined for the amylopullulanase produced by *Bacillus sp.* KSM-1378. The amylase and the pullulanase domains were located in the amino-terminal half and in the carboxy-terminal half of the enzyme, respectively, being separated by a tandem repeat of a sequence of 35 amino acids. Four regions, designated I, II, III, and IV, were highly conserved in each catalytic domain, and they included a putative catalytic triad Asp550-Glu579-Asp645 for the amylase activity and Asp1460-Glu1469-Asp1581 for the pullulanase activity. The purified enzyme was rotary shadowed at a low angle and observed by transmission electron microscopy; it appeared to be a “castanet-like” or “bent dumbbell-like” molecule with a diameter of approximately 25 nm.

Amyloytic enzymes, such as α-amylase (EC 3.2.1.1) and debranching pullulanase (pullulan 6-glucanohydrolase; EC 3.2.1.41), are industrially important for the liquefaction of starch and in saccharification processes (Norman, 1982). We have found and characterized some unique debranching enzymes, such as an alkaline pullulanase (Ara et al., 1992), an alkali-resistant neopullulanase (Igarashi et al., 1992), and an alkaline isomaltase (EC 3.2.1.68) (Ara et al., 1994), in strains of alkalophilic *Bacillus*, and these enzymes can be used as effective additives in dishwashing and laundry detergents under alkaline conditions. Amylopullulanases (APase) (Saha et al., 1989) or type II pullulanases (Spreinat and Antranikian, 1990) have frequently been found in cultures of thermophiles (Coleman, 1993). Recently, we also found and characterized the first known alkaline APase (the product of the *apuA* gene) in cultures of alkalophilic *Bacillus sp.* KSM-1378 (Ara et al., 1995b). This APase is unique in that it efficiently hydrolyzes the α-1,6 linkages of pullulan, as well as the α-1,4 linkages of amylose, amylpectin, and glycogen at alkaline pH values, whereas other APases, the type that have frequently been found in cultures of some thermophiles, are all active at acid or neutral pH.

There are many reports that demonstrate unequivocally that a single active site is involved in the α-1,4- and α-1,6-hydrolyzing activities of several APases, such as the enzymes from *Thermoactinomyces* (Sakano et al., 1982), *Thermus* sp. (Nakamura et al., 1989), *Clostridium thermohydrodsulfuricum* (Melasniemi, 1988; Saha et al., 1988), *Pyrococcus furiosus*, and *Thermococcus litoralis* (Brown and Kelly, 1993). For example, it has been shown that the specific conversion of an aspartic acid residue and a glutamic acid residue individually to their amide forms in the APase from *Thermoanaerobacter ethanoli- cus* 39E (formerly *C. thermohydrodsulfuricum* 39E; Lee et al., 1993a) caused the loss of both the α-amylase and the pullulanase activities, providing proof of the presence of a single active site in the enzyme (Mathupala et al., 1993). However, in the case of the alkaline APase from *Bacillus sp.* KSM-1378, all the kinetic data that we have obtained (Ara et al., 1995a, 1995b) can only be explained by assuming that the dual catalytic activity of this enzyme is associated with different active sites. In fact, we found that the 210-kDa APase of this organism could be cleaved by papain to generate a 114-kDa amylase-hydrolyzing polypeptide and a 102-kDa pullulan-hydrolyzing polypeptide (Ara et al., 1996). This report describes the molecular cloning of the *apuA* gene for the 210-kDa APase from *Bacillus sp.* KSM-1378 and the determination of its nucleotide sequence. Furthermore, the deduced amino acid sequence of the product of the *apuA* gene from this organism is compared with those of other acid and neutral APases, with emphasis on their respective catalytic properties and molecular structures.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains, Plasmids, and Propagation*—The source of the enzyme examined in this study was *Bacillus sp.* KSM-1378, which had
previously been isolated from a soil sample in our laboratory. It was grown on a nutrient medium composed of 7.5% (w/v) glucose, 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.2% (w/v) MgSO4 and 0.1% (w/v) FeCl3. The culture was incubated at 37 °C for 24 h.
Sequencing of Amylopullulanase Gene from Bacillus 24077

Brilliant Blue R-250 in 50% (v/v) methanol for 1 min, and then it was destained in 50% (v/v) methanol. The amino acid sequence of the protein band was directly determined in an amino acid sequencer (model 470A, Applied Biosystems) that was connected to an on-line PTH-derivative analyzer. Internal carboxyl-terminal sequences of proteins were determined after degradation with a lysyl endopeptidase in a protein sequencer (model PPSQ-10, Shimadzu) equipped with a carboxyl-terminal fragment fractionator (model CTFP-1, Shimadzu) in accordance with the manufacturer’s recommendations.

Electron Microscopic Examination of APase—The preparation of purified APase (or fragments after cleavage by papain) was diluted to 0.1 mg protein/ml with a 10 mM solution of Tris-HCl buffer (pH 8.0) that contained 60% (v/v) glycerol, and the solution was spread onto a freshly cleaved piece of mica with a spray gun (model SP-B, Olympus, Tokyo, Japan). The mica was dried quickly under a vacuum of 10–5 Torr and cleaved piece of mica with a spray gun (model SP-B, Olympus, Tokyo, Japan). The mica was dried quickly under a vacuum of 10–5 Torr and then rotary-shadowed at an angle of 3–10° with platinum/carbon evaporated from a platinum-carbon evaporator in a freeze-etching apparatus (model JFD-9010, JEOL, Tokyo, Japan) as described by Tyler and Branton (1980). After coating with a supporting film of carbon, the carbon film with the platinum replica was taken from the apparatus, mounted on a 200-mesh copper grid, and then examined with a transmission electron microscope (model JEM-1010, JEOL). The 114-kDa amylose- and the 102-kDa pullulan-hydrolyzing polypeptides, both of which were generated by limited proteolysis of intact APase with papain, were similarly examined under the electron microscope.

RESULTS AND DISCUSSION

Cloning of the Gene for Alkaline Pullulanase—A gene library for Bacillus sp. KSM-1378 was constructed by ligating PstI fragments of the genomic DNA into the PstI site of pBR322. After transformation of E. coli cells with the ligation mixture, an attempt was made to identify amylase- and pullulanase-positive transformants by the formation of halos on starch-azure and red pullulan agar plates, respectively. However, no transformants with both amylase and pullulanase activities nor any amylase-positive transformants were obtained under the cloning conditions that employed the E. coli pBR322 system.

A pullulanase-positive clone, designated pBP101 (10.6 kbp), was found to encode an active pullulanase on a 6.2-kbp PstI-PstI insert (fragment A), which had various other restriction sites, as shown in Fig. 1. Subcloning showed that a 4.2-kbp PstI-BamHI region of the insert contained the region essential for the pullulanase activity. Sequencing analysis indicated that neither a putative Shine-Dalgarno sequence nor a signal sequence was present in the 4.2-kbp PstI-BamHI region. In a separate experiment with pWE15 and E. coli NM554 (Gigapack II Plus Packaging Extract kit, Stratagene), a fragment containing the 6.2-kbp PstI-PstI region with the same restriction sites was found to have been inserted in the BamHI site of the cosmid, which was designated pWEK155. The sequence of pWEK155 included an additional 735-bp fragment in the flanking region upstream of the 5’-terminal PstI site of the first insert (Fig. 1).

The 2.8-kbp fragment between the EcoRV sites in the 735-bp flanking region and in the 6.2-kbp PstI-PstI region (probe 1, see Fig. 2) was found to hybridize with a 2.8-kbp EcoRV fragment of the genomic DNA from Bacillus sp. KSM-1378. In addition, the deduced amino acid sequence of the first 0.5-kbp PstI-BamHI region of fragment A, TVPLALVSGEVLSDKL, was identical to the amino-terminal sequence of a 102-kDa pullu-
FIG. 3. Complete nucleotide sequence and the deduced amino acid sequence of the apuA gene. The DNA sequence was determined completely for both strands. The deduced gene product is indicated in the one-letter code under the DNA sequence. Numbers on the right side of the nucleotide and amino acid sequences denote nucleotide and amino acid positions, respectively. The sequence similar to -35 and -10 consensus promoters of B. subtilis (sigA) is underlined. Shine-Dalgarno sequence also underlined is a putative ribosome-binding site. The apuA ORF extends from Met1 to Leu1938. The initial underlined deduced amino acid sequence (Glu33 through Pro46) refers to the amino-terminal end of the extracellular, mature APase secreted by Bacillus sp. KSM-1378. The second underlined amino acid sequence (Asp781 through Thr797) and the third underlined amino acid sequence (Thr1046 through Leu1061) refer to the internal carboxyl-terminal sequence of a 114-kDa amylose-hydrolyzing polypeptide and to the amino-terminal sequence of a 102-kDa pullulan-hydrolyzing polypeptide, respectively, which were generated by limited digestion of the intact APase with papain. Two sets of four highly conserved regions, designated I, II, III, and IV, in the ORF are enclosed in boxes. Two repeats of 12 amino acid residues found in the ORF are donated by single and double underlinings, respectively. Inverted repeats (palindromic sequence) downstream of the stop codon TAA of the ORF are donated by convergent arrows.
lan-hydrolyzing polypeptide generated by limited proteolysis of the intact APase with papain (see Fig. 3). It appeared, therefore, that the 735-bp fragment was part of an unidentified gene, located upstream of the gene for pullulanase but not necessarily required for the expression of the active pullulanase. The pBP101-encoded pullulanase was expressed in E. coli cells and was purified almost to homogeneity (see "Experimental Procedures"). The purified enzyme had a molecular mass of 105 or 103 kDa, as judged by SDS-PAGE or by HPLC on a column of TSK gel G3000 SWXL (Ara et al., 1995b), respectively. The specific activity for pullulan of the enzyme was 217 units/mg protein. The optimum pH and temperature for the reaction were around pH 9.5 and 50°C, respectively, and these values were almost identical to those for the pullulanase activity of APase secreted by Bacillus sp. KSM-1378. The recombinant enzyme efficiently hydrolyzed pullulan to generate maltooltriose as the major end product, but it did not hydrolyze amylose at all, as judged by thin-layer chromatography (data not shown).
not shown). It appeared, therefore, that the gene for the alkaline amylase domain of APase might possibly be located upstream of the gene for the alkaline pullulanase in pBP101.

**Genome Walking by Inverse PCR**—The sequencing strategy and restriction map of the entire apuA gene, which was used to determine the sequence, are summarized in Fig. 2. Sequences of primers (A through F) used in the PCR experiments are given under “Experimental Procedures.” First, to assist in the choice of restriction enzymes for inverse PCR (Triglia et al., 1988; Ochman et al., 1988), we performed Southern hybridization analysis. The initial 730-bp PstI-XbaI region (probe 2) of the 4.2-kbp PstI-BamHI region was labeled for use as the probe. The genomic DNA from Bacillus sp. KSM-1378 was first digested with XbaI, and the resultant digest was subjected to agarose gel electrophoresis, and then the DNA bands were transferred onto a nylon filter and allowed to hybridize with digoxigenin-labeled probe 2. A 1.5-kbp XbaI fragment hybridized with the probe, and the XbaI site was located 0.8 kilobases upstream of the 5′-terminal PstI site. The Bacillus sp. KSM-1378 genomic DNA (0.9 μg) was digested with XbaI and ligated under conditions that favored the formation of monomeric circles (Collins and Weissman, 1984) by T4 DNA ligase. The first inverse PCR was conducted to amplify a 0.8-kbp DNA fragment (fragment B), using the self-circularized molecules as template and primers A and B. The primers had been synthesized on the basis of the results of sequencing of the 4.2-kbp PstI-BamHI fragment. The sequence of the amplified 0.8-kbp DNA fragment included a sequence that encoded the deduced amino acid sequence DRYSGQEQVAEGSE, which was identical to the sequence of the internal carboxyl-terminal region of a 114-kDa amylase-hydrolyzing polypeptide that was generated by limited hydrolysis of intact APase with papain (see Fig. 3). However, neither the regulatory nor the structural gene for intact APase was present in the 0.8-kbp fragment. Therefore, we performed Southern hybridization analysis of the genomic DNA to clone the flanking region of the XbaI site.

Southern hybridization analysis revealed that an EcoRI site was found 1.2 kilobases upstream of the 5′-terminal XbaI site. Therefore, inverse PCR experiments were conducted to amplify a 1.2-kbp DNA fragment (fragment C) using EcoRI self-circularized DNA molecules as template and suitably synthesized primers C and D, and then to amplify a 1.1-kbp DNA fragment (fragment D) using XbaI self-circularized DNA molecules as template and appropriate primers E and F, so that we could detect the sequence that encoded the regulatory and amino-terminal regions of intact APase. The resultant fragment D was found to contain a putative regulatory region and a sequence that corresponded to the amino acid sequence ETGDK-RKIFS YERP, which was identical to the amino-terminal sequence common to the intact APase from Bacillus sp. KSM-1378 and to a 114-kDa amylase-hydrolyzing polypeptide generated by limited digestion of the intact enzyme with papain (see Fig. 3).

**The Entire Nucleotide Sequence of apuA**—The nucleotide sequence of apuA, extending from the 5′-terminal XbaI site (nucleotide 1) to the 3′ terminus at nucleotide 6,142, was determined and is shown in Fig. 3. Starting from an ATG initiation codon at nucleotide 145, there is a very long ORF of 5,814 bp that terminates in a TAA stop codon at nucleotide 5,959. Upstream of this ORF, there is a putative ribosome-binding site (Shine-Dalgarno sequence) with the sequence 5′- AAAGGGG-3′, followed after 8 bases by a potential ATG initiation codon. The sequence would have a free energy (ΔG) of −11.9 kcal/mol (−49.8 kJ/mol), as calculated by the method of Tinoco et al. (1978), when bound to the 3′ end of the 16 S rRNA from B. subtilis. The sequence from nucleotides 35 to 65 resem-bled the consensus sequence of sigma A-type vegetative promoters (sigA or σ70) of B. subtilis (Moran et al., 1982; Haldenwang, 1995). It consisted of 5′-TTTAC-3′ as the potential −35 region and 5′-TTAAT-3′ as the potential −10 region, separated by 19 nucleotides. A long inverted repeat sequence was found downstream of the stop codon of the ORF (from nucleotides 5,963 to 6,013). The ΔG value of this sequence for a stem-loop structure was calculated to be −31.1 kcal/mol (−130.1 kJ/mol), which would be sufficient for termination of transcription.

**Amino Acid Sequence Analysis**—The ORF in the nucleotide sequence encoded 1,938 amino acid residues, as shown under the nucleotide sequence in Fig. 3. The amino acid sequence deduced from the apuA gene contains a short hydrophilic region from amino acids 1 to 9, followed by a hydrophobic region that extends from amino acids 10 to 32. The hydrophilic region is slightly basic because of the presence of two lysine and two arginine residues. The hydrophilic-hydrophobic sequence is similar to signal peptides of Bacillus (Murphy et al., 1984; Mezes and Lampen, 1985). A deduced sequence that was identical to the amino-terminal 14 amino acid residues of the APase secreted by Bacillus sp. KSM-1378 was found at amino acids 33–46. The residues Ala20–Ala21–Ala22 in the hydrophobic region might be the recognition site of a signal peptidase (Perlman and Halvorson, 1983; Simonen and Palva, 1993). If this putative signal peptide were cleaved on the carboxyl-terminal side of Ala23, the molecular mass of the extracellular mature APase (from Ghu23 to Leu938) would be 211,450 Da, a value close to the 210 kDa determined by SDS-PAGE of the APase that was purified from the culture broth of Bacillus sp. KSM-1378 (Ara et al., 1995b).

As can unequivocally be seen in Fig. 3, the entire amino acid sequence of the product of apuA gene of Bacillus sp. KSM-1378 consists of the amylase domain in the amino-terminal half and the pullulanase domain in the carboxyl-terminal half. A long sequence with 35 amino acid residues, NTLVRHILQY-RKDTDN/N/A/S/D/YEN/G/L/WG/WLC/N/WG/E/DVA/EA/S/PSE (D)/N/G/WPS/N, is repeated between the two catalytic domains, with one copy extending from amino acids 834 to 868 and another from amino acids 944 to 978. The amino acid residues in parentheses are those found in the second sequence. The deduced amino acid sequences were found to exhibit strong homology to two repeated sequences, 844 NNIRIH YKREDNY/KNYGAWLNDVAPSNWPGO953 and 855 NTVRHITREA VYDDFIWNGDVA SPSDWPGT990, that were found in the carboxyl-terminal region of an alkaline amylase strain of Micrococcus (Kimura and Horikoshi, 1990). It exhibited no significant similarity to other sequences in the GenBank/SWISS-PROT/NBRF data bases. In the carboxyl-terminal region of this APase, three repeats of PGD GDG DTNP were also found, without interruptions, at amino acids 1,821–1,832, 1,833–1,844, and 1,845–1,856, and eight repeats of PGXG were found between amino acids 1,857 and 1,896, where X represents any of a number of amino acid residues. These short repeated sequences contain many α-helix-breaking proline and glycine residues, but we have no idea at present why and how, in terms of structure, these repeated sequences are located in the carboxyl-terminal region of this enzyme. Similar sequences have been reported in the internal amino acid sequences in a metaloproteinase precursor from Bacillus polymyxa (YGDG DG DSTP; Takekawa et al., 1991), a glucoamylase from Clastoridium (PWGDQG GDDTNGG; Ohnishi et al., 1992), and a chitinase from Streptomyces (PG TGGSADLP; NBRF accession number S32039), but these sequences are not repeated. There are many reports of the carboxyl-terminal repeats of amino acid residues, as in the
the two chromatography steps. The partially purified preparation of enzyme had pH optima of 5.2 and 5.9, respectively. The relative activities of the enzyme were found to copurify through the two chromatography steps.

The deduced amino acid sequence contained two sets of four highly conserved regions, designated I, II, III, and IV, one set being located between amino acids 1-1,110 and the other set between amino acids 1,396 and 1,637 in the pullulanase domain. The amino acid sequences of these four conserved regions were compared with those of representative bacterial α-amylases and pullulanases, as shown in Fig. 4. The catalytic residues of the α-amylase domain (and of the pullulanase domain) of AαPase from Bacillus sp. KSM-1378 were tentatively identified to be Glu\(^{1493}\) (Glu\(^{1493}\) in conserved region III, Asp\(^{550}\) (Asp\(^{1581}\) in conserved region IV (Matsuda and Horikoshi, 1990). The amino acid sequence of the pullulanase domain exhibited 34.0 (amino acids 1,317–1,637) and 23.7% (amino acids 386–500) identity with those of pullulanase domains from Thermus thermophilus (Peters et al., 1983), Bacillus subtilis (Tanaka et al., 1984), and Arthrobacteris (Tanii et al., 1985). However, the repeated amino acid sequences in our enzyme also exhibited no similarity to these reported sequences.

The deduced amino acid sequence of the α-amylase domain (and of the pullulanase domain) of AαPase from Bacillus sp. KSM-1378 was determined, and it was entirely identical to the sequence of the α-amylase domain (and of the pullulanase domain) of pAP101 (13.7 kbp). The α-amylase domain was determined fairly well with the band visualized by activity staining on the side of the amino acid sequence denote amino acid positions of the aligned amino acid sequence. The four regions indicated by numbers on the side of the amino acid sequence denote amino acid positions of the aligned amino acid sequence. The four regions indicated by asterisks on the side of the amino acid sequence denote amino acid positions of the aligned amino acid sequence.

The conserved recombinant plasmid was designated pHYPUL (9.1 kbp). pHYPUL was then expressed in E. coli cells and formed halos around the colonies of transformants on red pullulan agar plates. After these two recombinant plasmids had been digested with appropriate restriction enzymes and partially sequenced, pUCAMY and pHYPUL were both digested with PstI and connected by T4 DNA ligase to construct the entire αpuA gene in a plasmid designated pAP101 (13.7 kbp). During this procedure, the ampicillin resistance of pHYPUL was lost because of the presence of a PstI site on the recombinant plasmid. The sequence of the constructed αpuA gene in pAP101 was determined, and it was entirely identical to the sequence shown in Fig. 3.

The recombinant plasmid pAP101 was introduced into B. subtilis ISW1214 cells, and one of the transformants obtained was grown at 30 °C for 60 h with shaking in LB broth supplemented with tetracycline (15 μg/ml). The product of the αpuA gene was expressed extracellularly at a level of 60 units/liter, in terms of the alkaline pullulanase activity. The product of αpuA in the culture supernatant was purified by chromatography on a column of DEAE-cellulose and then by HPLC on a column of TSK G3000 SWXL. The α-1,4 and α-1,6 hydrolytic activities were found to copurify through the two chromatography steps. The partially purified preparation of enzyme had pH optima of around pH 8–9 for the amylose activity and at pH 9.5 for the pullulanase activity, values close to the pH optima for the respective enzymatic activities of the APase purified from cultures of Bacillus sp. KSM-1378 (Ara et al., 1995b). The molecular mass of the purified enzyme was estimated to be approximately 200–210 kDa by SDS-PAGE, a value close to the 210 kDa determined for the native enzyme produced by Bacillus sp. KSM-1378 (Fig. 6A). The purified enzyme hydrolyzed soluble starch (from potato), amylepectin (from corn), amylase (degree of polymerization, 17), and pullulan (from Aureobasidium pullulans) at a relative rate of 100:58:31:46, a value similar to the relative rate (100:52:55:31) for the APase activity of Bacillus sp. KSM-1378. In addition, the band of the purified enzyme protein, detected by staining after nondenaturing PAGE, coincided fairly well with the band visualized by activity staining on a starch-azure agar sheet or on a red pullulan agar sheet, indicating that the single αpuA protein encoded by pAP101 had
both α-amylase and pullulanase activities (Fig. 6B). There were additional protein bands, showing amylase activity and moving faster than the main APase band, which might be generated by proteolytic enzyme(s) contaminated in the enzyme preparations.

Electron Microscopic Observations of APase—The 210-kDa APase from Bacillus sp. KSM-1378 and its 114-kDa amylase- and 102-kDa pullulan-hydrolyzing fragments, generated by partial digestion with papain, were visualized after rotary shadowing by the method of Tyler and Branton (1980). Samples dried from a solution in glycerol were rotary shadowed with platinum/carbon at a low angle, and the resultant specimens, after coating with a supporting film of carbon, were observed by transmission electron microscopy, as shown in Fig. 7. The intact APase molecules were seen as “castanet-like” or “bent dumbbell-like” shapes with a diameter of approximately 25 nm (Fig. 7, A and B). Two globular (ovoid) heads of different sizes were clearly seen, and they were joined by a thin, short linker region. When the intact molecule was cleaved by limited proteolysis with papain, the amylase- and pullulan-hydrolyzing polypeptides were observed as a mixture of globular molecules of different sizes under the electron microscope (Fig. 7C). The joint region might correspond to the linker sequence between

![Fig. 5. Schematic representation of construction of the apuA gene in the plasmid, designated pAP101. The arrows in plasmids indicate the positions and the orientations of the sequences that encode resistance to antibiotics, tetracycline (Tet) and ampicillin (Amp). Fragment E encoding the alkaline amylase domain (hatched bar) and part of the 5'-terminal alkaline pullulanase domain (thick bar) was amplified by PCR using primers G and H and the genomic DNA from Bacillus sp. KSM-1378 as template (also see Fig. 2), and was inserted in the Smal site of pUC18 (pUCAMY). pUCAMY and pHYPUL that had been prepared from fragment A and shuttle vector pHY300PLK were both digested with PstI and connected by T4 ligase to construct the recombinant plasmid pAP101. Inside the circle of pAP101, the constructed apuA gene is stippled.](image)

![Fig. 6. Electrophoretic profiles of the product of apuA gene expressed in B. subtilis cells. Detailed methods for electrophoretic analyses are described under “Experimental Procedures.” A, SDS-PAGE. Lane 1, a purified preparation of APase produced by Bacillus sp. KSM-1378; lane 2, a partially purified preparation of apuA product encoded by pAP101; lane M, the molecular mass markers (calibration in kDa). B, nondenaturing PAGE of the native APase (lanes 1) and the apuA product (lanes 2). Left, staining for protein. Middle, staining for pullulanase. Right, staining for amylase. Migration was top (anode) to bottom (cathode), and bromphenol blue was used as a dye marker. The arrow indicates the position of the protein band of the native APase from Bacillus sp. KSM-1378.](image)

![Fig. 7. Electron microscopic visualization of APase molecules by rotary shadowing with platinum/carbon. Plates A and B represent the native APase molecules that was purified to homogeneity from the culture broth of Bacillus sp. KSM-1378. Plate C represents a mixture of the 114-kDa amylase-hydrolyzing and the 102-kDa pullulan-hydrolyzing polypeptides generated by limited proteolysis of the purified native APase with papain. The proteolytic conditions and the purification method of the two fragments are described under “Experimental Procedures.” The scale bar indicates 50 (B) and 100 nm (A and C).](image)
the amylase and the pullulanase domains, which were deduced from the nucleotide sequence (see Fig. 3). The bent structure, with two heads, of the intact APase might be related to the presence of this linker sequence because many β-turn-forming proline residues are concentrated in the linker region.

The alkaline APase from Bacillus sp. KSM-1378 has unique features, having α-1,4- and α-1,6-activity at different active sites and an unusual but noncontradictory molecular structure. The production of this enzyme is induced by pullulan but by neither amylose nor soluble starch. Because the pullulanase domain lies in the carboxyl-terminal half, we postulate that this APase might be the product of gene fusion caused by recombination of a gene for a pullulanase with a gene for an amylase. It is possible that a foreign gene for an amylase gene was introduced by chance, in frame, between the regulatory region and the structural gene of the original pullulanase in Bacillus sp. KSM-1378. Similar events might have been responsible for formation of genes for other bifunctional enzymes, such as the enzyme with endo- and exoglucanase activities from Caldacellum saccharolyticum (Saul et al., 1990) and the enzyme with xylanase and β(1,3-1,4)-glucanase activities from Ruminococcus flavefaciens (Flixt et al., 1993).

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Amino Acid Sequence and Molecular Structure of an Alkaline Amylopullulanase from \textit{Bacillus} That Hydrolyzes $\alpha$-1,4 and $\alpha$-1,6 Linkages in Polysaccharides at Different Active Sites

Yuji Hatada, Kazuaki Igarashi, Katsuya Ozaki, Katsutoshi Ara, Jun Hitomi, Tohru Kobayashi, Shuji Kawai, Tomoyoshi Watabe and Susumu Ito

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