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Molecular Cloning, Nucleotide Sequencing, and Expression of the *Bacillus subtilis* (*natto*) IAM1212 α-Amylase Gene, Which Encodes an α-Amylase Structurally Similar to but Enzymatically Distinct from That of *B. subtilis* 2633

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An α-amylase gene of *Bacillus subtilis* (*natto*) IAM1212 was cloned in a λEMBL3 bacteriophage vector, and the nucleotide sequence was determined. An open reading frame encoding the α-amylase (AMY1212) consists of 1,431 base pairs and contains 477 amino acid residues, which is the same in size as the α-amylase (AMY2633) of *B. subtilis* 2633, an α-amylase-hyperproducing strain, and smaller than that of *B. subtilis* 168, Marburg strain. The amino acid sequence of AMY1212 is different from that of AMY2633 at five residues. Enzymatic properties of these two α-amylases were examined by introducing the cloned genes into an α-amylase-deficient strain, *B. subtilis* M15. It was revealed that products of soluble starch hydrolyzed by AMY1212 are maltose and maltotriose, while those of AMY2633 are glucose and maltose. From the detailed analyses with oligosaccharides as substrates, it was concluded that the difference in hydrolysis products of the two similar α-amylases should be ascribed to the different activity hydrolyzing low-molecular-weight substrates, especially maltotriose; AMY1212 slowly hydrolyzes maltotetraose and cannot hydrolyze maltotriose, while AMY2633 efficiently hydrolyzes maltotetraose and maltotriose. Further analyses with chimeric α-amylase molecules constructed from the cloned genes revealed that only one amino acid substitution is responsible for the differences in hydrolysis products.

α-Amylase is an end-type enzyme that hydrolyzes α-1,4-glucosidic linkages from starch and various types of oligosaccharide with α-anomeric configuration. Two types, saccharifying and liquefying types, of bacterial α-amylases are known (4, 17, 26). α-Amylases of *Bacillus subtilis* species including *B. subtilis* 168 (Marburg strain), *B. subtilis* 2633 (hyperproducing strain), and *B. subtilis* (*natto*) IAM1212 (*amyEn*) belong to the saccharifying type, and those of *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus* belong to the liquefying type. These two types are distantly related to each other in terms of the primary structures and also related to other various amylases including those of eucaryotic origin (11, 21, 23).

Several types of *B. subtilis* α-amylases have been cloned and sequenced (1, 2, 15, 22, 29, 31–33). Comparative studies on the primary structures revealed that two subtypes exist for *B. subtilis* α-amylases showing different molecular weights. α-Amylase genes of *B. subtilis* 168 (32) and NA64 (31) encode 660 amino acid residues and produce secreted enzymes with molecular weights of about 55,000, although the 3′-terminal part has not been cloned for the *B. subtilis* NA64 gene. These α-amylases are known to produce mainly glucose and maltose as hydrolysis products of soluble starch (14). On the other hand, *B. subtilis* (*natto*) IAM1212 secretes a smaller molecular species of the enzyme (molecular weight, 34,000 to 45,000) (14). It was reported that the α-amylase of *B. subtilis* (*natto*) IAM1212 (AMY1212) produced maltose and maltotriose as hydrolysis products from soluble starch and failed to hydrolyze maltotriose, which can be hydrolyzed into glucose and maltose by the α-amylases of *B. subtilis* 6160 and NA64 (14, 30). Yamane et al. (29) determined the sequence of the α-amylase gene from *B. subtilis* N7, a derivative of *B. subtilis* 6160 obtained by DNA-mediated transformation with *B. subtilis* (*natto*) IAM1212 DNA, and found that the α-amylase gene of *B. subtilis* N7 encodes 477 amino acid residues, which is significantly smaller than the α-amylases of *B. subtilis* 168 and NA64. This difference is due to a deletion of 32 base pairs in the C-terminal region of the open reading frame, which causes a frameshift to generate a termination codon near the deletion point (29). From the observation that the α-amylase of *B. subtilis* N7 failed to hydrolyze maltotriose, Yamane et al. (29) proposed that the absence of the C-terminal part causes the inability to hydrolyze maltotriose. However, we recently cloned the α-amylase gene of *B. subtilis* 2633 (2), an α-amylase-extrahyperproducing derivative obtained by a series of DNA-mediated transformations and mutagenesis from *B. subtilis* 6160, and showed that the gene encodes 477 amino acid residues (1) but that the hydrolysis products from the α-amylase are mainly glucose and maltose (2). This means that the ability to hydrolyze maltotriose might be ascribed to another structural difference(s) than the C-terminal part of the enzyme.

In this report, we describe molecular cloning and nucleotide sequencing analyses of the α-amylase gene of *B. subtilis* (*natto*) IAM1212, a prototrophic strain which contains the *amyR2* promoter (29) and *amyEn* structural gene encoding a low-molecular-weight α-amylase not hydrolyzing maltotriose. Expression studies with cloned α-amylase genes of *B. subtilis* (*natto*) IAM1212 and *B. subtilis* 2633 and their chimeras revealed that the difference in hydrolysis products

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TABLE 1. Strains and plasmids used in this study

Strain, plasmid, or phage (reference)	Description
Bacillus subtilis M15 (28)	*amyE purB6 metB5*
Bacillus subtilis (*natto*) IAM1212 (16)	Prototroph, *amyR2 amyEn*
Escherichia coli MM294	F^- *supE44 endA1 thi-1 hsdR4*
Escherichia coli LE392	*hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*
Plasmid vectors	
pHY300PLK (10)	Ap^r TC^r
pUC118/119 (24)	Ap^r
Phage vector λEMBL3 (3)	

of the two α-amylases should be ascribed to only one amino acid substitution.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* MM294 was used as a host strain for manipulating recombinant plasmids in *E. coli*. *E. coli* LE392 was used as an indicator strain for propagating λEMBL3 phage.

Kits. Packaging mixture for constructing a gene library of *B. subtilis* (*natto*) IAM1212 was purchased from Stratagene Inc. (Giga Pack Gold). The DNA labeling kit was a product of Takara Shuzo Co.

Media. *E. coli*, *B. subtilis*, and *B. subtilis* (*natto*) were grown in L broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter) or BY medium (5 g of nutrient broth, 10 g of polypeptone, 2 g of NaCl, 2 g of yeast extract per liter) at 37°C. For infection with λEMBL3 phage, *E. coli* LE392 was grown overnight in NZCYM medium (10 g of NZ amine, 5 g of NaCl, 1 g of Casamino Acids [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract, 2 g of $MgSO_4 \cdot 7H_2O$). In several cases, appropriate antibiotics were added at the following concentrations: ampicillin (sodium salt), 30 μg/ml; tetracycline, 10 μg/ml.

α-Amylase activity. α-Amylase activity was quantitatively determined by the modified blue value method by Fuwa (5) as described previously. A plate assay for α-amylase activity was performed by the KI-I_2 detection method as previously described (2).

Construction of *B. subtilis* (*natto*) IAM1212 gene library. Chromosomal DNA of *B. subtilis* (*natto*) IAM1212 was extracted as described previously (19), partially digested with *Sau*3AI, and ligated to λEMBL3 phage DNA which had been double digested with *Bam*HI and *Eco*RI and subsequently treated with alkaline phosphatase. After packaging, recombinant phage were plated with indicator *E. coli* LE392 cells suspended in 0.5 culture volume of 10 mM $MgSO_4$.

Phage propagation, DNA extraction, and plasmid isolation. Recombinant phage were propagated on a lawn of *E. coli* LE392 cells, and phage DNA was extracted as described previously (13). Plasmid DNA was purified by an alkali-sodium dodecyl sulfate method as described previously (2, 13).

Isolation of DNA fragment encoding α-amylase. Recombinant phage plaques were transferred onto nylon filters (Hybond; Amersham Corp., Arlington Heights, Ill.) and fixed with uv light. The filters were prehybridized at 65°C for 4 h in a buffer containing 50 mM Tris hydrochloride (pH 8.0), 1 M NaCl, 10 mM EDTA, 0.2% Ficoll 400, 0.1% sodium dodecyl sulfate, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 200 μg of heat-denatured salmon sperm DNA per ml. After prehybridization, the filters were hybridized with a chemically labeled DNA fragment encoding the α-amylase gene of *B. subtilis* 2633 prepared with a Chemiprobe kit (Takara Shuzo Co.) (18) at 65°C overnight in the same solution as used for prehybridization. The filters were finally washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C and blocked in skim milk. The filters were colored with anti-modified DNA mouse immunoglobulin G, anti-mouse immunoglobulin G antibody conjugated with alkaline phosphatase (second antibody), and *p*-Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

DNA sequencing. Appropriate DNA fragments were inserted into the polylinker site of a plasmid vector, pUC18 or pUC118. Nucleotide sequencing was done by the modified chain termination method as described previously (8). Both strands of relevant DNA regions were completely determined.

Transformation of *B. subtilis* and *E. coli*. Plasmid transformation of *B. subtilis* M15 was performed by the competent transformation method (7). Transformation of *E. coli* MM294 with plasmids was done as described by Hanahan (6).

Preparation of crude α-amylase. Culture fluid after 72 h of incubation in BY medium was centrifuged to remove cells. The supernatant containing α-amylase was collected and concentrated by ammonium sulfate precipitation or with an Amicon concentrator in some cases. The α-amylase activity of the crude solution was adjusted to 100 U/ml, and it was used as a crude enzyme preparation.

Thin-layer chromatography. Crude enzyme preparations diluted from culture supernatant were incubated with 5% (wt/vol) of various substrates at 37°C in a buffer containing 50 mM sodium PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.2) and 3 mM $CaCl_2$. Hydrolysis products were analyzed by thin-layer chromatography on Kieselgel 60 with a solvent system of 1-butanol–1-propanol–water (3:5:4, vol/vol/vol) in a chamber at 65°C. The products were detected by coloring with 29% (vol/vol) H_2SO_2 in methanol.

Construction of full-length α-amylase gene of *B. subtilis* (*natto*) IAM1212 in pUC118 and pHY300PLK. A DNA fragment corresponding to the 5′-terminal three-fourths of the α-amylase gene of *B. subtilis* (*natto*) IAM1212 was subcloned into pUC118 as follows. λNAT12 DNA containing the α-amylase gene of *B. subtilis* (*natto*) IAM1212 was digested with *Sma*I and *Hin*dIII and ligated with T4 DNA ligase to pUC118 which had been digested with same enzymes; the resultant plasmid pNAT5-1 was obtained. The 3′ part of the gene was subcloned as follows. λNAT12 DNA was digested completely with *Sal*I and partially with *Sau*3AI and ligated to pUC118 digested with *Sal*I and *Bam*HI, and the resultant plasmid pNAT3-1 was obtained. Next, to obtain a plasmid encoding all of the α-amylase gene, the *Sal*I-*Sac*I fragment of pNAT3-1 was excised and ligated to pNAT5-1 which had been digested with the same enzymes and treated with bacterial alkaline phosphatase, and the resultant plasmid pNAT118 was obtained. For expression of the gene in *B. subtilis*, the insert of pNAT118 was excised and inserted into an *E. coli-B. subtilis* shuttle vector, pHY300PLK, as follows. pNAT118 was digested with *Sac*I and blunt ended with Klenow fragment and subsequently digested with *Hin*dIII to excise the insert, and then the insert

was ligated to pHY300PLK digested with *Hin*dIII and *Sma*I. The resultant plasmid was termed pNAT-PLK.

Construction of plasmids encoding chimeric α-amylases of *B. subtilis* (*natto*) IAM1212 and *B. subtilis* 2633. The chimera 1 plasmid encoding the N-terminal half of AMY2633 and the C-terminal half of AMY1212 was constructed as follows (see Fig. 4). pAM26 encoding AMY2633 was digested with *Hin*dIII and filled in and subsequently digested with *Sal*I to remove the C-terminal half. The DNA fragment encoding the C-terminal half of AMY1212 was prepared from pNAT118 which had been digested with *Sac*I, blunt ended, and digested with *Sal*I. Then these two DNAs were ligated, and the resultant plasmid encoding a chimeric α-amylase (chimera 1) was obtained.

The chimera 2 plasmid encoding the N-terminal half of AMY1212 and the C-terminal half of AMY2633 was constructed as follows. The *Hin*dIII-*Sal*I fragment of pNAT-PLK encoding the N-terminal half of AMY1212 was inserted into pHY300PLK digested with *Hin*dIII and *Sal*I. The resultant plasmid was digested with *Sal*I and ligated with the *Sal*I fragment of pAM26 encoding the C-terminal half of AMY2633.

The chimera 3 plasmid was constructed as follows. An *Eco*RI-*Sma*I fragment of pAM26 encoding the C-terminal three-fourths of AMY2633 was inserted into pHY300PLK. The resultant plasmid was digested with *Eco*RI and ligated with the *Eco*RI fragment of pNAT-PLK encoding the N-terminal one-fourth of AMY1212.

The chimera 4 plasmid was constructed as follows. pNAT-PLK was digested with *Kpn*I and blunt ended and then digested with *Hin*dIII to excise the insert. The excised insert was ligated to pHY300PLK which had been digested with *Eco*RI and filled in and subsequently digested with *Hin*dIII. The resultant plasmid was digested with *Eco*RI and *Sal*I to remove the internal part of AMY1212 and ligated with the *Eco*RI-*Sal*I fragment of pAM26 encoding the internal part of AMY2633.

In all these chimeras, no amino acid was altered, inserted, or changed.

RESULTS

Cloning of α-amylase gene of *B. subtilis* (*natto*) IAM1212. From a library of about 16,000 plaques of recombinant EMBL3 phage, one plaque termed λNAT12 hybridized with the DNA fragment encoding the α-amylase of *B. subtilis* 2633. The frequency of the appearance of the positive clone was extremely lower than the expected value calculated from the genome size of the bacterium, and the positive plaque obtained was very small. This may be because chromosomal regions adjacent to the α-amylase gene are toxic in *E. coli*, as previously described (32).

The phage λNAT12 contained a 12-kilobase-pair (kbp) inserted DNA fragment which consists of two *Sal*I fragments of 8 and 4 kbp. Since these two *Sal*I fragments still contained toxic regions, a *Sal*I-*Hin*dIII fragment (1.7 kbp) and a *Sal*I-*Sau*3AI fragment (0.7 kbp) corresponding to the 5′ and 3′ regions of the α-amylase gene, respectively, were finally subcloned into pUC vectors.

To ascertain that the cloned DNA fragments encode α-amylase, we constructed a plasmid (pNAT118) containing the total region of the gene by ligating the two fragments (1.7 and 0.7 kbp as described above) into pUC118 and examined its α-amylase-producing activity. All the colonies of *E. coli* carrying pNAT118 tested by the KI-I_2 method showed haloes in starch-containing plates. This clearly indicated that the α-amylase gene of *B. subtilis* (*natto*) IAM1212 was cloned and contained in the DNA fragment of about 2.5 kbp.

Nucleotide sequence of α-amylase gene of *B. subtilis* (*natto*) IAM1212. The complete nucleotide sequence of the DNA fragment encompassing the α-amylase gene was determined with both strands and is shown in Fig. 1. It contains a single open reading frame of 1,431 base pairs encoding 477 amino acid residues, which follows the *amyR2* promoter sequence and Shine-Dalgarno sequence. The nucleotide sequence of the *amyR2* region of *B. subtilis* (*natto*) IAM1212 perfectly matches that of *B. subtilis* WLN-15 (16) and contains promoter sequences and a stem-and-loop structure which has no direct effect on α-amylase transcriptional regulation but might function as a terminator of upstream genes (25). However, the sequence of *B. subtilis* (*natto*) IAM1212 differs from that of *B. subtilis* NA64, whose sequence was first determined as *amyR2* (31) at four points in the relevant region including nucleotide residue 201 (Fig. 1), which is important for glucose repression (23).

In the C-terminal region of the open reading frame, a deletion of 32 base pairs was observed compared with the sequences of *B. subtilis* 168 (32) and NA64 (31) in accordance with the cases of *B. subtilis* N7 (29) and 2633 (1). As a result of the deletion, a termination codon appeared at nucleotide residues 1747 to 1749, and the deduced amino acid sequence consisted of 477 residues, which is again the same in size as those of *B. subtilis* N7 (29) and 2633 (1).

Amino acid sequence of α-amylase of *B. subtilis* (*natto*) IAM1212 (AMY1212). The amino acid sequence of AMY1212 (α-amylase of *B. subtilis* [*natto*] IAM1212) was compared with that of AMY2633 (α-amylase of *B. subtilis* 2633) (1), both of which consist of the same number of amino acid residues (477 residues). Although the α-amylase of *B. subtilis* N7 also consists of 477 residues, precise comparison of the amino acid sequence of the *B. subtilis* N7 α-amylase with the others is difficult because a discrepancy between the amino acid sequence and the nucleotide sequence is observed at four points for the *B. subtilis* N7 α-amylase (29; NBRF Protein Database, release 20). The total homology between AMY1212 and AMY2633 is 99%, and only five residues are different (Fig. 2). A high degree of homology is also observed for comparison of the sequence of AMY1212 with those of α-amylases of *B. subtilis* 168 and N7 (data not shown), although the C-terminal five residues of AMY1212 or AMY2633 are replaced by longer sequences in the α-amylases of *B. subtilis* 168 (32) and NA64 (31).

Expression of cloned α-amylase genes in α-amylase-deficient *B. subtilis* strain. To clarify the enzymatic properties of α-amylases, the cloned genes of *B. subtilis* (*natto*) IAM1212 and *B. subtilis* 2633 were inserted into a *B. subtilis-E. coli* shuttle vector, pHY300PLK, and introduced into *B. subtilis* M15, an α-amylase-deficient strain, by plasmid transformation. All tetracycline-resistant transformants carrying pNAT-PLK or pAM26 (1, 2) formed haloes around the colonies on starch-containing plates by the KI-I_2 detection method. The amount of α-amylase secreted after 72 h of culture in BY medium was 294 U/ml for *B. subtilis* M15 carrying pNAT-PLK, which was about 30 times the value for *B. subtilis* (*natto*) IAM1212 (about 10 U/ml) and roughly corresponded to the expected value calculated from the copy number of plasmid pHY300PLK (about 50 copies per cell).

On the other hand, *B. subtilis* M15 carrying pAM26 produced 12,000 U of α-amylase per ml after 48 h of incubation. This value was not significantly larger than that of *B. subtilis* 2633, probably because α-amylase-producing activity was saturated and little gene dosage effect was

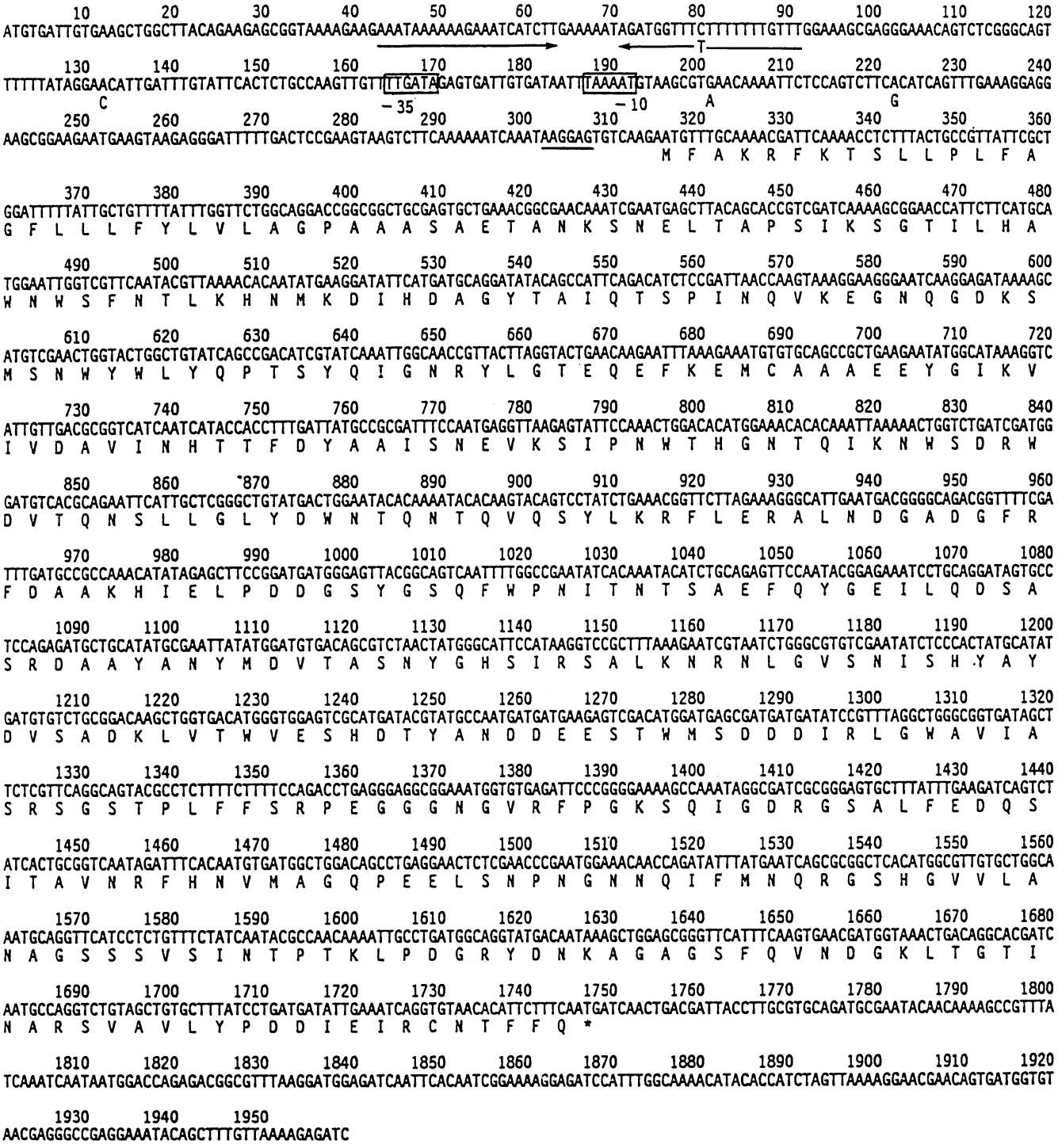

FIG. 1. Nucleotide and deduced amino acid sequences of DNA fragment encoding *B. subtilis* (*natto*) IAM1212 α-amylase and its flanking regions. The deduced amino acid sequence is shown below the nucleotide sequence. The possible −35 and −10 sequences in the promoter region and the possible ribosome-binding site (Shine-Dalgarno sequence) are boxed and underlined, respectively. The palindromic sequence of the *amyR2* promoter (29) is shown by arrows below the sequence. Four different residues of the *amyR2* region (nucleotide residues 1 to 300) of *B. subtilis* NA64 (31) are noted below the sequence of the *B. subtilis* (*natto*) IAM1212 α-amylase gene.

observed (2). However, the value was 40 times that for pNAT-PLK, indicating that the α-amylase gene of *B. subtilis* 2633 with the *amyR3* promoter has hyperproducing activity in itself, as described previously (2).

Substrate specificity of α-amylases of *B. subtilis* (*natto*) IAM1212 and *B. subtilis* 2633. As described in Fig. 2, the deduced amino acid sequences of the α-amylases of *B. subtilis* (*natto*) IAM1212 and *B. subtilis* 2633 are highly homologous and the residue numbers are identical. However, previous studies (2, 14) indicated that hydrolysis

```
          1                                                                                                  100
AMY1212   MFAKRFKTSLLPLFAGFLLLFYLVLAGPAAASAETANKSNELTAPSIKSGTILHAWNWSFNTLKHNMKDIHDAGYTAIQTSPINQVKEGNQGDKSMSNWY
AMY2633   ----------------------------------------------------------------------------------------------------

        101                                                                                                  200
          WLYQPTSYQIGNRYLGTEQEFKEMCAAAEEYGIKVIVDAVINHTTFDYAAISNEVKSIPNWTHGNTQIKNWSDRWDVTQNSLLGLYDWNTQNTQVQSYLK
          ----------------------------------------------S-----------------------------------------------------

        201                                                                                                  300
          RFLERALNDGADGFRFDAAKHIELPDDGSYGSQFWPNITNTSAEFQYGEILQDSASRDAAYANYMDVTASNYGHSIRSALKNRNLGVSNISHYAYDVSAD
          ----------------------------------------------------------------------------------------------S-----

        301                                                                                                  400
          KLVTWVESHDTYANDDEESTWMSDDDIRLGWAVIASRSGSTPLFFSRPEGGGNGVRFPGKSQIGDRGSALFEDQSITAVNRFHNVMAGQPEELSNPNGNN
          ----------------------------------------------------------------------A--------------H----------

        401                                                                    477
          QIFMNQRGSHGVVLANAGSSSVSINTPTKLPDGRYDNKAGAGSFQVNDGKLTGTINARSVAVLYPDDIEIRCNTFFQ
          -------------------------A----------------------------------------------------
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FIG. 2. Comparison of amino acid sequences of α-amylases of *B. subtilis* (*natto*) IAM1212 (AMY1212) and *B. subtilis* 2633 (AMY2633) (1). Dashes in the sequence of AMY2633 denote the same residues as those of AMY1212, and only different residues are shown.

products from soluble starch are different; hydrolysis products formed by AMY1212 are maltose and maltotriose, while those formed by AMY2633 are glucose and maltose. To certify that the difference is true for cloned genes when expressed in *B. subtilis* M15, we prepared crude enzyme preparations from culture fluids of *B. subtilis* M15 carrying pNAT-PLK or pAM26. Enzyme solutions diluted to 100 U/ml contained nearly the same amounts of α-amylase showing the same electrophoretic mobilities when these preparations were subjected to Western blot (immunoblot) analysis with anti-α-amylase antibody (data not shown). This indicates that the specific activities of the two enzymes are almost the same as described previously (14) and that the molecular weights of secreted enzymes are also the same.

Using these enzymes, hydrolysis products from soluble starch were examined. Major hydrolysis products produced by AMY1212 were maltose (G2) and maltotriose (G3), whereas those produced by AMY2633 were glucose (G1) and maltose (G2) under the same analytical conditions (Fig. 3). Thus, hydrolysis products made by the two α-amylases are clearly different, although the primary structures are very similar (Fig. 2).

To investigate the difference in details, we next analyzed hydrolysis products formed by AMY1212 and AMY2633 with soluble starch and various lengths of oligosaccharides as substrates (Table 2).

When soluble starch was used as a substrate, hydrolysis products made by AMY1212 were G2 and G3 (Table 2), and no glucose (G1) was produced even after longer incubation periods when an excess amount of the enzyme was used (data not shown). On the other hand, AMY2633 hydrolyzed soluble starch to produce G1 and G2. When G5 (maltopentaose) was used, profiles similar to those for soluble starch were observed for both AMY1212 and AMY2633 (Table 2). Major hydrolysis products were G2 and G3 for AMY1212 and G1 and G2 for AMY2633. When G4 (maltotetraose) was used, AMY1212 poorly hydrolyzed, and hydrolysis products after longer incubation periods (16 to 20 h) were G2 and G3. On the other hand, AMY2633 efficiently hydrolyzed G4, and major hydrolysis products were G1 and G2 (Table 2). And, when G3 (maltotriose) was used, hydrolyzing activities were completely different between AMY1212 and AMY2633 (Table 2). AMY1212 hardly hydrolyzed G3 even after 24 h of incubation, and only a small amount of G2 was observed, which should be derived from G6 produced from 2 mol of G3 by reverse reaction. On the contrary, AMY2633 efficiently hydrolyzed G3, although the rate was slower than rates when high-molecular-weight substrates were used.

Substrate specificity of chimeric α-amylases from AMY1212 and AMY2633. As described above, AMY1212 and AMY2633 showed distinct hydrolysis products, although the primary structures are almost the same except for five residues and the molecular weights are essentially the same. To examine the key amino acid residue(s) responsible for

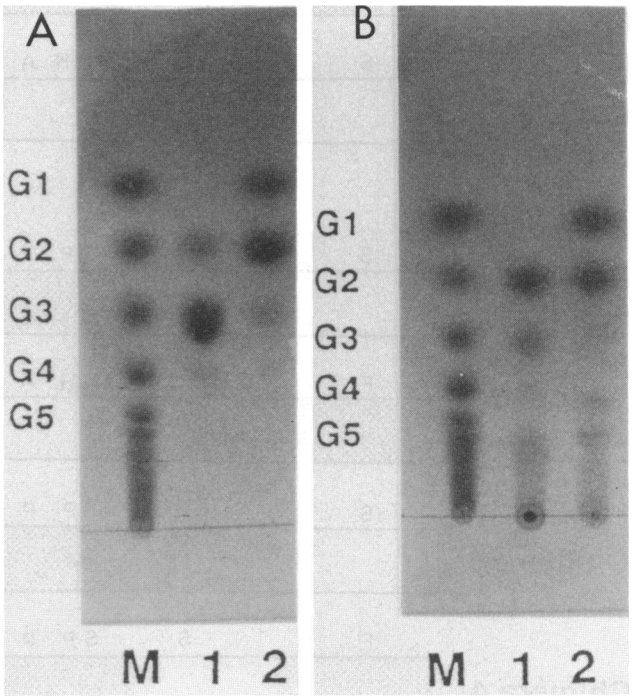

FIG. 3. Comparison of hydrolysis products produced from maltotriose (A) and soluble starch (B) by AMY1212 (lanes 1) and AMY2633 (lanes 2). Enzyme concentrations are 100 U/ml for each reaction. Incubation time was 24 h. Hydrolysis products were analyzed by thin-layer chromatography. Positions of oligosaccharides shown by the standard oligosaccharide mixture (lanes M) are G1 (glucose), G2 (maltose), G3 (maltotriose), G4 (maltotetraose), and G5 (maltopentaose).

TABLE 2. Hydrolysis products produced by AMY1212, AMY2633, and their chimeras from various substrates

α-Amylase	Hydrolysis products with the following substrate:			
	Starch	G5	G4	G3
AMY1212	G2, G3	G2, G3	G2, G3[a]	Not[b]
AMY2633	G1, G2	G1, G2	G1, G2	G1, G2
Chimera 1	G1, G2	ND[c]	ND	G1, G2
Chimera 2	G2, G3	ND	ND	Not
Chimera 3	G2, G3	ND	ND	Not
Chimera 4	G1, G2	ND	ND	G1, G2

[a] Very poorly hydrolyzed.
[b] Not, Not hydrolyzed.
[c] ND, Not determined (not analyzed).

different hydrolyzing activities, we constructed two chimeric α-amylases (chimeras 1 and 2, Fig. 4). Chimera 1 contains the N-terminal part of AMY2633 and the C-terminal part of AMY1212, and chimera 2 is vice versa. Recombinant plasmids encoding these two chimeras were introduced into *B. subtilis* M15, and crude enzyme preparations were obtained. Hydrolysis products of soluble starch and G3 were analyzed in the same way as described above, and the result is shown in Table 2. It is clear that chimera 1 could hydrolyze G3 and produced G1 from soluble starch. On the other hand, chimera 2 could neither hydrolyze G3 nor produce G1. Thus, one or both of two different residues located in the N-terminal half are responsible for the ability or inability to hydrolyze G3.

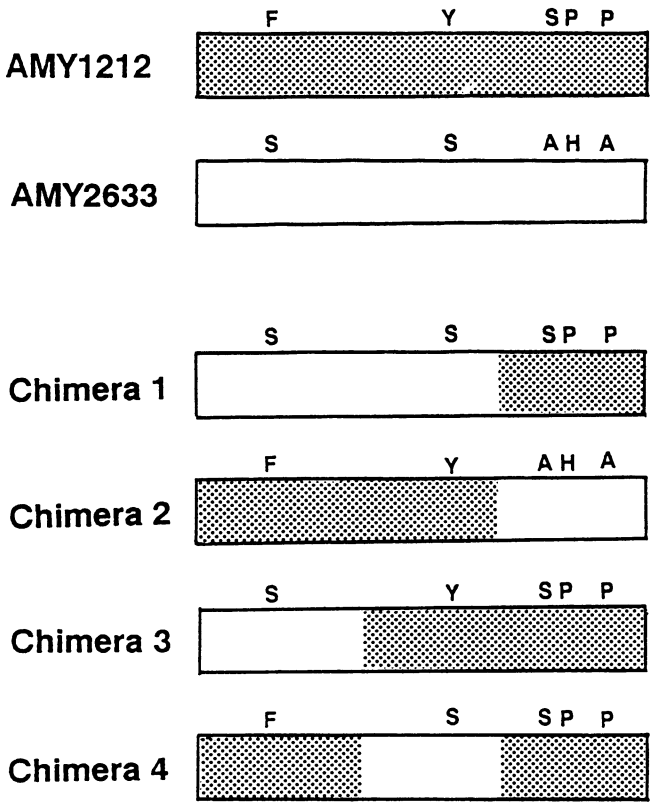

FIG. 4. Schematic structures of chimeric α-amylases constructed from AMY1212 and AMY2633 compared with AMY1212 and AMY2633. Open boxes represent regions derived from AMY2633, and shaded boxes are those from AMY1212. Each plasmid encoding each chimeric α-amylase was constructed as described in Materials and Methods. Relevant amino acid residues contained in each α-amylase are shown (see Fig. 2 for details).

Next, we determined which of the N-terminal two residues is responsible for the difference. For this purpose, two other chimeras (chimeras 3 and 4, Fig. 4) were constructed. Chimera 3 contains a Tyr residue at position 295 in place of the Ser residue of chimera 1. Chimera 4 contains a Phe residue at position 146 in place of the Ser residue of chimera 1. The two chimeras were expressed in the same way in *B. subtilis* M15 and assayed for their hydrolysis products with soluble starch and G3 as substrates. The result was clear (Table 2) that chimera 3 does not have G3-hydrolyzing and G1-producing activities, whereas chimera 4 has. Thus, it was clearly revealed that residue 295 of these *B. subtilis* α-amylases is responsible for the different hydrolyzing activities (see AMY1212 versus chimera 4 or chimera 1 versus chimera 3, Fig. 4 and Table 2).

DISCUSSION

Our present report describes the molecular cloning and nucleotide sequencing analysis of the α-amylase gene from a prototroph of *B. subtilis*, *B. subtilis* (*natto*) IAM1212, which has the *amyR2* promoter and *amyEn* structural gene. The nucleotide sequence of the gene revealed that the *B. subtilis* (*natto*) α-amylase gene contains an open reading frame consisting of 477 amino acid residues, which is the same in size as those of *B. subtilis* 2633 (1) and N7 (29) and smaller than those of *B. subtilis* 168 (32) and NA64 (31) (*amyEm* types).

The amino acid sequence of AMY1212 is very similar to that of AMY2633; only five residues are different, suggesting that both enzymes will show very similar enzymatic properties. However, hydrolysis products of soluble starch and oligosaccharides are remarkably different, although other enzymatic properties are similar (30; unpublished data). From the time course studies of the hydrolysis products from various substrates, it was concluded that AMY1212 poorly hydrolyzes G4 and cannot hydrolyze G3, and consequently, G1 (glucose) is never produced. On the other hand, AMY2633 efficiently hydrolyzes short oligosaccharides and produces G1 as a major hydrolysis product. Thus, only a few differences in the primary structures of α-amylases drastically alter the substrate specificity, probably due to changes in affinity with small substrates. Further, analyses with chimeric α-amylases revealed that the difference is ascribed to only one amino acid residue. But, as described in the previous report (30), specific activities of the two enzymes are similar when the substrate is soluble starch, indicating that the affinities for soluble starch are similar.

The affinity and catalytic process of α-amylases are often explained by the so-called subsite theory (9, 22). Determination of subsite affinities of AMY1212, AMY2633, and their chimeras may be important to interpret the differences in hydrolysis products. These problems together with the tertiary structures should be solved in future.

In any case, it became evident in this study that the difference in hydrolysis products is caused by only one amino acid substitution in the sequences of α-amylases as revealed from analyses with chimeric α-amylases (Fig. 4 and Table 2). The difference at residue 295 should be the key which determines the activity that hydrolyzes G3 and produces G1; α-amylase having a Ser residue at position 295

(AMY2633) can hydrolyze G3, whereas that having a Tyr residue (AMY1212) cannot. This rule also seems to be true for high-molecular-weight species of *B. subtilis* α-amylases. That is, α-amylases of *B. subtilis* 168 and NA64 are Ser-type enzymes in their primary structures and produce G1 from soluble starch and hydrolyze G3. α-Amylase of *B. subtilis* N7 is reported to have a Ser-type primary structure and not to hydrolyze G3 (29). In that study, however, discordance in the nucleotide sequence with the deduced amino acid sequence was observed at four points, and for hydrolysis product analysis, no detailed description such as enzyme amounts was reported. Thus, both careful nucleotide sequencing and well-defined analyses of hydrolysis products are needed for the α-amylase of *B. subtilis* N7.

Although the biological meanings of different hydrolysis products produced by very similar α-amylases as shown in this study are totally obscure, this naturally occurring mutation altering the enzymatic properties is very interesting not only for enzymology and bacteriology but also for protein engineering, which aims to change enzymes to more useful forms. For these purposes, more detailed enzymatic and protein physicochemical analyses such as X-ray crystallography are needed, along with recombinant DNA techniques including site-directed mutagenesis experiments.

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