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Cloning and Characterization of the Gene for Amylosucrase from *Neisseria polysaccharea*: Production of a Linear α-1,4-Glucan

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The gene for the amylosucrase from *Neisseria polysaccharea* (ATCC 43768) was cloned by use of a functional expression system in *Escherichia coli* XL1-Blue. The deduced amino acid sequence of the protein has homology to the sequences of the α-amylase class of enzymes, with the highest similarities being found to the sequences of the trehalose synthase from *Pimelobacter* sp. strain R48 (17) and amylomaltase from *Thermotoga maritima* (11). However, the regions of highest homology within the α-amylase class of enzymes, which are essential for the catalytic activity, are only scarcely found in the sequence of amylosucrase. By using the enzyme isolated from culture supernatants of transformed *E. coli* cells, it is possible to synthesize linear α-1,4-glucans from sucrose, indicating that the enzyme is not capable of producing α-1,6-glycosidic linkages on its own.

Glycogen is synthesized by many different bacterial species as well as in a wide range of eukaryotic organisms as an intracellular storage polysaccharide. It consists of mainly α-1,4-glycosidically linked glucose units and is branched via α-1,6-glycosidic bonds, which make up 7 to 13% of the total linkages in glycogen. In most of the bacteria analyzed, the biochemical pathway leading to glycogen formation involves three enzymes, namely, ADP-glucose pyrophosphorylase (generating ADP-glucose from glucose-1-phosphate and ATP), glycogen synthases (which transfer the glucose from ADP-glucose to a growing glucan chain), and the branching enzymes (which introduce the α-1,6-linkages into linear α-1,4-glucans) (19, 20). This pathway more or less resembles the synthesis of starch in higher plants.

In some bacteria, substances structurally similar to glycogen are synthesized without the involvement of a nucleotide sugar. In these cases, the polysaccharide is directly synthesized from maltose or sucrose through the enzymes amylomaltase (26) and amylosucrase (7, 12, 13, 14, 18), respectively. These enzymes catalyze the formation of linear α-1,4-glycosidically linked glucans by transferring one portion of the respective disaccharide to a growing polymer chain and releasing the other. They fall into the class of transglycosidases which are often involved in the synthesis of polysaccharides, such as levan (5) or dextran (22), which may occur as bacterial slimes and capsules.

The presence of amylomaltase has been reported in many bacterial species from different genera when they are grown on maltose or maltodextrins. In the case of *Escherichia coli*, this enzyme enables the cells to direct these substrates into intermediary metabolism, since they are not imported via a specific phosphotransferase system. The linear α-1,4-glucans (maltodextrins) synthesized by amylomaltase are degraded phosphorylytically through maltodextrin phosphorylase, which leads to the formation of glucose-1-phosphate (26). When mutants which are deficient for phosphoglucomutase are grown on maltose, high levels of linear maltooligosaccharides accumulate, which can be demonstrated by the deep blue staining of these cells when they are treated with iodine vapor (1).

The occurrence of amylosucrase is confined to the genus *Neisseria*. In most of the species investigated, the enzyme is located intracellularly and contributes to the synthesis of a polymer very similar to glycogen when the cells are grown on sucrose. In *Neisseria polysaccharea*, amylosucrase is secreted, and hence the polysaccharide is produced outside the cells and may serve as a protectant. In none of the studies on partially purified preparations of the enzyme did it become clear whether the enzyme is also capable of introducing branch points into the glucans or if a contaminating branching enzyme accomplished this.

Here we report the cloning and sequencing of the gene encoding amylosucrase from *N. polysaccharea*. Furthermore, we describe the preliminary characterization of the enzyme expressed in *E. coli*. The N-terminal sequence of the protein was determined, and the glucan produced by the enzyme was characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and molecular cloning techniques. Unless otherwise mentioned, *E. coli* XL1-Blue was grown on YT medium (16) overnight at 37°C with ampicillin (100 μg/ml) as a selectable marker. Protein expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside; 1 μM).

Genomic fragments were cloned into pBluescript II SK(−) (Stratagene, Heidelberg, Germany); PCR products were cloned into pMEX7 (4).

N. polysaccharea (ATCC 43768) was grown on Columbia blood agar supplemented with 5% sucrose. Genomic DNA was isolated by the method of Ausubel et al. (2), subjected to a partial *Sau*3A digest, and cloned into the *Bam*HI site of the pBluescript II SK(−).

Standard molecular cloning techniques were performed as described by Sambrook et al. (23). Nested deletions were created by the method described by Henikoff (8). DNA sequencing was performed by the dideoxy method (24) with T7 DNA polymerase (Pharmacia, Freiburg, Germany).

Staining for amylosucrase activity. *E. coli* XL1-Blue containing the genomic library of *N. polysaccharea* was grown on solid YT medium supplemented with 5% (wt/vol) sucrose. The colonies were stained with crystalline iodine sublimate after growth. Cells and the periphery of colonies expressing the amylosucrase stain blue. Supernatants of liquid cultures were filtered through a 0.2-μm-pore-size membrane and incubated on substrate-containing agarose plates (5% [wt/vol] sucrose plus 1.5% [wt/vol] agarose in 50 mM Na citrate [pH 6.5]) for at least 1 h at 37°C. The plates were subsequently stained with iodine vapor.

After electrophoresis of protein extracts on continuous 8% (wt/vol) acrylamide

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gels (10) under nondenaturing conditions, the gels were equilibrated in Na citrate buffer (100 mM, pH 6.5) and subsequently incubated in buffer containing 5% (wt/vol) sucrose for different time periods (from 1 to several hours). The glucans formed through the action of amylosucrase were readily visible as a white band and could also be stained with Lugol's solution (Sigma, Deisenhofen, Germany).

Purification of amylosucrase for N-terminal sequencing. The supernatant of a 200-ml culture of *E. coli* XL1-Blue transformed with the amylosucrase-secreting clone (pAS1) was filtered through a 0.2-μm-pore-size membrane to remove intact cells and concentrated 200-fold further in an ultrafiltration cell (Amicon, Danvers, Mass.) equipped with a membrane with a size exclusion limit of 30 kDa (YM 30). The concentrate was separated on a native polyacrylamide (PAA) gel. After incubating the gel in a buffer containing 5% (wt/vol) sucrose, a white band was visible and excised. The identity of the band was confirmed by staining a portion with Lugol's solution. Protein contained within the excised gel was eluted into sample buffer and separated under denaturing conditions in a gel containing 10% acrylamide (10). After staining with Coomassie brilliant blue (15), a single band was visible and excised. The protein was eluted into 0.1 M Tris (pH 8.0)–0.1% sodium dodecyl sulfate (SDS) and subjected to N-terminal sequencing through a commercial service (Hewlett-Packard, Waldbronn, Germany).

PCR amplification and cloning of pAS2 and pAS3. For the extracellular and intracellular expression of the amylosucrase in *E. coli*, two fragments of pAS1 were amplified by PCR and subcloned into pMEX7, one containing the sequence for the entire protein and the other containing the sequence for the predicted mature protein. The primers for the 5′ ends were 5′-GCACCATGGTGACCC CCACGCAGCA-3′ (ASN2) and 5′-CTCACCATGGGCATCTTGGACATC-3′ (ASN3), respectively, and the primer for the 3′ end was 5′-CTGCCATGGTTC AGACGGCATTTGG-3′ (ASC). The PCR fragments generated with the primer pairs ASN2-ASC and ASN3-ASC were cloned into the *Nco*I site of pMEX7.

Carbohydrate analysis. The sugar and oligosaccharide samples were separated on a high-performance anion-exchange chromatography (HPAEC) column (CarboPac PA100; Dionex, Idstein, Germany) and eluted with a nonlinear Na acetate gradient (0 to 1 M) in 100 mM NaOH. The samples were detected with pulsed amperometry (PED-2; Dionex).

Polysaccharides were separated by size exclusion chromatography on a PL-GFC 300-Å column (300 by 7.5 mm; Latek, Eppelheim, Germany) with 100 mM NaOH as the mobile phase and the same detection system. Here pullulans of different sizes (186 and 853 kDa; Polymer Laboratories, Amherst, Mass.) were used as molecular size standards.

For the hydrolysis of glucans with different enzymes, amylose was dissolved in dimethyl sulfoxide. After dilution, the hydrolysis with pullulanase (Boehringer GmbH, Mannheim, Germany), β-amylase (Boehringer), or amyloglucosidase (Boehringer) was performed with 16 mM Na acetate (pH 4.8). The isoamylolysis (isoamylase was purchased from Megazyme, Warriewood, Australia) was performed with 50 mM Na acetate (pH 3.5). To ensure the complete digestion of the samples, incubations were carried out for 24 h at 37°C with an excess of enzyme (80 U/mg of substrate).

Total hydrolysis of polysaccharides was performed by incubating the dissolved samples in 1.8 M H_2SO_4 at 95°C for 2 h. Neutralization was done by adding equimolar amounts of NaOH.

The determination of reducing ends was performed as described by Bernfeld (3) with the dinitrosalicylic acid reagent.

RESULTS AND DISCUSSION

Cloning and sequencing of the gene encoding amylosucrase from *N. polysaccharea*. A genomic library of *N. polysaccharea* was prepared and transformed into *E. coli* XL1-Blue. Thirty thousand independent transformants were plated on solid YT medium containing 5% sucrose and 0.1 mM IPTG. After growth, the cells were stained with iodine vapor. One clone, designated AS1, was selected; this clone synthesized blue-staining material in the periphery of the colony, indicating that active amylosucrase was secreted in these cells and an amylose-like material was produced. Plasmid DNA was isolated and retransformed into *E. coli* XL1-Blue. Independent transformants were plated in replicate and stained again. Ninety percent of the transformants gave rise to the same blue staining as that described above. The blue-staining halo surrounding the colonies after growth on sucrose-containing medium is easily visible in Fig. 1, quadrant 1.

Plasmid DNA was prepared and digested with several restriction enzymes. The analysis of plasmid pAS1 revealed an insert size of ca. 4.2 kb. Nested deletions were created from both ends by use of exonuclease III and nuclease S1. Competent *E. coli* XL1-Blue cells were transformed with the resulting plasmids and tested for the presence of amylosucrase activity as outlined above. The insert size of the plasmids conferring activity was determined. Plasmid pAS72, with an insert size of ca. 2.2 kb, still contained the full information to enable the expression of amylosucrase and was subjected to sequence analysis.

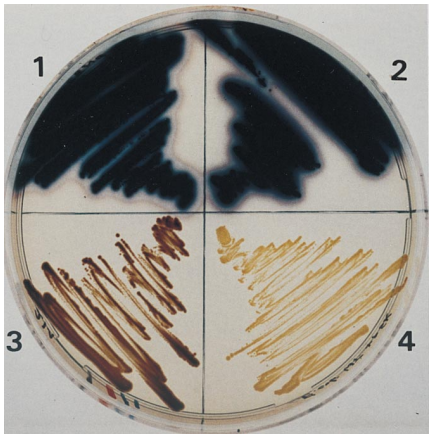

FIG. 1. *E. coli* strains (XL1-Blue transformed with different plasmids) were grown on YT agar plates containing 5% sucrose and 0.1 mM IPTG. After growth, the plates were stained with iodine vapor. Quadrants: 1, pAS1 (containing the genomic fragment for amylosucrase as isolated from a genomic library of *N. polysaccharea*); 2, pAS2 (containing the full coding region for amylosucrase); 2, pAS3 (containing the coding region for amylosucrase with a deletion of the 16 N-terminal amino acids); 4, pBluescript II SK(−).

The sequence of the insert of plasmid pAS72 is depicted in Fig. 2. It is 2,217 nucleotides (nt) long, and the coding region for amylosucrase is defined by an open reading frame of 1,842 bp coding for 614 amino acids. It is oriented in reverse direction to that of the *lac* promoter contained on the vector pBluescript SK II(−). This indicates that the genomic fragment cloned from *N. polysaccharea* contains not only the coding region for amylosucrase but also regulatory elements promoting gene expression. A putative promoter with the −35 region between nt −108 and −101 and the −10 region between nt −84 and −79 matches the consensus sequence of bacterial promoters. Furthermore, a putative Shine-Dalgarno sequence is located between nt −7 and −10. The induction of this putative promoter in *E. coli* is not dependent on the presence of sucrose, since amylosucrase is expressed in its absence. Cells transformed with pAS125 still show amylosucrase activity, whereas cells transformed with pAS55 do not, although this plasmid still contains the complete coding region for amylosucrase (Fig. 2). This indicates that regulatory elements are present in the regions mentioned above.

Sequence comparison of amylosucrase with related enzymes. The deduced amino acid sequence (Fig. 2) of amylosucrase was compared to entries in the SwissProt database by use of the algorithms BLAST and FASTA in the Genetics Computer Group sequence analysis package (6). Sequence homologies were found only to enzymes which fall into the α-amylase family of proteins. This family comprises α-glucan hydrolases such as α-amylases and α-glucosidase, which hydrolyze α-1,4-glucosidic linkages, and other enzymes capable of hydrolyzing α-1,6-glucosidic linkages such as pullulanase, isoamylase, dextran glucosidase, and oligo-1,6-glucosidase. Glucotransferases, such as amylomaltase, and glucanotransferases, such as cyclodextrin glucanotransferase or different branching enzymes, also belong to this group of proteins. All enzymes have the ability to hydrolyze α-glucosidic linkages

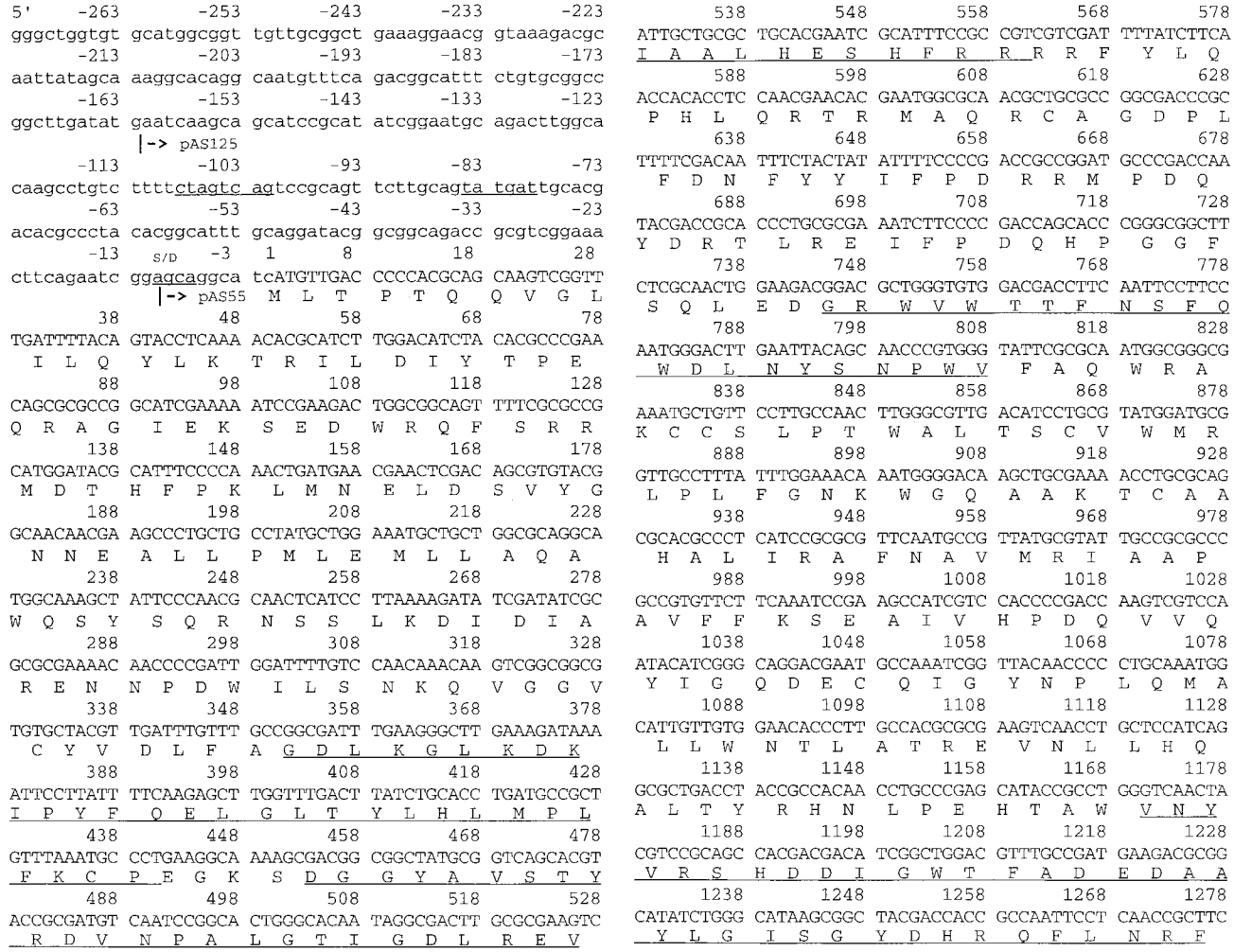

FIG. 2. Nucleotide and deduced amino acid sequences of the genomic fragment of *N. polysaccharea* containing the coding region for amylosucrase within the plasmid pAS72. The coding region is represented by uppercase letters; the noncoding region is represented by lowercase letters. Potential promoter sequences are underlined, and the putative Shine-Dalgarno sequence (S/D) is indicated. The 5′ ends of other deletion clones are indicated by arrows. The amino acid sequence of the amylosucrase is given in the single-letter code. Regions with homologies to the α-amylase class of enzymes are underlined.

either exclusively or as part of a transfer reaction. The extensive but patchwise homology of amylosucrase to some members of this family of proteins could be due to the fact that all of these different enzymes are able to interact with α-glucans, which is especially true for amylosucrase, since it has an extremely high affinity for its product (13). The four conserved regions in the active site of the α-amylase family are not present within the sequence of amylosucrase except for region 4 (27). The absence of these catalytically important residues in the amylosucrase is explainable since α-glucan hydrolysis is not necessary for the synthesis of amylose from sucrose.

The highest similarities were found to the trehalose synthase from *Pimelobacter* sp. strain R48 (accession no. D78198) (17) and to the amylomaltase from *Thermotoga maritima* (accession no. Z50813) (11). These enzymes catalyze very similar reactions; therefore, the homologies to amylosucrase should be expected. Surprisingly, no homologies were found to other hexosyltransferases, which either synthesize the same product (starch synthase or glycogen synthase), or which share the same substrate (dextransucrase, levansucrase, or inulinsucrase).

Isolation and N-terminal sequencing of amylosucrase. Amylosucrase is secreted when expressed in *E. coli*. To determine the N terminus of the mature enzyme, amylosucrase was purified from culture supernatants.

E. coli cells containing the plasmid pAS1 were grown overnight at 37°C in liquid YT medium. The cells were removed by both centrifugation and filtration. The supernatant was concentrated 200-fold by ultrafiltration through an Amicon cell with a size exclusion limit of 30 kDa. An additional protein of 70 kDa is visible in concentrated culture supernatants from *E. coli* cells transformed with pAS1 (Fig. 3, lane 3) in comparison to untransformed control samples (Fig. 3, lane 1). The proteins of the supernatant were separated electrophoretically on a native PAA gel. After equilibration, the gel was incubated overnight at 37°C in a sucrose-containing solution. A white band, which was stainable with Lugol's solution in a control experiment, was formed through the action of amylosucrase. This band was excised, and proteins were eluted into SDS-containing sample buffer. These were subjected to a second electrophoresis under denaturing conditions. Only one protein with the size of 70 kDa, which is in agreement with the pre-

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      1288        1298        1208        1218        1228
TTCGTCAACC GTTTCGACGG CACGTTCGCT CGTGGCGTAC CGTTCCAATA
F  V  N  R   F  D  G   T  F  A   R  G  V  P   F  Q  Y
      1338        1348        1358        1368        1378
CAACCCAAGC ACAGGCGACT GCCGTGTCAG TGGTACAGCC GCGGCATTGG
 N  P  S   T  G  D  C   R  V  S   G  T  A   A  A  L  V
      1388        1398        1408        1418        1428
TCGGCTTGGC GCAAGACGAT CCCCACGCCG TTGACCGCAT CAAACTCTTG
  G  L  A   Q  D  D   P  H  A  V   D  R  I   K  L  L
      1438        1448        1458        1468        1478
TACAGCATTG CTTTGAGTAC CGGCGGTCTG CCGCTGATTT ACCTAGGCGA
Y  S  I  A   L  S  T   G  G  L   P  L  I  Y   L  G  D
      1488        1498        1508        1518        1528
CGAAGTGGGT ACGCTCAATG ACGACGACTG GTGCCAAGCA GCAATAAGAG
E  V  G   T  L  N  D   D  D  W   C  Q  A   A  I  R  A
      1538        1548        1558        1568        1578
CGACGACAGC CGTTGGGCCA CCGTCCGCGC TACAACGAAG CCCTGTACGC
  T  T  A   V  G  P   P  S  A  L   Q  R  S   P  V  R
      1588        1598        1608        1618        1628
GCAACCGAAC GATCCGTCGA CCGCAGCCGG CAAATCTATC AGGGCTTGCG
A  T  E  R   S  L  D   A  A  G   Q  I  Y  Q   G  L  R
      1638        1648        1658        1668        1678
CCATATGATT GCCGTCCGCC AAAGCAATCC GCGCTTCGAC GGCGGCAGGC
H  M  I   A  V  R  Q   S  N  P   R  F  D   G  G  R  L
      1688        1698        1708        1718        1728
TGGTTACATT CAACACCAAC AACAAGCACA TCATCGGCTA CATCGCAACA
  V  T  F   N  T  N   N  K  H  I   I  G  Y   I  A  T
      1738        1748        1758        1768        1778
ATGCGCTTTT GGCATTCGGT AACTTCAGCG AATATCCGCA AACCGTTACC
M  R  F  W   H  S  V   T  S  A   N  I  R  K   P  L  P
      1788        1798        1808        1818        1828
GCGCATACCC TGCAAGCCAT GCCCTTCAAG GCGCACGACC TCATCGGTGG
 R  I  P   C  K  P  C   P  S  R   R  T  T   S  S  V  A
      1838        1848        1858        1868        1878
CAAAACTGTC AGCCTGAatc aggatttgac gcttcagccc tatcaggtca
  K  L  S   A  stop
      1888        1898        1908        1918        1928
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      1938
accgtttcag acggcat
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FIG. 2—*Continued.*

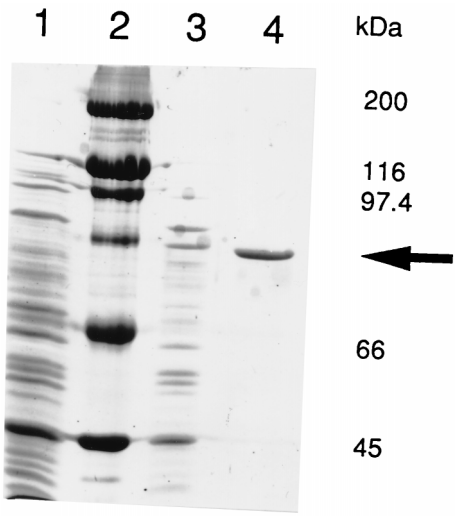

FIG. 3. SDS-PAA gel electrophoresis (10% [wt/vol] PAA) of different protein extracts (8). Lanes: 1, concentrated (200-fold) culture supernatant of untransformed *E. coli* XL1-Blue; 2, molecular size markers; 3, concentrated (200-fold) culture supernatant of *E. coli* XL1-Blue transformed with pAS1; 4, amylosucrase which was excised from a nondenaturing PAA gel.

dicted molecular size, was detectable (Fig. 3, lane 4) in the eluate from the gel slice. This band again was excised, and the protein was eluted into 0.1 M Tris (pH 8.0) containing 0.1% (wt/vol) SDS. The protein was subsequently sequenced at the N-terminal end through a commercial service (Hewlett-Packard). The sequence is identical to the N terminus of the deduced amino acid sequence from the immature (pre-) protein, indicating that in *E. coli* the signal sequence, even though it can be identified by sequence comparison (see below), is not processed.

Intra- and extracellular expression of amylosucrase in *E. coli*. To determine a potential cleavage site for the signal peptide, the sequence of the 60 N-terminal amino acids of amylosucrase was compared to that of other prokaryotic signal peptides by using the algorithm of von Heijne and Abrahmsén (29) (Sigcleave/Prok). A putative signal peptide cleavage site was predicted at position 16 of the immature protein with a high statistical significance. To enable a high level of intra- and extracellular expression of amylosucrase in *E. coli*, two types of primer pairs were synthesized for the PCR amplification of the complete coding region and the coding region for the putative mature protein, respectively. Through the introduction of *Nco*I restriction sites, a conservative exchange from leucine to serine at position 2 was introduced into the protein encoded by the PCR product containing the full coding region. An exchange from threonine to methionine and from arginine to glycine was introduced at positions 17 and 18 to promote expression of the mature protein. The PCR products were cloned into the expression vector pMEX7, giving rise to the plasmids pAS2 and pAS3. *E. coli* XL1-Blue containing the different plasmids was grown in the presence of 5% (wt/vol) sucrose and subsequently stained with iodine vapor (Fig. 1). Similar to the situation in the case of pAS1, plasmid pAS2 led to extracellular localization of the amylosucrase (Fig. 1, quadrants 1 and 2). XL1-Blue cells transformed with pAS3, however, did not form the halo surrounding the colonies, and furthermore, the glucans formed in these cells had an altered iodine staining pattern, from blue to brown (Fig. 1, quadrant 3). This can be taken as a clear indication of the intracellular localization of the glucans (and the amylosucrase). The iodine staining indicates that these glucans are branched, probably due to the action of the glycogen branching enzyme (GlgB) which is present in *E. coli*. In contrast, controls transformed with the vector alone did not give any, or very little, staining with iodine (Fig. 1, quadrant 4), indicating that they are not able to synthesize significant amounts of glycogen when they are grown in the presence of sucrose.

These experiments clearly prove the functionality of the signal sequence in promoting secretion of the amylosucrase into the medium in *E. coli* XL1-Blue, which is an *E. coli* K-12 derivative. However, *E. coli* K-12 is generally regarded as a strain which does not secrete proteins beyond the outer membrane (30), even though some reports on the recovery of heterologously expressed proteins directly from the growth medium have been made (9). The mechanism by which these proteins were translocated into the medium is not understood and is regarded as leakage from, or lysis of, the cell, but the phenomenon seems to be sequence specific. This could serve as an explanation for the fact that the signal sequence of amylosucrase is not processed. On the other hand, processing of a signal sequence is not a stringent requirement for protein export (25). Altogether, the mechanism by which amylosucrase is translocated into the medium remains unclear.

The results also indicate that sucrose is transported or diffuses into *E. coli* XL1-Blue without phosphorylation, even though this strain is not able to grow on sucrose as a sole carbon source. Interestingly, mutants of *E. coli* which are de-

fective for phosphoglucomutase accumulate linear α-1,4-glucans when grown on maltose (1). Maltose is taken up and converted into glucans through the action of amylomaltase, an enzyme very similar to amylosucrase. The only apparent distinction between the two systems besides the difference in the substrate disaccharide is the release of glucose from maltose in the one and the release of fructose from sucrose in the other. However, the release of glucose or the presence of maltose does not lead to the expression of glycogen branching enzyme as observed in the case of the release of fructose or the presence of sucrose. The glycogen biosynthetic genes are clustered in *E. coli* K-12 and are organized in two operons. In contrast to the operon containing *glgA* and *glgC* (encoding glycogen synthase and ADP-glucose pyrophosphorylase), no positive effectors which control the genetic regulation of the operon comprising *glgB*, the structural gene for glycogen branching enzyme, are known (19, 20). The detailed comparative analysis of the two systems with respect to metabolite levels might give some deeper insights into the genetic regulation of glycogen biosynthesis in *E. coli*.

Production and characterization of the glucan synthesized by amylosucrase in vitro. Earlier reports have documented that different species of *Neisseria* produce glycogen or amylopectin-like glucans due to the presence of an amylosucrase. However, whether the introduction of branch points is accomplished by a branching enzyme or by the amylosucrase itself remains an open question (7, 12, 13, 18). To resolve this question and to determine the molecular weight of the glucans synthesized, the product of amylosucrase action was further analyzed. Since *E. coli* does not secrete branching enzyme nor any other interfering activity, the characteristics of the synthesized glucans should therefore be specific for the catalytic action of amylosucrase.

E. coli cells containing the plasmid pAS1 were grown in liquid culture, and the supernatant was concentrated. The concentrate was analyzed qualitatively for amylosucrase activity by incubating an aliquot on a sucrose-containing agarose plate. The synthesis of glucans was detected with iodine vapor. The supernatant was then utilized for the production of glucans. The concentrated supernatant was incubated for 5 days at 37°C in 5% (wt/vol) sucrose-containing buffer. After 24 h, the solution became opaque and a white precipitate was formed; this precipitate was harvested by centrifugation and washed with water. The following studies were performed with this precipitate.

The glucans were dissolved in 100 mM NaOH, and Lugol's solution was added after neutralization with HCl. The absorption spectrum of the glucan-iodine complex displays its maximum (λ_{max}) at 615 nm, which can be taken as an indication for the linear or only slightly branched nature of the molecule. Iodine is capable of forming complexes with α-1,4-glucans, which have different absorption maxima depending on the average chain length. Glycogen, with an average chain length of 10 to 13, has a λ_{max} of ca. 500 nm, and amylose, an essentially linear molecule with a degree of polymerization (DP) of more than 1,000, has a λ_{max} of 620 to 650 nm, whereas amylopectin displays a λ_{max} between the two values (ca. 550 nm) (28). The slight shift towards wavelengths lower than that of amylose could be explained by the fact that shorter but still linear glucans are contained within the polysaccharide preparation (see below). In addition, the glucans were hydrolyzed with isoamylase or pullulanase, and the number of reducing ends was determined before and after treatment. No significant differences were observed. Furthermore, equal amounts of the glucans were digested with either amyloglucosidase or β-amylase and reducing ends were determined. The value for

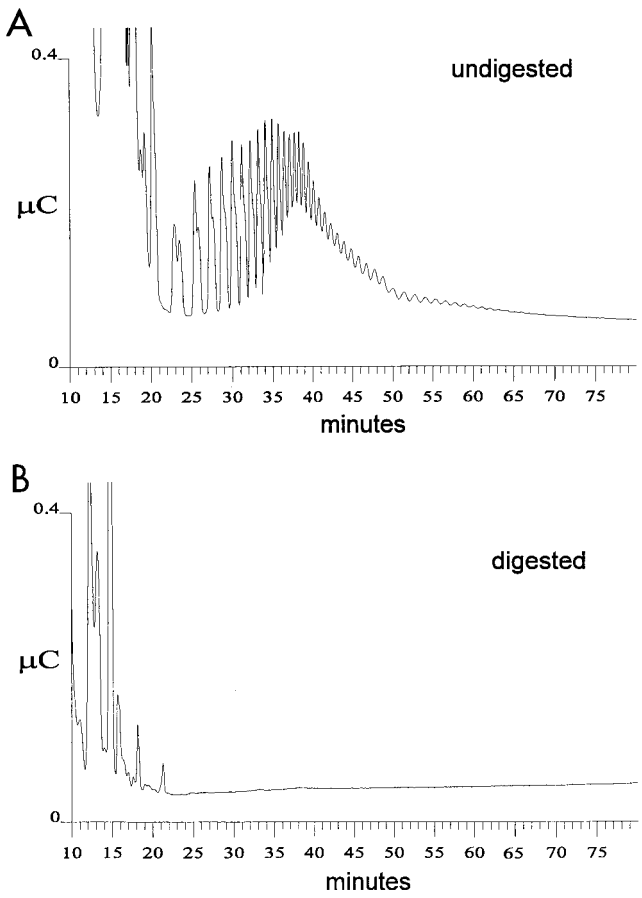

FIG. 4. HPAEC of amylose produced by recombinant amylosucrase (A) and the β-amylase digest of the same preparation (B). μC, microcoulomb.

the β-amylase digest amounts to 52% of the amyloglucosidase treatment. From this, it can be concluded that branch points occur only to an extent below the resolution limit of the analytical procedure. Amyloglucosidase hydrolyzes α-1,4- as well as α-1,6-glycosidic linkages and is therefore capable of the complete hydrolysis of α-1,4-glucans (containing branch points) down to the glucose monomers. β-Amylase is an exoamylase which hydrolyzes maltose units from the nonreducing ends of α-1,4-glucans but is not able to cleave or bypass α-1,6-glycosidic branch points. If there are no or very few branch points, then β-amylase should release 50% of the reducing power from the polymer compared with that released by amyloglucosidase. This is very similar to the result observed.

Further experiments to prove the linear nature of the glucans were undertaken. The glucans were separated by HPAEC on a CarboPac PA-100 column before and after hydrolysis with the different enzymes. Glucans with a DP of up to 50 glucose units could be detected in the untreated sample (Fig. 4A). No additional glucans were detectable after hydrolysis with isoamylase (data not shown), which is in agreement with the measurements described above. In addition, the glucans digested with β-amylase were separated on the same column. The main carbohydrates which were detectable were maltose, the product of the β-amylase digest, sucrose, and fructose, which are present as contaminants in the glucan preparation, and some traces of higher maltooligosaccharides up to maltotetraose or maltopentaose (Fig. 4B). However, no β-limit dextrins of a higher DP were detected, again indicating that the

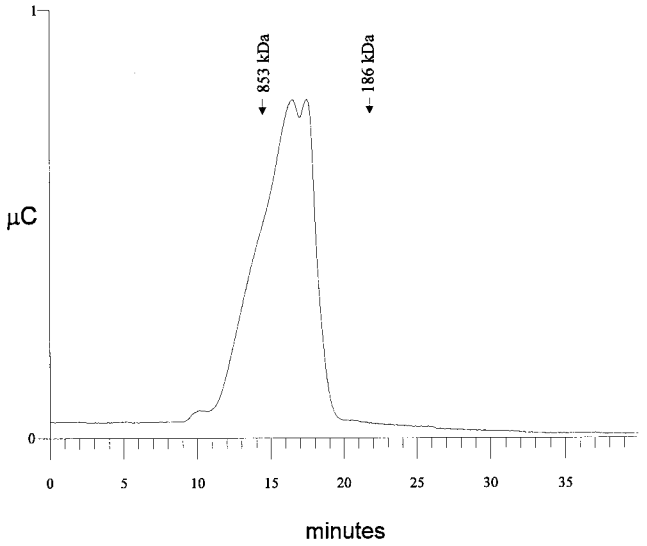

FIG. 5. Size exclusion chromatography (using a PL-GFC 300-Å column with a mobile phase of 100 mM NaOH and a pulsed amperometry detector) of amylose produced by amylosucrase. The retention times and the sizes of the pullulans used as molecular size standards are indicated. μC, microcoulomb.

glucans synthesized by amylosucrase are essentially linear. In addition, in other experiments using ^{13}C nuclear magnetic resonance to characterize the glucans produced by amylosucrase from *N. polysaccharea* expressed in *E. coli*, no evidence for the presence of α-1,6-glycosidic linkages has been obtained (21). Taken together, none of the experiments undertaken to prove the linear nature of the α-1,4-glucan synthesized by amylosucrase indicated the presence of α-1,6-glycosidic linkages in the polymer. However, it cannot be excluded that these linkages occur to a very low extent; clearly, the previous reports on the synthesis of a glycogen-like polymer by amylosucrase have to be reevaluated. In these reports, the preparations of amylosucrase investigated may have contained a contaminating branching enzyme which was responsible for the generation of branch points. A second explanation might be that the amylosucrases from different *Neisseria* species differ with respect to their catalytic activity. The amylosucrase from *N. polysaccharea* investigated here is an extracellular enzyme, whereas all the amylosucrases studied on the biochemical level were derived from organisms which synthesize the glucan intracellularly. Nevertheless, the glucan produced by *N. polysaccharea* is branched to a similar degree as the ones synthesized in the other *Neisseria* species.

The average molecular weight of the glucans was determined by measuring the reducing power before and after total acid hydrolysis. From this, an average DP of 1,528 glucose units, corresponding to a molecular size of 276 kDa, was calculated. To analyze the size distribution of the glucans in the high-molecular-weight range, size exclusion chromatography was performed. By using calibrated pullulans of 186 and 853 kDa as standards, one double peak with sizes of 489 and 630 kDa was detected; these sizes would correspond to average chain lengths of ca. 3,000 and 3,800 glucose units, respectively (Fig. 5). It is assumed that a major proportion of the glucans synthesized have a DP of between 2,000 and 5,000 glucose units. Taking into account that a significant proportion of low-molecular-weight glucans are present in the preparation, as demonstrated by HPAEC, the lower average DP can be explained. Therefore, it can be concluded that amylosucrase is capable of synthesizing a polymer very similar to amylose, the fraction of starch, which is held to be essentially linear (0.1% branch points).

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REFERENCES

1. **Adhya, S., and M. Schwartz.** 1971. Phosphoglucomutase mutants of *Escherichia coli* K-12. J. Bacteriol. **108:**621–626.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. J. Wiley & Sons, Inc., New York, N.Y.
3. **Bernfeld, P.** 1955. Amylases, α and β. Methods Enzymol. **1:**149–158.
4. **Büttcher, V., A. Rühlmann, and F. Cramer.** 1990. Improved single-stranded DNA producing expression vectors for protein manipulation in *Escherichia coli*. Nucleic Acids Res. **18:**1075.
5. **Cote, G. L., and J. A. Ahlgren.** 1993. Metabolism in microorganisms. I. Levan and levansucrase, p. 141–168. *In* M. Suzuki and N. J. Chatterton (ed.), Science and technology of fructans. CRC Press, Inc., Boca Raton, Fla.
6. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
7. **Hehre, E., D. M. Hamilton, and A. S. Carlson.** 1949. Synthesis of a polysaccharide of the starch-glycogen class from sucrose by a cell-free, bacterial enzyme system (amylosucrase). J. Biol. Chem. **177:**267–279.
8. **Henikoff, S.** 1984. Unidirectional digestion with exonucleaseIII creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
9. **Hockney, R. C.** 1994. Recent developments in heterologous protein production in Escherichia coli. Trends Biotechnol. **12:**456–463.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
11. **Liebl, W., R. Feil, J. Gabelsberger, J. Kellermann, and K.-H. Schleifer.** 1992. Purification and characterization of a novel thermostable 4-α-glucanotransferase of *Thermotoga maritima* cloned in *Escherichia coli*. Eur. J. Biochem. **207:**81–88.
12. **MacKenzie, C. R., K. G. Johnson, and I. J. McDonald.** 1977. Glycogen synthesis by amylosucrase from *Neisseria perflava*. Can. J. Microbiol. **23:** 1303–1307.
13. **MacKenzie, C. R., I. J. McDonald, and K. G. Johnson.** 1978. Glycogen metabolism in the genus *Neisseria*: synthesis from sucrose by amylosucrase. Can. J. Microbiol. **24:**357–362.
14. **MacKenzie, C. R., M. B. Perry, I. J. McDonald, and K. G. Johnson.** 1978. Structure of the D-glucans produced by *Neisseria perflava*. Can. J. Microbiol. **24:**1419–1422.
15. **Meyer, T. S., and B. L. Lamberts.** 1965. Use of Coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. Biochim. Biophys. Acta **107:**144–145.
16. **Miller, J. H.** 1983. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Nishimoto, T., M. Nakano, T. Nakada, H. Chaen, S. Fukuda, T. Sugimoto, M. Kurimoto, and Y. Tsujisaka.** 1996. Purification and properties of a novel enzyme, trehalose synthase, from *Pimelobacter* sp. R48. Biosci. Biotechnol. Biochem. **60:**640–644.
18. **Okada, G., and E. J. Hehre.** 1974. New studies on amylosucrase, a bacterial α-D-glucosylase that directly converts sucrose to a glycogen-like α-glucan. J. Biol. Chem. **249:**126–135.
19. **Preiss, J.** 1996. Regulation of glycogen synthesis, p. 1015–1024. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
20. **Preiss, J., and T. Romeo.** 1994. Molecular biology and regulatory aspects of glycogen biosynthesis in bacteria, p. 299–329. *In* W. E. Cohn and K. Moldave (ed.), Progress in nucleic acid research and molecular biology, vol. 47. Academic Press, Inc., San Diego, Calif.
21. **Remaud-Simeon, M., F. Albaret, B. Canard, I. Varlet, P. Colonna, R. M. Willemont, and P. Monsan.** 1995. Studies on a recombinant amylosucrase, p. 313–320. *In* S. B. Petersen, B. Svenson, and S. Pedersen (ed.), Carbohydrate bioengineering. Elsevier Science B.V., Amsterdam, The Netherlands.
22. **Robyt, J. F.** 1995. Mechanism and action of glucansucrases, p. 295–312. *In* S. B. Petersen, B. Svenson, and S. Pedersen (ed.), Carbohydrate bioengineering. Elsevier Science B.V., Amsterdam, The Netherlands.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

25. **Schatz, P. J., and J. Beckwith.** 1990. Genetic analysis of protein export in Escherichia coli. Annu. Rev. Genet. **24:**215–248.
26. **Schwartz, M.** 1987. The maltose regulon, p. 1482–1502. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
27. **Svensson, B.** 1994. Protein engineering in the α-amylase family: catalytic mechanism, substrate specificity and stability. Plant Mol. Biol. **25:**141–157.
28. **Thorn, W., and S. Mohazzeb.** 1990. Molecular weights, length and distribution of side-chains in α-D-polyglucanes. Starch/Stärke **42:**373–376.
29. **von Heijne, G., and L. Abrahmsén.** 1989. Species-specific variation in signal peptide design. Implications for protein secretion in foreign hosts. FEBS Lett. **244:**439–446.
30. **Wandersman, C.** 1996. Secretion across the bacterial outer membrane, p. 955–966. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.