Role of the *Escherichia coli* glgX Gene in Glycogen Metabolism

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A role for the *Escherichia coli* glgX gene in bacterial glycogen synthesis and/or degradation has been inferred from the sequence homology between the glgX gene and the genes encoding isoamylase-type debranching enzymes; however, experimental evidence or definition of the role of the gene has been lacking. Construction of *E. coli* strains with defined deletions in the glgX gene is reported here. The results show that the GlgX gene encodes an isoamylase-type debranching enzyme with high specificity for hydrolysis of chains consisting of three or four glucose residues. This specificity ensures that GlgX does not generate an extensive futile cycle during glycogen synthesis in which chains with more than four glucose residues are transferred by the branching enzyme. Disruption of glgX leads to overproduction of glycogen containing short external chains. These results suggest that the GlgX protein is predominantly involved in glycogen catabolism by selectively debranching the polysaccharide outer chains that were previously repressed by glycogen phosphorylase.

The synthesis of glycogen in *Escherichia coli* occurs when carbon is abundant but another nutrient required for growth is limiting. Although glycogen production or accumulation is not required for growth under laboratory culture conditions, the presence of glycogen may increase the viability of *E. coli* under adverse conditions or in specific ecological niches (reviewed in reference 29). Various *E. coli* mutants with mutations in glycogen synthesis and catabolism genes have been isolated (4, 7, 10, 28, 30). These mutants fell in three phenotypic classes with respect to the iodine staining phenotype of the bacterial colonies. Class A mutants were devoid of iodine-staining polysaccharide, class B mutants displayed blue staining that revealed the presence of moderately branched polysaccharides, and class C mutants displayed darker staining than the wild type owing to glycogen overproduction (7). These genetic and biochemical studies demonstrated that two activities are absolutely required for the synthesis of α-1,4-glucosyl-glucan, ADP-glucose pyrophosphorylase (EC 2.7.7.17; encoded by glgC) and glycogen synthase (EC 2.4.1.21; encoded by glgA). A third biosynthetic activity, glycogen branching enzyme (EC 2.4.1.18; encoded by glgB), is responsible for the introduction of α-1,6 linkages into the polymer. In *E. coli* these genes are located in a gene cluster which includes the glgC, glgA, and glgB genes and two other genes thought to be primarily involved in glycogen degradation, glgX and glgP (also known as glgY), encoding a putative glycogen debranching enzyme (EC 3.2.1.-) and glycogen phosphorylase (EC 1.4.1.1), respectively (30). The cluster of genes is expressed as two tandemly arranged operons, one comprised of glgC, glgA, and glgB and the other comprised of glgP and glgX (29). The transcription of these operons is subject to complex regulation (31, 33).

The glgX gene was identified as an open reading frame in plasmid pOP12 (30), which was originally shown to contain the glgC, glgA, and glgB genes (25). Sequence analysis suggested that GlgX was a glucan hydrolase, probably belonging to the isoamylase family of debranching enzymes (31). Evidence supporting this hypothesis was then obtained both by inactivating or overexpressing glgX, although not selectively, and by monitoring the total debranching activity in crude extracts (33). Debranching enzymes are usually classified as either isoamylases or pullulanases. Pullulanases hydrolyze pullulan and amylopectin but have lower activity with glycogen, whereas isoamy- lases do not hydrolyze pullulan and have high activity with glycogen. However, the isoamylase-type debranching enzyme characterized from *E. coli*, while unable to hydrolyze pullulan, is not a typical isoamylase as it has only weak activity with amylopectin or glycogen but high activity with β-amylase-limit dextrin and phosphorylase-limit dextrin (GPLD) (15).

Debranching enzymes from a range of bacterial and plant sources have been characterized. In addition to intracellular *glg* operon-encoded isoamylases homologous to *E. coli* GlgX, some bacteria produce an extracellular isoamylase that is able to cleave substrates such as glycogen and amylopectin but not pullulan. Examples are the extracellular isoamylases produced by *Pseudomonas amylofera* (2) and *Flavobacterium odoratum* (17). In higher plants, three classes of isoamylase genes and one class of pullulanase genes are found. Mutants lacking type 1 isoamylase activity typically have reduced starch contents and accumulate a soluble glycogen-like polysaccharide known as phytoglycogen. Examples of type 1 mutants are the *sugary-1* maize mutant, whose mutation is best known as the mutation underpinning sweet corn; the *sugary* rice mutant, which has a similar phenotype (24); and the *sta7* isoamylase-deficient mutant of *Chlamydomonas reinhardtii* (22), which lacks granular starch but produces a phytoglycogen-like molecule. Analysis of these mutants led to the formulation of a hypothesis which suggests that debranching activities are required to modify the structure of the growing amylopectin...
molecule in starch to a more ordered structure in order to facilitate its participation in the formation of the crystalline regions of amylopectin which are essential for the integrity of the starch granule (23). In bacterial glycogen synthesis, however, the role of the GlgX-class glycogen-debranching enzyme mutants has not been defined, and so it is not clear whether the enzyme is involved only in glycogen degradation or, as in starch biosynthesis, also acts during synthesis to shape the final structure of the glycogen produced. In this report, we describe the creation by homologous recombination of E. coli strains having defined mutations in glgX, and we describe the accumulation and structure of the glycogen produced by these mutants and relate these properties to the properties of the purified recombinant enzyme.

**MATERIALS AND METHODS**

Chemicals and reagents. Amyloglucosidase and the GOPOD reagent were purchased from Lambda Fluoreszenztechnologie (Graz, Austria). Columns and buffers for capillary electrophoreses were purchased from Beckman Instruments, Fullerton, Calif.

**Bacterial strains, plasmids, and growth conditions.** The *E. coli* strains used in this study are listed in Table 1. Plasmid pMAK705 was generously provided by S. Kushner, and pUC4-Kiss (containing the *nptII* gene) was purchased from Pharmacia. Luria-Bertani (LB) medium contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 0.2% glucose. M9 medium contained 0.4% glucose, 0.4% Casamino Acids, 0.1 mM CaCl₂, 0.2% MgSO₄·7H₂O, 0.6% NaHPO₄, 0.3% KH₂PO₄, 0.5% NaCl, and 0.1% NH₄Cl. Antibiotic selection plates contained either 50 \( \mu \)g of ampicillin per ml, as required. The iodine staining characteristics of colonies on M9 medium to express the lacZ gene.

**Enzyme purification.** The glgX gene was cloned in frame with a glutathione S-transferase (GST) or six-His N-terminal tag by using the procedure described in the instructions for the Gateway system from Invitrogen. Briefly, the PCR product was cloned in plasmid pDONR201, which allowed recombination with pDEST15 (for the GST tag) or pDEST17 (for the six-His tag). The resulting plasmids were then transferred to the expression host BL21AI. The fusion proteins were expressed by induction with 0.2% arabinose for 3 ha at 37°C in LB21AI (Stratagene) cultures containing the plasmid of interest and were purified with commercially available kits for GST-tagged proteins (MagneGST protein purification system; Sigma) or six-His-tagged proteins (Ni-nitrilotriacetic acid agarose; Invitrogen) by following the recommendations of the manufacturers.

**Complementation.** The *glgX* open reading frame was amplified by performing standard PCR with primers having XbaI restriction sites on each side. The PCR product obtained was then cloned in pUC19 as a translational fusion with lacZ, which allowed induction of glgX by isopropyl-b-D-thiogalactoside (IPTG).

**Assay of glycogen content.** The cell pellet from 20 ml of culture was suspended in 50 mM Tris-EDTA-acetate buffer (pH 7.8) and centrifuged at 5,000 x g for 5 min. The pellet was resuspended in 200 mM sodium acetate buffer (pH 4.5; 3 ml of buffer/g of cells) and sonicated after addition of 10 \( \mu \)l of 0.1 M Pefabloc per ml. The crude extract was then divided into aliquots for glycogen and protein analyses. The glycogen assay was performed by using a modification of the method described in the instructions for the Gateway system from Invitrogen.

**Indole staining.** The indole staining characteristics of colonies on M9 medium plates were analyzed by exposing the plates to indole vapor as described (7). The carboxylase and debranching enzyme activities were determined by using a standard curve covering the range from 0 to 200 nmol of glycogen released per min.

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**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW25113</td>
<td>lacP mbrT14 ΔlacZyclAI hisdR514 ΔaraBAD433 ΔhisBAD1L78</td>
<td>This study</td>
</tr>
<tr>
<td>BWX1</td>
<td>BW25113 glgX::Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>BWX2</td>
<td>BW25113 ΔglgX</td>
<td>This study</td>
</tr>
<tr>
<td>DH5α</td>
<td>F' endA1 hisdR17(p′<del>lacI</del>) supE44 thi-1 recA1 gyrA (Nal') relAΔ(lacYZA-argF)U169 deoR ΔpacYΔlacZ)M15</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21AI</td>
<td>F' ompT hisdR17 ΔglgX hisdR17 ΔglgX gal dcm araB::Tn5::araBAD-lacY1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>RR1</td>
<td>F' hissd20(p′<del>lacI</del>) recA1 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mil-1 supE44 ΔlacB</td>
<td>This study</td>
</tr>
<tr>
<td>MS201</td>
<td>F' hissd20(p′<del>lacI</del>) recA1 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mil-1 supE44 ΔlacB</td>
<td>This study</td>
</tr>
<tr>
<td>iK5</td>
<td>RR1 glgX::Kan'</td>
<td>This study</td>
</tr>
</tbody>
</table>

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**Plasmid pMAK705, yielding pMAK-iK3. This plasmid was transformed into *E. coli* strain RR1, and the procedure of Hamilton et al. (11) was used to resolve strains in which the genomic glgX gene had been replaced by homologous recombination with the kanamycin-disrupted glgX gene from pMAK-iK3. A second glgX allele was obtained by transformation of the BW25113 wild-type strain with a PCR product obtained from the pKD4 plasmid (8). The amplification product was composed of the kanamycin resistance gene flanked by yeast recombination sites. The primers used contained 45 and 43 nucleotides in 5' and 3' regions, respectively, corresponding to homologous regions in glgX. The resulting strain (BWX1) contained a glgX gene with a 1.5-kb deletion and a kanamycin resistance gene inserted at the site of the deletion. BWX1 was transformed with the pCP20 plasmid encoding a yeast recombination, which allowed eviction of the kanamycin cassette and retention of the BWX2 strain, in which the major part of the glgX open reading frame was deleted.

**PCR.** For strain iK5, PCR was used to verify that the homologous recombination procedure had disrupted the target gene. First, PCR amplification of the unique fragment produced by the desired homologous recombination event was performed by using a primer annealing to a region adjacent to the glgX gene but not included in the pMAK-iKX3 construct and a region within the *nptII* gene. In a second PCR assay we used primers which annealed to the 5' and 3' ends of the glgX gene. For BWX1 and BWX2, the primers used to check for replacement of the glgX gene by the kanamycin gene (in BWX1) or the subsequent removal of the kanamycin gene (in BWX2) annealed 200 bp 5' and 3' from the disruption region, respectively.
0 to 50 μg of glucose per ml. Control reaction mixtures lacking amyloglucosidase were used for all samples, but they had negligible glucose contents.

**Debranching enzyme assay procedures.** (i) Glucan solubilization assay. An extract from RR1 and IK5 cells (0.2 mg) was incubated with 1 mg of polysaccharides in 100 mM acetic buffer (pH 5.0) containing 5 mM dithiotreitol (DTT) at 37°C for 1 h. At the end of this incubation, 3 volumes of ethanol was added, and the mixture was centrifuged for 5 min. The supernatant (0.2 ml) was freeze-dried, and the glucan content was determined with a starch assay kit (Sigma). The freeze-dried material was resuspended in 0.2 ml of 33 mM citrate buffer (pH 4.6), added to an equal volume of starch assay reagent (containing amyloglucosidase), and incubated at 60°C for 15 min. After 1 min of centrifugation, the amount of glucose in 0.1 ml of supernatant was assayed with a second reagent (0.5 ml) containing hexokinase/glucose-6-phosphate dehydrogenase, ATP, and NADP and incubated at room temperature for 15 min. The absorbance at 340 nm was measured, and the amount of glucose produced was estimated by using a glucose standard curve.

(ii) Branch linkage assay. The debranching activity of GlgX was determined by measuring the amount of reducing ends produced by using the dinitrosalicylic acid colorimetric method (19). An extract of E. coli cells (0.4 mg of protein) was incubated at 37°C with 1 mg of substrate in 100 mM acetic buffer (pH 5.0) containing 5 mM DTT in a 300-μl (total volume) mixture for 1 h. Samples were then boiled for 2 min, and each supernatant was diluted to 450 μl (total volume) mixture for 1 h. Samples were then boiled. After centrifugation for 5 min, the supernatant was lyophilized, and then freeze-dried, and the glucan content was determined with a starch assay kit (Sigma) as described above but at pH 4.5 and in the absence of DTT by using a glucose standard curve.

**Results**

**Construction of a glgX-deficient E. coli strain by homologous recombination.** One aim of this study was to characterize an E. coli strain with specific inactivation of the glgX gene. Two different methods to create a defined deletion-insertion lesion in the glgX gene by homologous recombination were used. This was done to compare different isogenic pairs in different genetic backgrounds. Indeed, we observed that glycojen accumulation levels were very sensitive to the genetic backgrounds of the strains used. The first mutant strain, strain IK5, was obtained by homologous recombination with pMAK-IK3 by using a previously described procedure (11).

The second mutant strain, strain BWX2, was obtained by direct transformation with a PCR product of the BW25113 wild-type strain containing the pKD46 plasmid by using an established protocol (8). The resulting strain, designated BWX2, had a 1.5-kb deletion of the glgX gene and a scar due to the kanamycin resistance gene excision. This second technique that allowed removal of the kanamycin gene reduced the risk of a polar effect of the mutation on neighboring genes. Confirmation that the desired homologous recombination events had occurred was obtained by PCRs.

**Iodine-staining properties of glgX-deficient strain.** Iodine staining is frequently used as a diagnostic tool to determine the amount and structure of starch or glycogen produced by microorganisms or unicellular algae. Cells were grown on minimal medium (M9 medium) supplemented with glucose to stimulate glycogen synthesis. The light red-brown color observed for E. coli RR1 was typical of the color reaction of E. coli cells containing a wild-type glycogen biosynthetic pathway (Fig. 1). The BW25113 wild-type cells were indistinguishable from RR1 cells by iodine staining (data not shown). IK5 cells consistently stained a distinctly different darker brown color than RR1 cells (Fig. 1A), and the BWX1 and BWX2 mutant strains had a darker brown phenotype than BW25113. IK5 stained a darker color than BWX1 or BWX2, possibly due to the different genetic backgrounds in which the mutations were generated.

To check this hypothesis, transduction with phage P1vir was carried out by using the method of Miller (20). The glgX::kan mutation in IK5 was transferred by P1 phage into strain BW25113. As shown in Fig. 1A, the transduced strain containing the IK5 mutation in BW25113 yielded the same iodine staining phenotype that the BWX1 or BWX2 mutant cells had. To check if the iodine staining phenotype was due to the glgX mutation, the three mutant strains (IK5, BWX1, and BWX2) were transformed with the pX plasmid containing a translational fusion between the eight first amino acids of lacZ and the glgX gene in pUC19. The resulting ampicillin-resistant clones were used for iodine staining on M9 medium containing 5 mM IPTG. Both the mutants and the wild type displayed the same light iodine staining, suggesting that there was complementation of the mutant defects (Fig. 1B). No effect of the pX plasmid was observed without addition of IPTG. These data showed that the glgX deficiency in E. coli was responsible for the darker phenotype observed in the iodine staining test.
Isoamylase activities of wild-type, glgX-deficient, and complemented strains. The isoamylase activities of the wild-type and glgX-deficient strains were measured by using mid-log-phase cells grown in LB medium. Two assay methods were used to determine the amount and nature of the debranching activity present in IK5 and RR1.

Figure 2A shows the results when the activities of IK5 and RR1 were assayed with a variety of substrates by using a glucan solubilization assay. In this assay, the release of ethanol-soluble maltooligosaccharides was monitored after incubation of the substrate with enzyme. Figure 2B shows the results of quantification of reducing sugars released from various substrates by extracts of IK5 and RR1. Both assays demonstrated that the major debranching enzyme activity of E. coli was absent from IK5 cells and confirmed that the spectrum of activity of the enzyme in the RR1 cells was consistent with previous reports (15), which showed that the E. coli debranching enzyme has a much higher activity with GPLD as the substrate than with glycogen or amylopectin (Fig. 2). The glgX-deficient strain, IK5, did not contain levels of debranching enzyme above the baseline level with the phosphorylase-limit dextrin of glycogen, glycogen, or amylopectin. Both the BWX1 and BWX2 mutant strains also lacked measurable debranching enzyme activity in these assays (Fig. 2C). The debranching activity was also monitored with crude extracts of the mutant or the wild-type reference strain transformed with plasmid pX. In this case, the culture conditions were the same, except that 100 µg of ampicillin per ml was added. All the strains expressing GlgX at high levels exhibited three to four times more activity with GPLD than untransformed bacterial strains exhibited (Fig. 2C). These high levels of the debranching enzyme were consistent with the yellow phenotype observed after iodine staining (Fig. 1B). These data, together with complementary studies (33), support the conclusion that the E. coli isoamylase activity characterized in detail by Jeanningros et al. (15) is encoded by the glgX gene. Evidence that the activity observed was catalyzed by a debranching enzyme and not by another exo- or endoamylase was provided by 1H-NMR (Fig. 3).

Assay of isoamylase activity by 1H-NMR. The substrate utilized was the phosphorylase-limit dextrin of glycogen (Fig. 3A), in which the chemical shifts assigned to the H-1,4 (5.42 ppm) and H-1,6 (5.0 ppm) linkages could be clearly observed. Figure 3B shows the spectrum for the product of incubation of the substrate with isoamylase, which resulted in almost complete removal of the H-1,6 chemical shift and the appearance of a chemical shift corresponding to the H-anomer of the released reducing sugar at 5.27 ppm. Figure 3C shows the spectrum for the product of incubation of the substrate with extract from E. coli RR1 cells, which revealed the decrease in the chemical shift for the α-1,6 linkage and the appearance of a chemical shift corresponding to the α-anomer of the released reducing sugar at 5.27 ppm.

Figure 3C shows the spectrum for the product of incubation of the substrate with extract from E. coli RR1 cells, which revealed the decrease in the chemical shift for the α-1,6 linkage and the appearance of the chemical shift at 5.27 ppm. There was no evidence of any debranching activity when the substrate was incubated with IK5 cell extract (Fig. 3D). Table 2 shows the integrated data quantifying these observations. Complete debranching of the phosphorylase-limit dextrin substrate by RR1 extract was not observed because the GlgX enzyme was apparently unable to efficiently hydrolyze the α-1,6 linkages of side chains longer than four glucosyl residues, and its action was therefore confined to the outer chains of the limit dextrin, leaving the core structure intact.

Accumulation of glycogen. The amount of glycogen accumulated by IK5, BWX1, or BWX2 was two to three times greater than the amounts of glycogen accumulated by the parental RR1 and BW25113 strains, and this difference was observed throughout the E. coli growth curve (Fig. 4), supporting the hypothesis that GlgX plays a role in glycogen degradation.
Twofold overaccumulation of glycogen was also observed for the dark-brown iodine staining class C mutants deficient for allosteric regulation of ADP-glucose pyrophosphorylase reported previously (7), while MS201, the \textit{glgB}-deficient strain, had a very small quantity of glucan. These results are consistent with a major role for the branching enzyme and GlgX in glycogen synthesis and degradation, respectively.

**Glycogen structure.** While iodine staining provided a rapid indication of the structure of glycogen or starch, it did not provide the detailed information on structure required to define the role of the GlgX debranching enzyme. Chain length analysis by the fluorophore-assisted carbohydrate electrophoresis technique (21, 26) provided an accurate method for determining the impact of a specific mutation on glycogen.
structure. This technique involved debranching the extracted glycogen with a highly purified isoamylase and then labeling the reducing ends of the oligosaccharide population by reductive amination with the charged fluorophore APTS. The labeled oligosaccharides were then separated and detected by capillary electrophoresis. Comparison of the glycogens produced by the wild-type RR1 strain and IK5 showed that the major differences between these glycogens were in the chain lengths with DP between 3 and 7.

When this analytical system was used, significant differences in the glycogens produced in the presence and in the absence of the glgX gene were observed; on a molar basis, the glycogen of IK5 had significantly more short chains than the wild-type glycogen (Fig. 5A). A plot of the normalized distribution of the chain lengths of IK5 subtracted from the chain lengths of RR1 is shown in Fig. 5B; 9.6% of the chains in RR1 had a DP of 3 to 5, whereas 17.4% of the chains in IK5 fell in this range.

**GlgX purification.** The GlgX protein was tagged at its N terminus with GST or six-His, which allowed purification of the enzyme. Each construct was introduced into BL21AI cells, and production of the proteins of interest was induced for 3 h by using 0.2% arabinose in LB medium supplemented with 100 μg of ampicillin per ml at 37°C. The production of both tagged proteins was highly enhanced by arabinose. Most of the GST-tagged enzyme was found in the soluble phase. Around 10% of the six-His-tagged GlgX was in the soluble extract, which allowed purification of the enzyme from this fraction. The remaining six-His-tagged GlgX was in inclusion bodies. The purified enzymes were checked on a 7.5% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel stained with Coomassie brilliant blue. The GST-tagged GlgX appeared as a single band at 110 kDa (data not shown). A few low-molecular-weight contaminating proteins were always purified with the six-His-tagged GlgX, which appeared as a faint band at 76 kDa due to the small amount of protein in the soluble phase used for purification. Protein samples from both expression systems were assayed with GPLD as described above, and this allowed us to estimate the yields and purification factors for each enzyme. GST-GlgX was purified with a yield of 2% and a purification factor of 30-fold, and the six-His-tagged protein was purified 51-fold with a yield of 9% compared with the activities determined for the soluble fraction obtained from the induced crude extracts used for purification. The low yields observed may have been due to the instability of the enzymes after purification.

Both purified proteins were immediately used in incubation assays with rabbit liver glycogen (Sigma, St. Louis, Mo.) by using the glucan solubilization assay procedure described previously, but the debranched oligosaccharides obtained were labeled and analyzed by capillary electrophoresis. Both purified GST- and six-His-tagged GlgX debranched oligosaccharides with degrees of polymerization of three or four glucose residues.

**TABLE 2. Debranching activity measured by 1H-NMR spectroscopy**

<table>
<thead>
<tr>
<th>Source of debranching enzyme</th>
<th>Ratio of branching (%)</th>
<th>% of reducing ends</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR1</td>
<td>9.7</td>
<td>1.02</td>
</tr>
<tr>
<td>IK5</td>
<td>9.1</td>
<td>0.00</td>
</tr>
<tr>
<td>Isoamylase</td>
<td>10.8</td>
<td>10.30</td>
</tr>
<tr>
<td>No enzyme</td>
<td>10.1</td>
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</tr>
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</table>
residues with high efficiency. These two purified enzymes hydrolyzed the α-1,6 linkages of longer chains with very low efficiency (Fig. 6). Oligosaccharides with DP greater than six glucose residues were not debranched by the purified enzymes. Some chains with DP of five or six were observed, but they did not account for more than 1% of the total debranched oligosaccharides. The same activity pattern was observed for both purified tagged proteins. The debranching activity encoded by the glgX gene of E. coli therefore defines an isoamylase type of debranching enzyme with high specificity for hydrolysis of branched maltotriose or maltotetraose.

DISCUSSION

Although previous studies had shown that the glgX gene was not absolutely required for synthesis of linear α-1,4-glucosylglucan (30), no E. coli strain specifically lacking the glgX gene alone was available prior to this study. Our results clearly indicate that the absence of the glgX gene product leads to glycogen overproduction, which suggests that the gene has an important function in storage polysaccharide catabolism.

Evidence from sequence comparisons (30) and a preliminary study of the glgX gene product (33) suggested that glgX was likely to encode a functional debranching enzyme and that this enzyme was likely to be the enzyme studied in detail by Jeanningros et al. (15). The absence of isoamylase-type debranching enzyme from the glgX-deficient mutant and the substrate specificity of the debranching enzyme present in wild-type cells (which showed higher reaction rates with phosphorylase-limit dextrin than with glycogen) provide strong evidence which suggests that glgX encodes the enzyme characterized by Jeanningros et al. (15). To definitively establish this, we expressed a recombinant glgX gene product and purified it to homogeneity. The pure protein displayed the same specificity for debranching chains consisting of three or four glucose residues that the enzyme studied by Jeanningros et al. displayed.

It has been suggested previously that the role of GlgX may be in the catabolism of glycogen in bacteria (15, 33). The results presented here demonstrate that disruption of the glgX gene alters the accumulation pattern and structure of the glycogen produced in E. coli throughout the growth of a culture. A role in glycogen degradation is supported by the accumulation phenotype. It is thought that glycogen is first acted on by glycogen phosphorylase (encoded by glgP), yielding glycogen-containing short external chains consisting of approximately four glucosyl units (32). This phosphorylase-limit dextrin is then acted upon by the GlgX isoamylase, yielding a population.
of linear maltooligosaccharides with a distribution of DP centered around maltotetraose. This population of maltooligosaccharides is thought to be disproportionated by amylomaltase (encoded by malQ), providing a substrate that is depolymerized by the malP-encoded maltodextrin phosphorylase, yielding glucose-1-phosphate (G1P) (32).

The results presented here are consistent with the mechanism that was first proposed by Palmer et al. (27) and was expanded upon by Krebs and Preiss (16). Briefly, in the absence of the glgX-encoded isoamylase, glycogen with short outer chains, resulting from the action of the GlgP glycogen phosphorylase, accumulates, inducing a shift in chain length distribution toward lower DP. Analysis of the glycogen in the glgX-deficient strain IKS showed that the level of external chains in glycogen is increased severalfold, which is consistent with the lack of GlgX activity and with its specificity for removing short external chains of glycogen. In addition, because neither glycogen phosphorylase nor amylomaltase can act on phosphorylase-limit dextrins, glycogen degradation is impaired in the absence of GlgX, and glycogen accumulates. While the role of GlgX is clearly predominantly to facilitate degradation, the data presented here also indicate that the enzyme remains active during glycogen synthesis, shaping the structure of the glycogen produced by reducing the frequency of short external chains. This activity may facilitate the production of larger glycogen molecules as the presence of very short chains has been predicted to lead to allosteric crowding and inhibition of synthesis (18).

The complex regulation of genes in the E. coli glycogen operons described previously (13, 29, 33) indicates that there is complex transcriptional control of the expression of the genes for glycogen biosynthesis and catabolism. However, the impact of the glgX mutation in induction of both glycogen accumulation and an altered glycogen structure suggests that glycogen biosynthesis and degradation occur concomitantly in an E. coli culture and that these pathways may both be active and in balance in an individual cell. It has been demonstrated that a key enzyme for glycogen degradation, glycogen phosphorylase (GlgP), is present throughout growth when there is net glycogen accumulation (5, 6). The substrate specificities and regulatory properties of the synthesis and degradation enzymes suggest that both synthesis and degradation pathways may be present in the same cell but kept in balance through the specificities of the respective enzyme systems and the availability of carbon flux. During synthesis, the pattern of action of E. coli branching enzyme, transferring chains larger than six glucosyl residues, produces external chains that are longer than those that can be acted on efficiently by GlgX, ensuring that there is not an extensive futile cycling between branching and debranching enzyme activities. Glycogen phosphorylase-mediated phosphorolysis of external chains should be limited relative to glycogen synthase-mediated extension of external chains due to the availability of ADP-glucose (promoting glycogen synthesis via ADP-glucose pyrophosphorylase and inhibiting glycogen degradation through inhibition of glycogen phosphorylase [6]), high G1P levels, and low free P, and AMP levels in the cell (AMP is a positive allosteric effector of glycogen phosphorylase in E. coli [5]). Therefore, the narrow specificity of GlgX, which cleaves only glycogen chains with external chains with DP of four or less, allows this activity to coexist in the cell with net glycogen synthesis and is consistent with the hypothesis that the two genes of the glgB-glgX operon are tandemly expressed, as suggested by Romeo et al. (30).

Isoamylases with broad specificity, such as plant isoamylases and extracellular bacterial isoamylases, would be inconsistent with net glycogen synthesis as they would induce a futile cycle; thus, they would need to be extremely tightly controlled at the transcriptional or translational level to allow net glycogen synthesis. The data presented in this report suggest that even during periods of net glycogen synthesis, components of the glycogen degradation pathway are active, but they also suggest that it is only when growth ceases, ADP-glucose becomes limiting, G1P levels fall, and P, accumulates that there are significant rates of net glycogen degradation.

Debranching enzymes belong to family 13 of the glycosylhydrolases, which are defined by the presence of a conserved domain (domain A) that assumes a barrel shape whose sides are a succession of eight β sheets and α helices. The loops connecting adjoining α helices and β sheets are numbered according to their positions within the polypeptide chain.

Differences in the length of the loop 4 region of isoamylases have been suggested to underpin differences in substrate specificity between isoamylases (1, 14). Both the plant isoamylase type 1 genes and P. amyloderamosa genes have a longer loop 4 region than GlgX; it has been suggested that this region is required to accommodate the longer side chains of amylopectin and glycogen compared to the short side chains of phosphorylase-limit dextrins preferentially hydrolyzed by GlgX. It has recently been shown that plant type 3 isoamylases have loop 4 regions whose lengths are similar to those of E. coli GlgX and which utilize phosphorylase-limit dextrins preferentially over substrates with longer external side chains (14). All of the recently reported isoamylase-like sequences with the exception of plant isoamylase type 1 and the P. amyloderamosa family of secreted isoamylases have predicted loop 4 regions whose lengths are similar to those of E. coli GlgX. Confirma-
tion that a short loop 4 region is indicative of substrate specificity favoring phosphorylase-limit dextrins over substrates with longer external chains requires additional evidence.

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