A \textit{glgC} Gene Essential Only for the First of Two Spatially Distinct Phases of Glycogen Synthesis in \textit{Streptomyces coelicolor} A3(2)

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Received 5 August 1997/Accepted 14 October 1997

By using a PCR approach based on conserved regions of ADP-glucose pyrophosphorylases, a \textit{glgC} gene was cloned from \textit{Streptomyces coelicolor} A3(2). The deduced \textit{glgC} gene product showed end-to-end relatedness to other bacterial ADP-glucose pyrophosphorylases. The \textit{glgC} gene is about 1,000 kb from the leftmost chromosome end and is not closely linked to either of the two \textit{glgB} genes of \textit{S. coelicolor}, which encode glycogen branching enzymes active in different locations in differentiated colonies. Disruption of \textit{glgC} eliminated only the first of two temporal peaks of ADP-glucose pyrophosphorylase activity and glycogen accumulation and prevented cytologically observable glycogen accumulation in the substrate mycelium of colonies (phase I), while glycogen deposition in young spore chains (phase II) remained readily detectable. The cloned \textit{glgC} gene therefore encodes an ADP-glucose pyrophosphorylase essential only for phase I (and it is therefore named \textit{glgC}). A second, phase II-specific, \textit{glgC} gene should also exist in \textit{S. coelicolor}, though it was not detected by hybridization analysis.

Glycogen is a branched homopolysaccharide of \(\alpha\)-1,4-linked glucose subunits with \(\alpha\)-1,6-linked glucose at the branching points. Many bacteria accumulate glycogen when their growth is nutrient limited in the presence of an excess of carbon source (40). The biosynthesis of bacterial glycogen from glucose-1-phosphate is catalyzed by three enzymes: ADP-glucose pyrophosphorylase, glycogen synthase, and branching enzyme, the products of the \textit{glgC}, \textit{glgA}, and \textit{glgB} genes, respectively (38, 40). These genes, together with \textit{glgY} (= \textit{glgP}), coding for glycogen phosphorylase, and \textit{glgX}, which probably encodes a glycogen debranching enzyme, are organized in two adjacent operons, \textit{gglBX} and \textit{gglXY} in \textit{Escherichia coli} (38). In \textit{E. coli}, glycogen synthesis is regulated at the enzymatic level by allosteric regulation of ADP-glucose pyrophosphorylase and at the mRNA abundance level by positively acting factors, such as cyclic AMP, ppGpp, and the stationary-phase factor \(\sigma^s\) (see references 28, 38, and 41 for recent reviews), and by the negatively acting product of a pleiotropic carbon storage regulatory gene, \textit{cstA} (27, 50). In the developmentally more complex organism \textit{Bacillus subtilis}, intriguingly, a single \textit{ggl} operon containing all the essential \textit{ggl} genes is apparently controlled by a sporulation mother-cell-specific form of RNA polymerase (\textit{Er}\textsuperscript{ss}), suggesting that glycogen metabolism is developmentally and spatially controlled in \textit{B. subtilis} (22).

\textit{Streptomyces} spp. are gram-positive bacteria that undergo particularly complex morphological differentiation. Initially, the spores germinate to form a highly branched substrate mycelium, which is firmly attached to the solid medium. Later, the aerial mycelium emerges, and spores are generated by septation of the aerial hyphae (14, 15, 31, 32, 49). During \textit{Streptomyces} differentiation, glycogen accumulates in two temporally and spatially distinct phases (4, 6, 37). The first phase (phase I) takes place in substrate hyphae around the time of emergence of aerial hyphae, and the second phase (phase II) occurs in the tips of aerial hyphae at the initiation of their septation into spore compartments. These observations suggest that glycogen accumulation and degradation may play a role in morphological differentiation of streptomycetes (6, 10, 37). A study of the genetic determination of glycogen branching enzyme in the genetically amenable organism \textit{Streptomyces coelicolor} A3(2) revealed that there are two \textit{gglB} genes, widely separated on the chromosome (8). Each was shown to be involved in a separate phase of glycogen synthesis: \textit{gglB1} in phase I and \textit{gglB2} in phase II. Evidence that this is a more or less general situation among streptomycetes was obtained by Southern blotting and by work with \textit{Streptomyces aureofaciens} (18) which showed that disruption of a \textit{gglB} gene affected glycogen synthesis only in phase II. Because \textit{gglB} mutants retained the ability to make and to degrade \(\alpha\)-1,4-polyglucan deposits, it was not possible to assess the importance of glycogen metabolism for normal colony development with these mutants.

The finding of developmentally differentially expressed isoforms of branching enzyme resembles the situation for plants (9) and raises the possibility that other genes for glycogen synthesis might also be duplicated in \textit{S. coelicolor} A3(2), with each copy being specific for one phase of deposition. Here we report the cloning and characterization of a gene of \textit{S. coelicolor} A3(2) encoding an ADP-glucose pyrophosphorylase specifically needed for phase I, but not phase II, glycogen accumulation, implying that another \textit{gglC} gene must be responsible for phase II. Disruption mutants were morphologically normal, ruling out an essential developmental role for phase I glycogen synthesis.

\textbf{MATERIALS AND METHODS}

\textit{Bacterial strains, plasmids, phases, and culture conditions.} \textit{S. coelicolor} J1501 (\textit{hisA1 ureA1 ggl SCP1 SCP2}) (11), a derivative of \textit{S. coelicolor} A3(2), was...
used as a source of chromosomal DNA for cloning, and J1508 (hisA1 araA1 strA1 gpl SCP1\'^{T9} SCP2\') (20) was used in the construction of J1888 and E002 (see below), which contain disrupted versions of gplC. Streptomyces lividans 66 (29) was the host for \( \delta C31 \) propagation and for the transformation of proteolysins. E. coli TGI (11) was used as the host for subcloning, and E. coli ED8767 (recA56 supE44 supF8 hsdS4 (rKm\(^{+}\) mKm) gsp21 gfl22 metB13) (34) was used to construct the cosmids library. E. coli ET100D1 (\( \delta \) dam+33-18Xac dcm-6 hsdRd recF143 zif-260-glpD +1 lacI21 hisG4 leuB6 L. ton315 pl136 histA21 thiB-ras-78 mitB1 glnA4/v2) was used to prepare nonmethylated plasmid DNA for use in the transformation of \( S. coelicolor \) (24). The bifunctional cosmid pKC505 (45) was obtained from the Eli Lilly Co. The Streptomyces phage vector KC361 was used for single-crossover gene disruption (7). The pUC18 derivative plI2925 (21) and the pBluescript-derived phagemid pBIC KS\(^{+} \) (Strategene) were used for subcloning, and the M13 derivatives mp18 and mp19 were used for DNA sequencing (51).

Media and culture conditions for \( S. coelicolor \) strains were described in reference 19. The media were as follows: MM (minimal agar medium, used for cultures to be assayed for \( ADP \)-glucose pyrophosphorylase or glycogen to be tested by electron microscopy), R2YE (rich agar medium for protoplast regeneration and routine culture), YEME (rich liquid medium used to obtain mycelium for protoplast preparation or DNA isolation); and Difco Nutrient Agar, containing 0.5% glucose, 8 mM Ca(NO\(_3\))\(_2\), and 10 mM MgCl\(_2\) (for phage propagation). Standard media and growth conditions were used for \( E. coli \) (44). When protoplast-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 50 \( \mu \)g of tetracycline per ml, 10 \( \mu \)g of tobramycin per ml, 200 \( \mu \)g of hygromycin per ml, 200 \( \mu \)g of carbenicillin per ml, 25 \( \mu \)g of chloramphenicol per ml, and 100 \( \mu \)g of ampicillin per ml.

Construction of a cosmid library of \( S. coelicolor \). A library of J1501 chromosomal DNA was prepared in cosmids pKC505. Chromosomal DNA was partially digested with Sau3AI, and fragments of about 30 kb (separated by sucrose gradient centrifugation as described in reference 19) were dephosphorylated by alkaline phosphatase treatment. The cosmid DNA was ligated with HpaI, dephosphorylated, and digested with BamHI to generate both cosmid arms. Insert DNAs and vector were ligated and packaged in vitro with a commercial packaging kit (Amersham International). The phages were used to infect \( E. coli \) ED8767, and transductants were selected on Trypticase soy agar plates incubated at 32°C for 48 h. Conditions for colony hybridization with a PCR-generated probe (see below). Positive colonies were confirmed by Southern blot analysis.

DNA hybridization. DNA fragments were transferred from agarose gels to nylon membranes as described in reference 19. The blots were hybridized to \( ^{32} \)P-labelled probes at 68°C for 16 h in 3\( \times \)SSC (0.5 M NaCl, 0.015 M sodium citrate, pH 7.0)–4\( \times \)Denhardt’s solution–100 \( \mu \)g of salmon sperm DNA per ml. Following hybridization, filters were washed twice with 2\( \times \)SSC–0.1% sodium dodecyl sulfate (SDS) and twice with 0.2\( \times \)SSC–0.1% SDS at 68°C before autoradiography. In some cases, the probes were nonradioactively labelled with the DIG System of Boehringer Mannheim. For colony hybridization, \( E. coli \) colony hybridizations were carried out as described in reference 19. Strains were grown on Trypticase soy agar plates incubated at 30°C for 3 d. The DNA from the whole colony was extracted using the alkaline lysis method (2). Restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other manipulations were performed according to standard procedures for \( E. coli \) (44) and \( S. coelicolor \) (19).

DNA manipulation procedures. DNA restriction fragments were subcloned from Streptomyces and from \( \delta C31 \) phage derivatives as described in reference 19. Plasmid DNA was prepared from \( E. coli \) by the alkaline lysis method (2). Restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other manipulations were performed according to standard procedures for \( E. coli \) (44) and \( S. coelicolor \) (19). Preparation of Streptomyces protoplasts and their transformation or transfection were carried out as described in reference 19.

PCR. A PCR approach was taken for the cloning of a gplC gene from \( S. coelicolor \). In the amino acid sequences of bacterial ADP-glucose pyrophosphorylases, two potential catalytic site residues, Lys-195 and Tyr-114 (\( S. coelicolor \) strain J1888), were confirmed by Southern blot analysis. These bands were excised from agar plates and inserted into the Small site of pJMN66 (11a) to generate pDSC1b, then digested with \( \delta C31 \) and SacI, and ligated into the att-deleted Streptomyces \( \delta C31 \)-based phage vector K861 to give \( \delta C31 \) MC1. Phage MC1 was used to transfect J1501 (\( \delta \) dam+33-18Xac dcm-6 hsdRd recF143 zif-260-glpD +1 lacI21 hisG4 leuB6 L. ton315 pl136 histA21 thiB-ras-78 mitB1 glnA4/v2) as host for subcloning and the M13 derivatives mp18 and mp19 were used for DNA sequencing (51).

Transformation of \( S. coelicolor \) strains. \( S. coelicolor \) strains were transformed by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories). Transformation of \( S. coelicolor \) strains was carried out as described in reference 19. After transformation, the colonies were incubated at 30°C for 3 d. DNA manipulation procedures were performed as described in reference 19.

Nucleotide sequence analysis. Nucleotide sequence analysis was performed in multiple colonies of the deleted and amplified forms of the gplC gene of strain J1508 (data not shown). Nucleotide sequence analysis was performed in multiple colonies of the amplified and deleted forms of the gplC gene of strain J1508 (data not shown). Nucleotide sequence analysis was performed in multiple colonies of the amplified and deleted forms of the gplC gene of strain J1508 (data not shown).

Assays of ADP-glucose pyrophosphorylase activity. \( S. coelicolor \) strains were grown at 28°C from spore suspensions spread on sterile cellophane films placed on plates containing 35 ml of solid MM (19) with mannitol (0.5% [w/v]) as carbon source. At various growth times, samples of mycelium were scraped...
from the cellophane, washed twice with distilled water, and suspended in 1 ml of 50 mM Tris-HCl buffer (pH 8.0)–1 mM EDTA–1 mM dithiothreitol. The mycelia were then disrupted in an MSE Soniprep 150 ultrasonic disintegrator (Sanyo-Gallenkamp, Leicester, United Kingdom) (10 pulses of 30 s each with intermittent cooling). The cell extracts were used to assay for ADP-glucose pyrophosphorylase activity, by determination of the ADP-[14C]glucose formed from ATP and [14C]glucose-1-phosphate as described previously (39). Protein concentration was assayed as described in reference 3.

**Assays of glycogen levels.** Cultures for glycogen analysis were grown in the same way as described above for ADP-glucose pyrophosphorylase assays. Extraction of polysaccharides and the assay of amyloglucosidase-releasable glucose were performed as described in reference 5.

**Electron microscopy.** Sample preparation and transmission electron microscopy of thin sections of colonies stained for polysaccharide deposits were performed as described previously (4, 37, 48).

**Nucleotide sequence accession number.** The sequence of the **glgC** gene cloned from *S. coelicolor* was deposited in the EMBL data bank under accession no. X89733.

**RESULTS**

**Cloning of a glgC gene from S. coelicolor.** The cloning of a **glgC** gene from *S. coelicolor* was based on the existence of two highly conserved regions in the amino acid sequences of the **glgC** genes from different bacteria. These sequences were used to design oligonucleotides for use as PCR...
primers. The PCR experiment generated a band of ca. 500 bp, as predicted. This fragment was used as a probe to screen a library of *S. coelicolor* DNA in the cosmids pKC505. Two hybridizing cosmids were obtained, but only one (pUO9001) proved fruitful as a source of *glgC*. A *Bam*HI fragment of 7 kb from pUO9001 specifically hybridized with the probe. This fragment was cloned in pJ2925 to give pUO9021 (Fig. 1). The 2-kb *Bam*HI-*Sac*I fragment of pUO9021 hybridized with the PCR-generated probe. Its sequence (Fig. 2) contained a single predicted protein-encoding gene. The deduced product of this gene showed significant similarities with ADP-glucose pyrophosphorylases of different origin (47). The *S. coelicolor* gene product was most similar to the presumptive ADP-glucose pyrophosphorylases of gram-positive bacteria, particularly that of *Mycobacterium leprae* (59.6% identity), and was much more distantly related to the cyanobacterial enzymes and the large or small subunits of plant enzymes (Fig. 3).

In *E. coli* and plants, ADP-glucose pyrophosphorylases are under allosteric control. Figure 2 indicates three amino acid residues that have been identified as being important in activator, inhibitor, and substrate binding in the *E. coli* enzyme (25, 26, 36). The *S. coelicolor* gene product contains one of these residues, Lys-195, located in the motif Glu-Lys-Pro, which appears to be conserved in all ADP-glucose pyrophosphorylases as well as in related enzymes that also activate glucose-1-phosphate by nucleotidylation. In contrast, Tyr-114, a residue found in most bacterial enzymes and thought to be close to the adenosine ring of the substrate, is replaced by Phe in *S. coelicolor*, as in plants and cyanobacteria. Lys-39, which is part of the allosteric activator binding site in the *E. coli* enzyme, appears to be weakly conserved in other ADP-glucose pyrophosphorylases and is replaced by Ala in the *S. coelicolor* enzyme. However, a high degree of conservation is present in the primary sequence surrounding this residue in both bacterial and plant enzymes.

**Location of *glgC* in the chromosome of *S. coelicolor***. Hybridization of *glgC* probes to Southern blots of *S. coelicolor* DNA digested with the rare-cutting enzyme *Ase*I and separated by pulsed-field gel electrophoresis (blots kindly supplied by H. M. Kieser) showed that the *glgC* gene was located in *Ase*I fragment M (110 kb) (23). It was subsequently located in cosmid M11 of an ordered set of cosmids (42), about 1,000 kb from the left-hand end of the linear chromosome. It is therefore separated by large intervals from the two *glgB* genes of *S. coelicolor*, which are located about 2,500 kb (*glgB1*) and 450 kb (*glgB1I*) from the right-hand end (42).

**Disruption of *glgC* causes loss of the earlier of two peaks of ADP-glucose pyrophosphorylase activity and glycogen deposition during colony development**. To examine the role of *glgC* in *S. coelicolor*, we carried out insertional inactivation. An internal fragment of *glgC* was cloned in an *attP* site-deleted derivative of the temperate phage *φ*C31. The resultant phage, *φ*C31 MC1, was used to disrupt *glgC* by single-crossover integration, to give strain E002 (lacking the 66 C-terminal amino acid residues of the *glgC* product). The mutant showed no obvious changes in its overall growth, development, and pigment (antibiotic) production. To investigate whether strain E002 was defective in ADP-glucose pyrophosphorylase, the activity of this enzyme was measured at different phases of the life cycle, by using crude extracts of J1508 and E002 (Fig. 4). The wild-type strain showed two periods of high enzyme activity, one coinciding with the start of aerial mycelium formation and the other coinciding with sporulation. The specific activities, which were very reproducible, were very low compared to those reported for other bacteria (e.g., in reference 13), possibly because only a small proportion of hyphal compartments were engaged in glycogen synthesis in the differentiating mycelium. In addition, the reaction mixture may not be optimal for the *Streptomyces* enzyme, since it was developed for use with the enzyme from enteric bacteria (39). Remarkably, the *glgC*-disrupted strain specifically lacked the first peak of ADP-glucose pyrophosphorylase but retained the second, albeit delayed and at a slightly lower level than in the *glgC*+ strain. Since ADP-glucose pyrophosphorylase provides the activated precursor for glycogen synthesis, the *glgC* mutant should be deficient in glycogen at times corresponding to the first peak of wild-type activity. Indeed, a small early peak of amyloglucosidase-degradable polysaccharide was detected in J1508 and absent from E002 (Fig. 4). As with ADP-glucose pyrophosphorylase activity, the second peak of glycogen accumulation in the mutant was lower than that in the control.

**Cytological evidence that the *glgC* gene is specifically associated with phase I glycogen synthesis**. To provide spatial evidence that the first peak of ADP-glucose pyrophosphorylase activity corresponds to phase I glycogen deposition, a *glgC* mutant was analyzed cytologically by electron microscopy. In this case, the mutant (J1888) contained *glgC* disrupted by a *hyg* cassette, such that the highly conserved N-terminal 109 amino acids were dislocated from the rest of the protein, including several other highly conserved regions. Colonies of J1888 grown for 4 days on MM plus mannitol were prepared for transmission electron microscopy, and thin sections were stained with silver proteinate for polysaccharides. This procedure was previously shown to be largely glycogen specific in *S. coelicolor* by revealing striking and spatially specific changes in the appearance of polysaccharide deposits when *glgB1* and *glgB1I* (branching enzyme) mutants were studied (8). In agreement with the results of ADP-glucose pyrophosphorylase assays, phase II glycogen was readily detected in immature spore chains (Fig. 5), whereas phase I glycogen was entirely absent from the mycelial parts of the colony.

**DISCUSSION**

The use of PCR primers based on conserved regions of various ADP-glucose pyrophosphorylases led to the amplifica-
against Southern blots of *S. coelicolor* genomic DNA has not revealed candidate sequences, suggesting considerable sequence divergence. Likewise, the genes for phase I- and phase II-specific branching enzyme isoforms in various *Streptomyces* spp. appear in some, but not all, cases to have diverged enough to prevent their simultaneous detection with a single *glgB* probe (8, 18). Surprisingly, *glgCI* is located far from either of the two known *glgB* genes, whereas, in all other bacteria analyzed, *glgC* and *glgB* are very closely linked, forming part of a cluster of all the major *glg* genes (22). It is not yet known whether a *glgA* gene is linked to *glgCI* or to either of the *glgB* genes.

Neither of the two *glgCI*-disrupted mutants (E002 and J1888) showed any obvious deficiency in normal development and pigmented antibiotic production. We can therefore rule out, at least in the conditions tested, an essential role for phase I glycogen in either of these processes, though it may well make a contribution. In this context, the data in Fig. 4 may suggest that a lack of phase I glycogen in the *glgC* mutant limits the supply of carbon for both aerial growth (causing reduced specific activity of phase II-associated ADP-glucose pyrophosphorylase) and phase II glycogen deposition (as reflected in the reduced contribution of phase II glycogen to the dry weight of the total mycelium). Probably, it will be necessary to inactivate the production of other carbon storage compounds, such as triacylglycerol (35), before the developmental role of storage metabolism can be properly evaluated.

These results in this paper, following the earlier discovery of genetically distinct, tissue-specific isoforms of glycogen branching enzyme, reinforce the idea of the *Streptomyces* colony as a multicellular, differentiating organism. It would not be surprising if all the steps in glycogen synthesis, and quite likely other pathways involved in localized metabolism of other storage compounds, such as triacylglycerol (35, 37) and polyphosphates (37), turn out to involve duplicated enzyme isoforms.

**ACKNOWLEDGMENTS**

This work was funded by grants from the EC (contracts BIOTCT910255 and ERBCHBICT941012) and the Comisión Interministerial de Ciencia y Tecnología (CICYT) (BIOT 94-1025), a BBSRC ISIS award, and a grant-in-aid from the BBSRC to the John Innes Centre. M.C.M. also received a postdoctoral fellowship from the Fondo para la Investigación Científica y Técnica del Principado de Asturias (FICYT).

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