

A New Piece of an Old Jigsaw: Glucose Kinase Is Activated Posttranslationally in a Glucose Transport-Dependent Manner in *Streptomyces coelicolor* A3(2)

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Key Words

Phosphotransferase system · GlcP · Posttranslational modification · Glucose transport · Catabolite control

Abstract

Members of the soil-dwelling prokaryotic genus *Streptomyces* are indispensable for the recycling of complex polysaccharides, and produce a wide range of natural products. Nutrient limitation is likely to be a major signal for the onset of their development, resulting in spore formation by specialized aerial hyphae. Streptomycetes grow on numerous carbon sources, which they utilize in a preferential manner. The main signaling pathway underlying this phenomenon is carbon catabolite repression, which in streptomycetes is totally dependent on the glycolytic enzyme glucose kinase (Glc). How Glc exerts this fascinating dual role (metabolic and regulatory) is still largely a mystery. We show here that while Glc is made constitutively throughout the growth of *Streptomyces coelicolor* A3(2), its catalytic activity is modulated in a carbon source-dependent manner: while cultures growing exponentially on glucose exhibit high Glc activity, mannitol-grown cultures show negligible activity. Glc activity was

directly proportional to the amount of two Glk isoforms observed by Western blot analysis. The activity profile of GlcP, the major glucose permease, correlated very well with that of Glk. Our data are consistent with a direct interaction between Glk and GlcP, suggesting that a Glk-GlcP permease complex is required for efficient glucose transport by metabolic trapping. This is supported by the strongly reduced accumulation of glucose in glucose kinase mutants. A model to explain our data is presented.

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Introduction: The Story So Far

The soil-dwelling, Gram-positive streptomycetes undergo complex morphological development (spore germination – vegetative mycelium – aerial mycelium – spores) [Chater, 1998; Claessen et al., 2006; Schauer et al., 1988; Willey et al., 1991]. Perhaps the most important signal for the onset of development is nutrient limitation, suggesting a direct link between carbon-sensing systems and developmental control. Many streptomycetes sporulate poorly on glucose-containing agar media when com-

pared to those containing mannitol as sole carbon source. Furthermore, many of the early developmental mutants (called bald or *bld* due to their failure to produce an aerial mycelium) are able to circumvent the developmental arrest when grown on mannitol-containing media [Merrick, 1976; Pope et al., 1996]. While several lines of evidence suggest that catabolite control forms an important checkpoint towards the onset of development, mutants devoid of carbon catabolite control produce viable spores and respond normally to nutritional stimuli [Angell et al., 1994].

In unicellular bacteria such as *Escherichia coli* and *Bacillus subtilis*, the phosphoenolpyruvate-dependent phosphotransferase system (PTS) plays a dominant role in carbon regulation [Brückner and Titgemeyer, 2002; Saier and Reizer, 1994]. Its phosphotransferases confer global control to carbon utilization operons by triggering the action of master regulators, known as CcpA (catabolite control protein A) in low-GC Gram-positive bacteria and Crp (cAMP-receptor protein) in Gram-negative enteric bacteria. The genes of the PTS have been also studied in detail in *Streptomyces coelicolor* and other high-GC Gram-positive bacteria (see this issue Moon et al.) [Butler et al., 1999; Moon et al., 2005; Nothaft et al., 2003a, b; Parche et al., 1999, 2001]. In *S. coelicolor*, null mutants of the global *pts* genes (*ptsH*, *ptsI* and *crr*) have a bald phenotype, which is the first genetic evidence of a direct link between carbon utilization and the control of development [Nothaft et al., 2003a; Rigali et al., 2006]. The importance of the PTS for the control of development is further underlined by the observation that addition of the major PTS substrate *N*-acetylglucosamine locks streptomycetes in the vegetative state [Rigali et al., 2006]. However, a direct role of the PTS in carbon regulation remains obscure [Butler et al., 1999; Nothaft et al., 2003a; Parche et al., 1999; Rigali et al., 2006].

In contrast, glucose kinase (Glk; gene *glkA*) appears to be a central player in the game of carbon catabolite repression in *S. coelicolor* [Angell et al., 1992, 1994; Hodgson, 1982, 2000; Kwakman and Postma, 1994]. Mutants lacking a functional *glkA* gene are unable to grow on glucose as sole carbon source and are deregulated in glucose repression of several catabolite-controlled genes and operons including those for the utilization of agar, glycerol, maltose and galactose [Angell et al., 1992; Brawner et al., 1997; Hindle and Smith, 1994; Kwakman and Postma, 1994]. Additionally, restoration of glucose kinase activity by introduction of a functional glucose kinase gene from *Zymomonas mobilis* failed to restore catabolite control

[Angell et al., 1994]. It was further demonstrated that glucose repression operates at the transcriptional level. The active form of glucose kinase is most likely a stable tetrameric complex [Imriskova et al., 2005]. Due to the lack of a DNA-binding motif, Glk has been proposed to interact with transcription factors, such as GylR and MalR, which are responsible for both specific and global catabolite control of the glycerol and maltose regulons [Hindle and Smith, 1994; Kwakman and Postma, 1994; Mahr et al., 2000; van Wezel et al., 1997]. Despite extensive research, the mechanism through which Glk exerts its dual role is still largely a mystery.

In this paper we provide evidence that the activity of glucose kinase is modulated in a carbon source- and growth phase-dependent manner. We show further that the activity profiles of Glk and GlcP are very similar and suggest that they interact with each other to form an active Glk/GlcP permease complex. The detection of three apparent glucose kinase isoforms leads to the suggestion that the role of glucose kinase in carbon regulation is triggered by posttranslational modification.

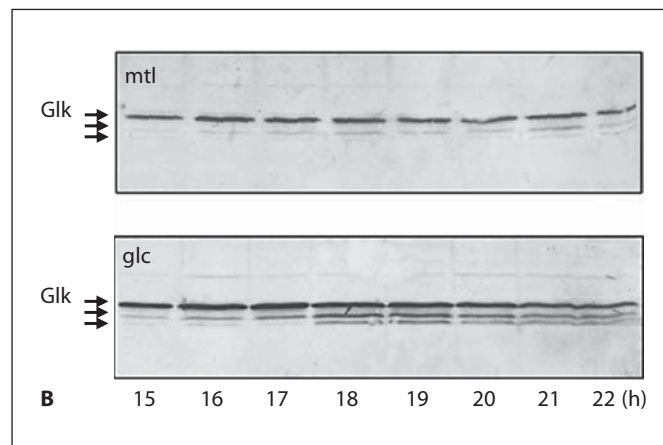
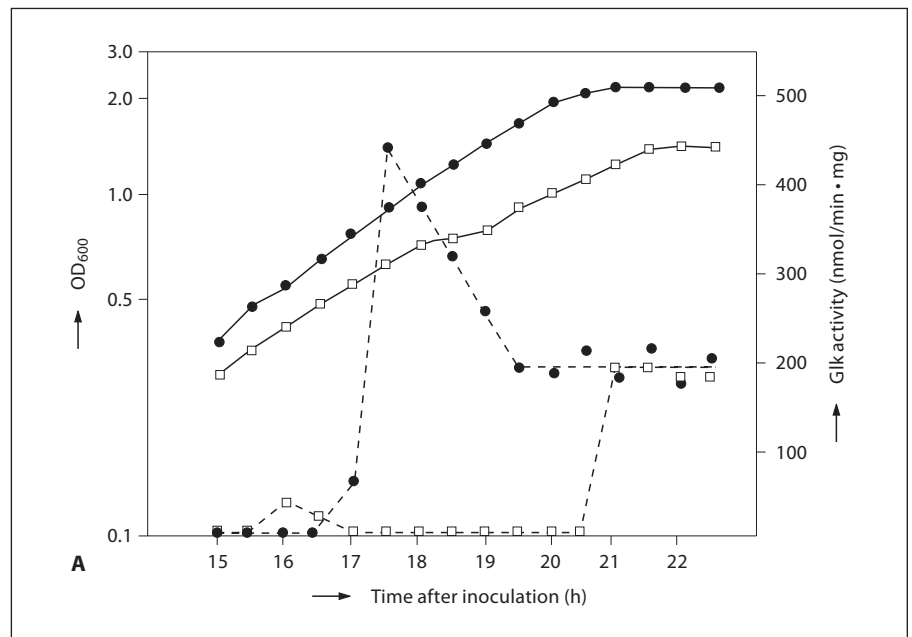
Results and Discussion: Novel Insights

Carbon Utilization Affects the Enzymatic Activity of Glucose Kinase

Glucose kinase is made constitutively in submerged cultures, independent of the carbon source used [Mahr et al., 2000]. Glucose-independent control of *glkA* expression is consistent with the observation that glucose kinase is not only involved in catabolite repression exerted by glucose, but also by carbohydrates that do not require the presence of a catalytically active glucose kinase for their metabolism [Kwakman and Postma, 1994].

To assess the growth-phase dependence of glucose kinase activity, *S. coelicolor* M145 was grown in NMMP minimal medium with casamino acids and glucose or mannitol as carbon source. *S. coelicolor* grew more efficiently in glucose-grown cultures, and reached a higher final optical density (fig. 1). Protein extracts prepared from mycelium of the same growth curves were analyzed using a glucose kinase activity assay. Interestingly, most samples obtained from exponentially growing mannitol-grown cultures showed negligible activity (below 5 nmol/min·mg), but this activity increased to around 200 nmol/min·mg when the stationary phase was reached. In contrast, we observed a sharp rise in Glk activity (up to approximately 500 nmol/min·mg) during the mid-exponential phase in the glucose-grown cultures (fig. 1). On

Fig. 1. Glucose-dependent activation and modification of glucose kinase. **A** Growth curves (solid lines) and glucose kinase activities (dashed lines) of *S. coelicolor* M145 grown in NMMP supplemented with glucose (closed circles), or mannitol (open squares) are depicted. Note that Glk activity is induced during exponential growth in glucose- but not in mannitol-grown cultures. Glk activities were standardized against total protein content, and corrected for Glk concentrations. **B** Western blot analysis of *S. coelicolor* M145 extracts from submerged cultures. The growth curves and Glk activities are shown in figure 1A. All protein extracts were standardized against total protein content. Clearly visible are two additional bands with a migration rate faster than the major Glk immunosignal. It is anticipated that Glk is activated in the presence of glucose by posttranslational modification.



transition to the stationary phase, activity dropped to a significantly lower level, comparable to the maximum values observed in mannitol-grown cultures.

While glucose kinase activity varied enormously dependent on carbon source and growth phases, Western blot analysis showed that glucose kinase was produced constitutively in both cultures, although total protein levels were somewhat higher in glucose-grown cultures (fig. 2). Surprisingly, two minor bands were observed, migrating slightly faster than the main Glk band. These bands were particularly strong during mid- and late-exponential growth in the presence of glucose (17–24 h), but much weaker in the mannitol-grown cultures. Such constitutive expression is in line with earlier observations

[Angell et al., 1994; Mahr et al., 2000]. However, no minor bands were detected in our previous study [Mahr et al., 2000]. This difference can be explained by the fact that we used (for the Western blot shown in fig. 1B) a gel system that produced very sharp and focused protein bands (compare also figures 1 and 4). The measured glucose kinase activity correlated with the abundance of the faster migrating minor Glk band, particularly after approximately 18 h of growth. Thus, there was no direct relationship between the amount of glucose kinase in the protein extracts and the enzymatic activity of the protein. This implies either proteolytic processing or posttranslational modification. For example, phosphorylation of Glk could be involved in modulating its activity. Such phosphoryla-

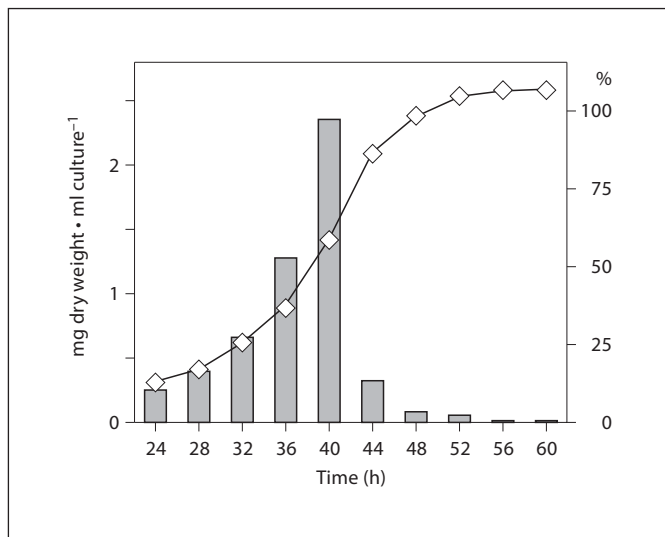


Fig. 2. Glucose transport activity profile. The figure shows the initial glucose uptake rates of *S. lividans* TK24 (grey bars; nmol glucose/mg dry weight/min) along the growth curve (diamonds; mg dry weight ml⁻¹).

tion events can be detected by Western blot, as shown for the carbon regulation PTS factor enzyme IIA^{Glc} of *E. coli* [Hogema et al., 1998].

In a control experiment, we analyzed the glucose kinase mutants M480 and J1915. Cross-reactivity with bands around 35–40 kDa was not observed with extracts from these mutants (not shown). *S. coelicolor* has a second gene encoding a protein with glucose kinase activity, designated GlkII (SCO6260), which is inactive in normal cells and whose expression can be induced at high frequency in *glkA* mutants grown for a prolonged period in the presence of glucose [Angell et al., 1994]. SCO6260 is part of an operon that encodes an ABC-type permease for ribose or ribonucleosides and therefore is likely to be a sugar kinase for one of these substrates [Bertram et al., 2004]. SCO6260 (382 aa, 41 kDa) is significantly larger than Glk (317 aa, 33 kDa), with 46% amino acid identity (68% similar) between the major Glk (full aa sequence) and GlkII (aa 36–348). Considering the much larger size of GlkII it cannot correspond to the faster migrating bands detected in the Western blot (fig. 1B). Therefore it is likely that these bands represent isoforms of Glk.

Relationship between Glucose Kinase Activity and Glucose Transport

We showed recently that *S. coelicolor* internalizes glucose by the proton symporter GlcP and not by a PTS as

most other bacteria do [van Wezel et al., 2005]. While there are two *glcP* genes with identical gene products, only *glcP1* is expressed and encodes the major glucose uptake system [van Wezel et al., 2005]. We were curious to compare the Glk activity profile with the profile of glucose transport. For this, we used the closely related *Streptomyces lividans* TK24 that has the identical Glk/GlcP system [van Wezel et al., 2005]. Interestingly, the glucose transport rates measured from cells taken along the growth curve showed a very similar profile to that of Glk activity (compare figure 1A with figure 2), suggesting a tight functional relationship.

When we assayed glucose uptake in the *glkA* deletion strain M480, we found that accumulation of glucose was strongly reduced (fig. 3A). The kinetic curves presented in figure 3A show that glucose uptake was inducible in both strains, when transport from mycelia grown in the absence and presence of glucose was compared. However, glucose uptake stopped after 1 min in the *glkA* mutant, while *glkA*⁺ mycelia continued to transport glucose (fig. 3A). Quantitative real-time RT-PCR experiments showed that the basal level as well as the inducibility of *glcP1* transcription was at least as high (if not higher) in the *glkA* mutant as in the parental strain (fig 3B). Hence, the difference in glucose transport activities between M145 and M480 is not caused by differences in the expression of *glcP1*. A likely explanation is that glucose uptake in the *glkA* mutant is inhibited by the intracellular accumulation of glucose. A reduction in glucose uptake has been also observed in a glucose kinase mutant of *Streptomyces peucetius* [Ramos et al., 2004]. The authors inferred from their study that glucose might play an indirect role in glucose repression due to this effect.

The above data suggest that GlcP and Glk operate in concert by metabolic trapping and perhaps interact with each other. To address this point, we analyzed whether Glk binds to GlcP. We grew M145 in NMMP minimal medium with glycerol (no GlcP) and glucose (GlcP present) and measured the amount of cytoplasmic and membrane-associated Glk by Western blot analysis (fig. 4). While in glycerol-grown cultures all Glk was found in the cytoplasmic fraction, a significant amount of Glk protein was found in the membrane-associated fraction prepared from glucose-grown cultures. Interestingly, the amount of Glk found in the membrane-associated fraction increased dramatically in cells harboring multiple copies of *glcP* (resulting from overexpression of the GlcP glucose transporter). This observation strongly suggests that Glk interacts directly with GlcP.

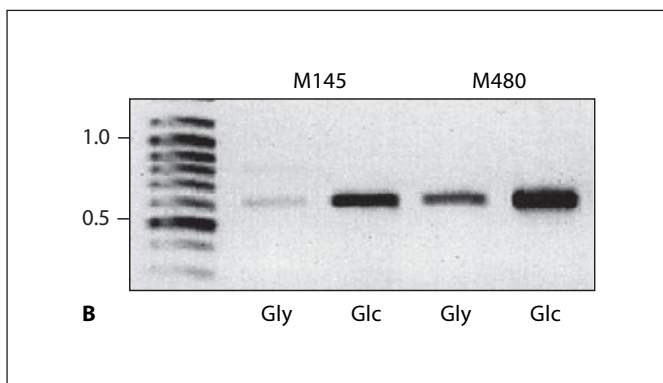
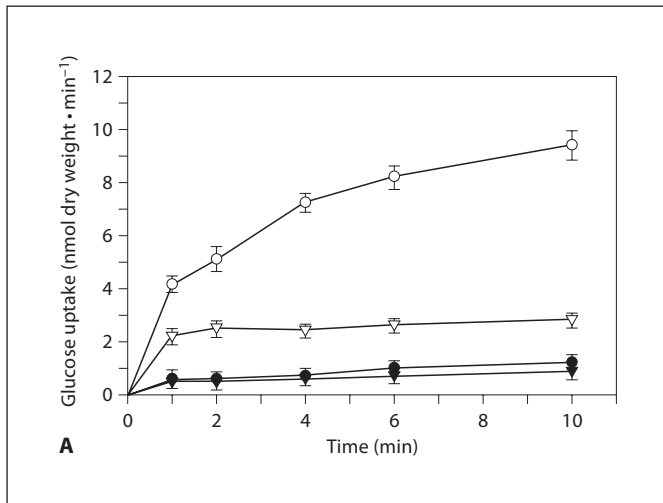


Fig. 3. Glucose transport depends on Glk. **A** Glucose uptake in *S. coelicolor* M145 and M480. M145 (*glkA*⁺; circles) and M480 (*glkA* mutant; triangles) were grown in liquid mineral medium supplemented with 50 mM glycerol (gly; closed symbols) or 50 mM glycerol plus 50 mM glucose (glc/gly; open symbols). Standard deviations of triplicate data points are presented by error bars. **B** Quantitative RT-PCR data are shown from RNA isolated from liquid-grown mycelia of M145 and M480. Total RNA was prepared from exponentially growing NMMP cultures supplemented with 50 mM glycerol and 50 mM glycerol plus glucose of M145 or M480. Amplification products were separated on a 1% agarose gel. Note that inducibility of *glcP1* transcription by glucose does not depend on the presence of glucose kinase.

Conclusions

The model presented in figure 5 summarizes our current understanding on the role of glucose kinase in glucose metabolism and global carbon catabolite repression. While the amount of Glk protein is more or less independent of the growth phase, its catalytic activity depends on the presence of glucose. In this scenario, the Glk tetramer

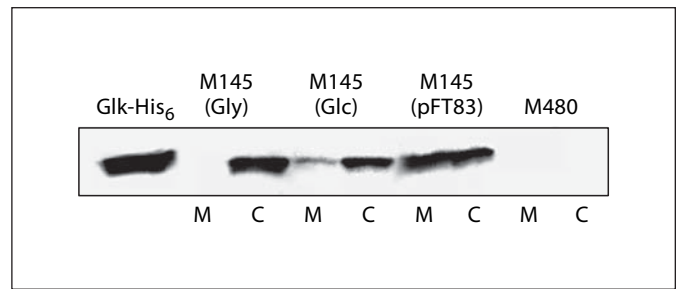


Fig. 4. Localization of glucose kinase. The Western blot of a 7.5% SDS-polyacrylamide gel with Glk-specific antibodies shows the amount of Glk in 50 μ g of the membrane (M) and cytoplasmic (C) protein fractions. Cells were grown on glycerol (Gly) or glucose (Glc). Glk is present in the membrane fraction under conditions when GlcP is expressed. This is especially obvious in extracts of M145(pFT83 *glcP*⁺) where GlcP is overproduced, which suggests a GlcP-Glk protein interaction.

is in complex with GlcP for efficient glucose uptake by metabolic trapping [Soupeine et al., 1998; Voegelé et al., 1993], while the cytoplasmic form will act in response to glucose, ATP, or other yet unknown metabolites. Our data suggest that Glk activity is modulated through metabolite-dependent activation and/or posttranslational modification (Glk^{*}). This may be similar to the mechanism of carbon catabolite repression in low-GC Gram-positive bacteria, where the metabolite-activated HPr kinase/phosphatase triggers the global repressor complex CcpA/HPr-Ser-P through serine phosphorylation of the PTS phosphotransferase HPr [Brückner and Titgemeyer, 2002; Reizer et al., 1998]. However, the exact nature of the modifications that (de)activate Glk are unknown but will have to be determined to fully appreciate the function of Glk in mediating carbon catabolite repression in streptomycetes. Intriguingly, Glk belongs to a subclass of Gram-positive glucose kinases that harbor a set of cysteines that are crucial for activity [Mesak et al., 2004]. This cysteine-rich motif may also play a key role in the functional control of Glk and thus should be targeted for mutational analysis. Recent studies on glucose repression of production of the antibiotic anthracycline in *S. peucetius* provided evidence that the gene upstream of *glkA* (SCO2127 in *S. coelicolor*) also influences glucose transport [Guzman et al., 2005a, 2005b]. Hence, it will be of primary interest to elucidate the function of SCO2127.

Despite the wealth of data gathered over the past 25 years, the veil on the glucose kinase mystery, namely how Glk can exert both global carbon catabolite repression

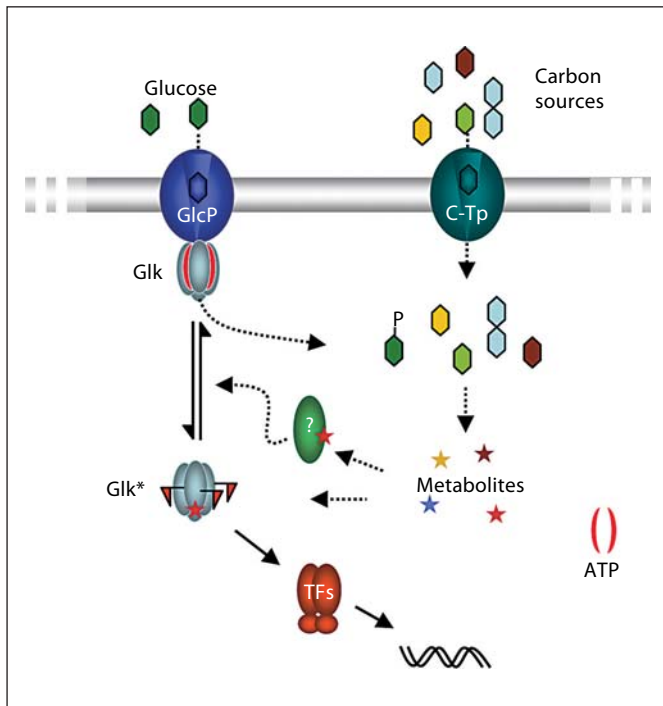


Fig. 5. Model on the function of glucose kinase. Glk interacts with GlcP in the presence of glucose to immediately convert the incoming sugar to Glc-6-phosphate. Glucose and other carbon sources, which enter the cell via specific carbohydrate permeases (C-Tp), generate metabolites during catabolism. These trigger Glk (Glk*) and/or an unknown nutrient sensor protein that is required for posttranslational modification of Glk. Glk* interacts with transcription factors (such as MalR, GylR, BldB, etc.) to exert carbon catabolite repression.

and its catalytic activity, has still not been fully lifted. Additional pieces of the jigsaw belonging to one of the longest standing debates in *Streptomyces* molecular biology still have to be uncovered.

Experimental Procedures

Bacterial Strains and Plasmids

Strains of *S. coelicolor* used in this communication were M145 (SCP1⁻ SCP2⁻ [Kieser et al., 2000] and its Δglk mutant derivatives M480 and J1915 [Angell et al., 1992; Kelemen et al., 1998]. *S. lividans* TK24 was used to generate the glucose uptake activity profile [Kieser et al., 2000]. *E. coli* DH5 α was used for propagation and isolation of plasmid DNA [Sambrook et al., 1989]. Plasmid pFT83 is a derivative of the multi-copy shuttle vector pUWL-KS, and harbors the promoter region of *glkA* fused to the coding region of *glcP1* resulting in overproduction of GlcP [Wehmeier, 1995].

Glucose Kinase Activity Assays

S. coelicolor strains were grown in 500 ml minimal medium (NMMP) [Kieser et al., 2000] supplemented with 1% (w/v) glucose or mannitol, under vigorous shaking at 28°C. Cells were harvested at 30-min intervals, washed twice and then resuspended in cold standard buffer (50 mM Tris pH 7.4, 5 mM MgCl₂, 40 mM NH₄ acetate, 50 mM NaCl, 1 mM DTT). Crude extracts were prepared by sonication and subsequent removal of cell debris by centrifugation. Glucose kinase activity in cell extracts was assayed using 50 μ g of total protein in a reaction mixture containing 50 mM Tris-Cl (pH 7.0), 20 mM glucose, 25 mM MgCl₂, 0.5 mM NADP, 1 mM ATP, and 0.7 U glucose-6-P dehydrogenase.

Glucose Growth Curve and Transport Assay

Spores of *S. lividans* TK24 were germinated and used at 7.5×10^7 ml⁻¹ to inoculate 50 ml R2YE lacking sucrose; the cultures were grown for 15 h at 30°C [Kieser et al., 2000]. Cells were washed twice in R2YE medium and 100 mg of mycelia were inoculated in 1 l of R2YE without sucrose in Erlenmeyer flasks supplemented with 1% (w/v) glucose or glycerol. Cells were grown under vigorous shaking at 30°C. Aliquots of 10 ml were taken every 4 h and centrifuged. Pellets were dried in a speed vacuum concentrator for 30 min at 50°C and weighed after drying. Dry weight (mg dry weight ml culture⁻¹) was plotted against time to generate a growth curve. Transport assays were performed as described previously [Nothaft et al., 2003b]. Cells were harvested by centrifugation, washed twice in transport buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM KCl), adjusted to 1.0–1.5 mg dry weight ml⁻¹, and preincubated for 5 min at 30°C. Uptake was initiated by addition of [U-C¹⁴]glucose at a final concentration of 20 μ M (5 mCi mmol⁻¹). Samples were taken at different times (10 s to 10 min) and treated as described [Nothaft et al., 2003b]. Initial glucose uptake rates were calculated from data obtained within the first 60 s when the transport was linear. Data were derived from at least three independent experiments.

Western Blot Analysis

S. coelicolor strains were grown as described for the glucose kinase activity assay. Proteins present in the cell extracts were separated by SDS-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel and transferred to a Hybond-C super (Amersham) by electroblotting. Glk was detected with a rabbit polyclonal antiserum raised against Glk(His₆) of *S. coelicolor*. Glk antibodies were visualized using the ECL Western blot analysis system (Amersham) [Mahr et al., 2000].

Localization of Glucose Kinase

Strains M145, M145(pFT83 *glcP*⁺), and M480 ($\Delta glkA$) were grown in 100 ml NMMP minimal medium with 0.1% casamino acids and 50 mM carbon source (and antibiotic when required) at 28°C to mid-exponential phase. They were harvested by centrifugation and washed in 50 mM NaCl, 50 mM Tris-HCl pH 7.5 and 10% glycerol. Pellets were resuspended in 4 ml of the same buffer and subjected to sonication. Cell debris was removed by centrifugation to obtain the cell-free extracts. These were separated into the soluble, cytoplasmic protein fraction and membrane fraction (pellet) by ultracentrifugation at 65,000 g for 1 h at 4°C. The oily membrane pellet was solubilized by sonication in 300 μ l buffer. The presence of Glk in the cytoplasmic and membrane protein fractions was determined by subjecting 50 μ g

of protein to polyacrylamide gel electrophoresis and subsequent Western blot analysis using Glk-specific polyclonal antibodies [Mahr et al., 2000].

Reverse Transcriptase Polymerase Chain Reactions

RNA was isolated from mycelium of *S. coelicolor* M145 and M480 (Δ glkA). NMMP cultures containing 0.1% casamino acids and 50 mM glycerol or 50 mM glucose were inoculated with spores and grown to mid-exponential phase (36–40 h). Total RNA was prepared as described and RT-PCR analyses were carried out us-

ing the one-step RT-PCR kit from Qiagen [van Wezel et al., 2005]. The quality of the RNA preparations was confirmed by the presence of equal amounts of constitutively expressed 16S rRNA using oligonucleotides 16SRT-for and 16SRT-rev. RT-PCR experiments without prior reverse transcription were performed as a control for DNA contamination. For quantitative analysis, samples were taken at three-cycle intervals between cycles 18 and 36 to compare non-saturated PCR product formation. Data were verified by experimental reproduction.

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