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Publication date
1994

Published in
Journal of Bacteriology

[Link to publication](#)

Citation for published version (APA):

Kwakman, J. H. J. M., & Postma, P. W. (1994). Glucose kinase has a regulatory role in carbon catabolite repression in *Streptomyces coelicolor*. *Journal of Bacteriology*, 176, 2694-2698.

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Glucose Kinase Has a Regulatory Role in Carbon Catabolite Repression in *Streptomyces coelicolor*

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Received 6 December 1993/Accepted 15 February 1994

A glucose kinase (*glkA*) mutant of *Streptomyces coelicolor* A3(2) M145 was selected by the ability to grow in the presence of the nonmetabolizable glucose analog 2-deoxyglucose. In this *glkA* mutant, carbon catabolite repression of glycerol kinase and agarase was relieved on several carbon sources tested, even though most of these carbon sources are not metabolized via glucose kinase. This suggests that catabolite repression is not regulated by the flux through glucose kinase and that the protein itself has a regulatory role in carbon catabolite repression. A 10-fold overproduction of glucose kinase also results in relief of catabolite repression, suggesting that excess glucose kinase can titrate the repressing signal away. This could be achieved directly by competition of excess glucose kinase with its repressing form for binding sites on DNA promoter regions or indirectly by competition for binding of another regulatory protein.

Streptomycetes are prokaryotic mycelial soil organisms with a complex life cycle in which the bacteria can go through different morphological stages. When the bacteria enter stationary phase, they start to produce a variety of metabolites which are not used for creating biomass. These secondary metabolites possess a large spectrum of biological activities (e.g., antibiotic, immunosuppressant, and insecticidal), which makes these organisms interesting both industrially and scientifically. During the last three decades, research has focused mainly on the production of secondary metabolites and their biosynthetic pathways in a vast number of distantly related streptomycetes. This has led to scattered knowledge about metabolism and its regulation in these organisms. One of the aims of this project is to investigate in more detail the process of catabolite repression in the genetically most-characterized streptomycete, *Streptomyces coelicolor* A3(2).

Catabolite repression is a widespread phenomenon in nature which can be defined as the repression of enzyme activities by the presence of a catabolite in the growth medium. Although this can be any catabolite, most interest has focused on the mechanism of glucose repression. Organisms used for such studies are the yeast *Saccharomyces cerevisiae* and several gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*) and gram-positive (*Bacillus subtilis* and *S. coelicolor*) bacteria, and they seem to have different mechanisms for glucose repression.

In *S. coelicolor* an enzyme responsible for phosphorylation of glucose seems to play a central role in glucose repression both at the level of transcription and at the level of inducer exclusion. This was concluded from the lack of glucose repression in 2-deoxyglucose-resistant strains which lacked glucose kinase activity (2, 5, 8, 11, 18). Most of the mutants had lost the ability to grow on glucose as a sole source of carbon (8). A fragment of 2.9 kb that complemented the growth defect on glucose was cloned (11), and this sequence contained one partial open reading frame (ORF1) and two complete open reading frames coding for proteins of 30 kDa (ORF2) and 33

kDa (ORF3) (2). Subcloning experiments revealed that ORF3 alone could restore glucose kinase activity in the 2-deoxyglucose-resistant *S. coelicolor* strains and in *E. coli glk* mutants and that ORF3 encodes the *S. coelicolor* glucose kinase (*glkA* gene).

Carbon catabolite repression is also exerted by carbon sources other than glucose, as was shown for *S. coelicolor* at the level of glycerol kinase activity (18). In order to investigate the role of glucose kinase in carbon catabolite repression, a glucose kinase mutant of *S. coelicolor* A3(2) was tested for pleiotropic effects on enzyme activities by several carbon sources. Furthermore, the effect of overexpression of glucose kinase on carbon catabolite repression was tested on several carbon sources by testing strains of *S. coelicolor* A3(2) containing the *glkA* gene on a multicopy vector. Both deletion and overexpression of *glkA* seem to deregulate catabolite repression on several carbon sources, even though these carbon sources are not metabolized via glucose kinase. In this paper we propose possible models for the role of glucose kinase in carbon catabolite repression in *S. coelicolor* A3(2).

MATERIALS AND METHODS

Bacterial strains. *S. coelicolor* A3(2) M145 (prototrophic, SCP1[−] SCP2[−] [10]) was used as the wild-type strain in this study. *E. coli* ET 12567 (*recA dam::Tn9 dcm-6*) was used to generate unmethylated DNA, which could be directly used to transform *S. coelicolor* A3(2) M145. Transformations were performed by the protoplast method (10).

Constructs. The high-copy-number plasmid containing the *glkA* gene (pIJ2427) was kindly donated by Sue Angell (1). The construct contains a 1.2-kb fragment containing *glkA* in the high-copy-number *Streptomyces* vector pIJ702 (10). It contains 78 bp of sequence upstream from the start codon of *glkA*, the complete ORF of *glkA*, and 121 bp of sequence downstream from the stop codon.

Transposon mutagenesis. In order to construct a defined glucose kinase mutant of *S. coelicolor*, a genomic mutant collection was generated from *S. coelicolor* M145 by insertional mutagenesis with a derivative of the *Streptomyces lividans* insertional element IS493, Tn5096 (20). All 2-deoxyglucose-resistant mutants lacked glucose kinase activity, and they were unable to utilize glucose as a sole carbon source on silica-based

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plates. Southern analysis and PCR showed, however, that the transposons were not inserted into the glucose kinase gene, implying that these were spontaneous *glkA* mutants. A mutant which was characterized as carrying a point mutation or a small deletion mutation of *glkA* (KJ3) was used for further study of carbon catabolite repression.

Growth conditions. For the agarase spot tests, minimal (MM) agar plates containing 15 mM $(\text{NH}_4)_2\text{SO}_4$, 3 mM K_2HPO_4 , 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 40 μM FeSO_4 , and 2% Difco bacteriological agar were used. In order to avoid growth on agar, utilization of carbon sources was tested on silica-based plates (7a). These plates consisted of 50% ludox HS40 (purchased from Stokvis Chemicalien b.v., Barendrecht, The Netherlands, and dialyzed twice against 10 mM phosphate buffer [pH 6.8] and once against demineralized water), 15 mM phosphate buffer (pH 6.8), 7.5 mM $(\text{NH}_4)_2\text{SO}_4$, 0.8 mM MgSO_4 , 40 μM FeSO_4 , 0.6 M NaCl, and 55 mM carbon source. Setting of the silica was started after addition of the NaCl, and the plates were solidified after 16 h at room temperature. Submerged cultures for the induction of glycerol kinase were made by allowing spores to grow uninduced to a young mycelial state by inoculating ca. 10^8 washed spores in a 250-ml baffled flask with 100 ml of minimal liquid medium (NMM) [15 mM $(\text{NH}_4)_2\text{SO}_4$, 2.5 mM MgSO_4 , 15 mM phosphate buffer (pH 6.8), and 0.5% (wt/vol) Casamino Acids] containing 55 mM mannitol as a carbon source. After 16 h of growth at 30°C and 200 rpm in an orbital shaker, the mycelium was collected by centrifugation (5 min at $8,000 \times g$), washed once with NMM, and resuspended in 10 ml of NMM. Induction was started by adding 1 ml of the inoculum to 30 ml of NMM containing 100 mM glycerol with or without 55 mM repressing carbon source. For glucose kinase measurements, an inoculum of 5×10^6 spores was grown for 24 h at 30°C at 200 rpm in 30 ml of NMM containing 50 mM mannitol and treated similarly.

Enzyme activities. Cell extracts were made by a modification of the protocol of Seno and Chater (18). The mycelial cultures (30 ml) were collected by centrifugation, washed twice with ice-cold 150 mM NaCl, and resuspended in 1 ml of PED buffer (75 mM KH_2PO_4 , 2 mM dithiothreitol, and 1 mM EDTA, adjusted to pH 7.0 with KOH). The mycelia were disrupted by sonification of the bacterial suspension (three times for 1 s each at maximal output on a Branson B-12 sonifier). Cell debris and membranes were pelleted by centrifugation for 10 min in a Beckman Airfuge ($150,000 \times g$) at 4°C. The supernatant was preincubated for 30 min at 25°C prior to enzyme activity measurements. Glucose kinase and glycerol kinase activities were determined by measuring the formation of ADP in a coupled assay containing phosphoenolpyruvate, pyruvate kinase, NADH, and lactate dehydrogenase. NADH oxidation was monitored at 340 nm on a COBAS-BIO automated spectrophotometer. Glycerol kinase reactions were allowed to equilibrate for 90 s prior to activity measurements. The mixtures for kinase activity assays (200 μl per cuvette) contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 2 mM ATP, 1 mM phosphoenolpyruvate, 5 U of pyruvate kinase, and 10 U of lactate dehydrogenase at 25°C. The reaction was started by adding 0.3 mM NADH (ATPase background), 10 mM glucose plus 0.3 mM NADH (glucose kinase), or 10 mM glycerol plus 0.3 mM NADH (glycerol kinase). Protein concentrations were determined with the bicinchoninic acid method (19). Specific activities were expressed as nanomoles of NAD formed per minute per milligram of protein.

Agarase spot test. Spores were taken from a glycerol stock, washed with 1 ml of H_2O , and resuspended in H_2O to a final concentration of about 10^6 spores per μl . Spore suspension (10 μl) was spotted on MM agar plates and allowed to dry in a

laminar flow. The plates were then allowed to incubate for 72 h at 30°C, which was followed by polysaccharide staining with ca. 5 ml of a 0.1% KI-I₂ solution. Extracellular agarase activity was scored by measuring the size of the halo.

Southern analysis. *S. coelicolor* chromosomal DNA was isolated by using the small-scale alkaline lysis procedure (10). DNA (10 μg) was digested and run on a 0.9% agarose gel in 0.04 M Tris-acetate buffer containing 1 mM EDTA (TAE buffer). Subsequently it was vacuum blotted onto an uncharged Qiagen nylon membrane. Probes were labeled by using the Boehringer digoxigenin-11-dUTP labeling kit with the mixed hexanucleotide primer and the Klenow fragment of DNA polymerase. Hybridizations were performed at 42°C in 50% formamide-5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% lauroylsarcosine-0.02% sodium dodecyl sulfate (SDS)-1% blocking solution (Boehringer). Washing was performed twice for 20 min each at 65°C with 2 $\times$ SSC-0.1% SDS. Hybridizing DNA was visualized by staining of digoxigenin antibodies with lumigen purified protein derivative (Boehringer Mannheim) followed by fluorography.

PCR. Chromosomal DNA was isolated by using the small-scale alkaline lysis procedure (10). DNA (2 μg) was denatured by boiling for 5 min in 49 μl of 6% glycerol-1 $\times$ PCR buffer (from the Biozym Replitherm kit) in the presence of 200 ng of two oligonucleotides directed to the 5' end of *glkA* (G759 [5'CAGCGCATCGACCTGGAC3'], positions -75 to -58 from the start of ORF3) and to the 3' end of *glkA* (G1276 [5'CAGTTCCCCGCGCCCTC3'], positions 989 to 1006 of ORF3). The DNA was immediately put on ice, and PCR was started with 0.25 mM deoxynucleoside triphosphates and 1 U of Replitherm DNA polymerase (Biozym) in a total volume of 50 μl . The mixture was covered with 50 μl of mineral oil and put through 30 cycles of 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C.

Chemicals. Apramycin (Apralan) was obtained from the A.U.V., Cuyk, The Netherlands, and thiostrepton was obtained from Sigma. Bacteriological agar from Difco was used as the agar source.

RESULTS

Catabolite repression. One of the few known genes which is regulated by catabolite repression in *S. coelicolor* is the gene encoding glycerol kinase (18). In previous studies (18), the level of induction and repression of glycerol kinase activity reached a plateau about 8 h after induction had started, and under our conditions we observed similar behavior of the enzyme (Fig. 1A). Since the level of repression of glycerol kinase remained constant after 8 to 24 hours of growth, we chose to sample the cultures after 16 h of growth, which corresponded to the end of exponential phase. The induction of glycerol kinase was repressed about 2- to 10-fold by five different carbon sources tested in wild-type *S. coelicolor* (Fig. 1B). In 10 independent experiments the level of repression of glycerol kinase activity by glucose or arabinose varied about 10%.

The second reporter, agarase, is an extracellular enzyme which breaks down the agar into disaccharides (5). Activity of the enzyme results in a clear halo around a colony or mycelial spot, which can be visualized by iodine staining, so if a set amount of spores is spotted and the haloes are scored at a fixed time point (72 h after spotting), this phenotype can be used as a rough indication of catabolite repression. Many carbohydrates (glucose, galactose, mannitol, xylitol, and arabinose) and tricarboxylic acid cycle intermediates (malate, citrate, and succinate) caused repression of agarase activity (Fig. 2). Al-

FIG. 1. Glycerol kinase catabolite repression. (A) A young mycelial culture of *S. coelicolor* M145 was induced with 10 mM glycerol in NMM as described in Materials and Methods. Glucose repression of glycerol kinase activity was monitored over time by growth in the presence (hatched bars) or absence (solid bars) of 55 mM glucose. (B) Glycerol kinase activities after 16 to 24 h of induction of *S. coelicolor* M145 with 100 mM glycerol in the absence of a repressing carbon source (bar 1) or in the presence of 1% glucose (bar 2), arabinose (bar 3), galactose (bar 4), citrate (bar 5), or glutamate (bar 6). (C) Glycerol kinase activities after 16 to 24 h of induction of a *glkA* mutant (KJ3) with 100 mM glycerol in the absence (bar 1) or presence (bars 2 to 6) of a repressing carbon source (1%) as described for panel B.

though it has been reported that disaccharides containing glucose can repress glycerol kinase activity in submerged cultures (11), there was no significant repression of agarase on agar plates by maltose, sucrose, lactose, or melibiose in *S. coelicolor* M145 (Table 1). One explanation may be that this repression in submerged cultures has been due to partial hydrolysis of the disaccharides, resulting in catabolite repression by free external glucose, which can repress glycerol kinase at a concentration of 3 mM in submerged cultures (data not shown). At this concentration of free glucose, repression of agarase is not detectable on plates (data not shown).

FIG. 2. Agarase catabolite repression. Well-washed spores of a *glkA* mutant, *S. coelicolor* M145 (wild type), or *S. coelicolor* M145 with the *glkA* gene on a high-copy-number vector (pIJ2427) were spotted on an MM agar plate (Agar) containing 1% repressing carbon source (glucose [Glc], galactose [Gal], or glycerol [Gly]). After 3 days, agarase activity was visualized as a transparent halo around the bacterial spot by 0.1% KI-I₂ staining. The relative glucose kinase (glk) activities of the strains are indicated at the right.

These results and previous experiments (5, 18) show that in *S. coelicolor*, glycerol kinase and agarase activities are repressed by a variety of carbon sources, including C₆ sugars, C₅ sugars, amino acids, and other metabolic substrates. It was also known that in a *glkA* deletion mutant repression by glucose was lost, but the effects of other carbon sources were not tested. In order to investigate whether glucose kinase could have a more general role in catabolite repression, the effects of carbon

TABLE 1. Carbon catabolite repression of agarase in *S. coelicolor*^a

Compound	Repression ^b			Growth ^c (M145)
	KJ3 (<i>glkA</i>)	M145	M145/pIJ2427	
Agar	—	—	—	++ ^d
Melibiose	—	—	—	±
Lactose	—	—	—	—
Maltose	—	—	—	±
Sucrose	—	—	—	+
Fructose	—	+	—	+
Mannose	—	+	—	ND
GlcNAc ^e	—	+	—	ND
Arabinose	—	+	—	++
Mannitol	—	+	—	+
Glycerol	—	+	—	++
Galactose	—	+	—	++
Glucose	—	+	—	+
Gluconate	—	+	—	++
Xylitol	—	+	+	±
Succinate	—	+	+	ND
Glutamate	+	—	+	++
Malate	+	+	+	±
Citrate	+	+	+	—

^a M145, wild-type *S. coelicolor*; KJ3, glucose kinase mutant; pIJ2427, multicopy plasmid containing the gene encoding glucose kinase (*glkA*).

^b Agarase activity was scored on MM agar plates as shown in Fig. 2. A strain was scored as derepressed for agarase (—) on the indicated carbon source when the halo was the same as or larger than that on the control plate (agar). Agarase repression (+) by a carbon source was scored only when the halo was much smaller than that on the control plate (agar).

^c Growth on the indicated catabolite as a sole carbon source was assayed by replica plating well-sporulating colonies from rich medium (R5) to synthetic plates (ludox HS40). —, no growth; ±, growth after 7 days; +, growth after 4 days; ++, growth after 2 days; ND, not determined.

^d Growth on agar was scored on an MM agar plate.

^e GlcNAc, *N*-acetyl glucosamine.

sources other than glucose in three independent glucose kinase mutants were tested. These mutants showed the same phenotype for carbon catabolite repression, but only the results obtained with strain KJ3 (see Materials and Methods) are shown.

Both glycerol kinase (Fig. 1) and agarase (Fig. 2 and Table 1) activities could be fully induced in the *glkA* mutants in the presence of most carbon sources which repress synthesis in a wild-type strain. Thus, not only repression by glucose is abolished but also repression by carbon sources which are not metabolized via glucose kinase. This suggests that glucose kinase plays a more general role in catabolite repression than previously assumed, and therefore we have investigated the effect of overexpression of glucose kinase on catabolite repression. For this purpose the *glkA* gene was cloned on a multicopy *Streptomyces* vector (pIJ702 [10]) and introduced into the wild-type strain *S. coelicolor* M145 by transformation. Although the copy number of this type of vector has been estimated to be around 50, the glucose kinase activity was measured to be only 10-fold higher ($1.4 \mu\text{mol/min} \times \text{mg}$ of protein) than that of wild-type M145 ($0.15 \mu\text{mol/min} \times \text{mg}$ of protein).

Overexpression of glucose kinase resulted in normal induction of agarase activity (Fig. 2) in the presence of all carbohydrates tested (even on glucose), which is virtually the same phenotype as in the *glkA* mutant. Only Krebs cycle intermediates (succinate, malate, and citrate), glutamate, and xylitol showed some degree of repression.

DISCUSSION

Carbon catabolite repression can be exerted by many compounds, which are metabolized via different routes. In spite of their biochemical diversity, the presence of these molecules in the growth medium can lead to a decrease in the specific activities of enzymes which are involved in catabolism of other carbon sources. Although this repression can potentially be performed at all levels of expression (e.g., transcription, RNA processing, translation, and protein modification), the cells must contain a component(s) which can sense the presence of such repressing compounds in the growth medium, giving rise to a signal which eventually results in repression of the enzyme activity (e.g., inducer exclusion, transcriptional repression, or protein breakdown).

Proteins involved in phosphorylation of glucose are often regulators of catabolism of certain carbon (energy) sources. Well known is the bacterial phosphoenolpyruvate:glucose phosphotransferase system, in which one of the proteins in the glucose phosphorelay, IIA^{Glc} , regulates the expression of genes by activation of adenylate cyclase or by inhibiting uptake of a carbon source, resulting in exclusion of the inducing compound (15). In this particular case, the ratio between the phosphorylated and the unphosphorylated forms of IIA^{Glc} determines whether catabolic genes are transcribed, resulting in fine-tuned expression in response to environmental stimuli. Mutations in IIA^{Glc} give rise to strains which are catabolite repression resistant. In the yeast *S. cerevisiae* there are three proteins which are responsible for phosphorylation of glucose, i.e., hexokinase PI, hexokinase PII, and glucose kinase. It has been known for a long time that deletion of hexokinase PII results in a loss of catabolite repression and that this function can be taken over by the structurally related (75% identical) hexokinase PI when overexpressed (16, 21). Because glucose kinase overexpression in *S. cerevisiae* cannot suppress loss of catabolite repression in a PI/PII null mutant, it cannot simply be the glucose flux which is regulating catabolite repression.

Instead, it has been suggested that the hexokinase PI and PII proteins themselves play a role in gene regulation (7).

In the bacterium *S. coelicolor*, two enzymes are responsible for glucose phosphorylation. The enzyme which is normally active, the gene product of *glkA*, is an ATP-dependent glucose kinase (8, 11). This protein has been suggested to play a role in catabolite repression, since deletion mutants were defective in glucose repression of glycerol kinase (18) and agarase (5). Since catabolite repression studies with the deletion mutants were performed with glucose as the repressing carbon source, a simple explanation could be the absence of a glucose flux through the enzyme glucose kinase. The activities of the enzymes glycerol kinase and agarase, however, were also shown to be repressed by several other carbon sources (5, 18), and in this paper we show that this repression by other carbon sources was relieved in the absence of glucose kinase. Since these carbon sources are not catabolized via glucose kinase, this observation leads to the conclusion that, like in the yeast system, it is most likely the protein itself rather than the flux through it which is involved in catabolite repression. Furthermore, a strain in which glucose kinase was overexpressed had the same phenotype as the glucose kinase mutant, i.e., no catabolite repression (Fig. 2). This observation raises the possibility that glucose kinase in mutant KJ3 could be catalytically inactive but still possess its regulatory function. This regulatory function could be lost by a second mutation, which causes overexpression of the protein. It is very unlikely, however, that these two types of mutations occurred simultaneously in the three independent glucose kinase mutants we tested. The results we obtained are in agreement with a soon-to-be-published report (1) in which the authors describe the induction of a second glucose-phosphorylating activity in a *glkA* deletion mutant. The presence of this new glucose kinase, however, was not sufficient to restore glucose repression of agarase transcription. In addition, those authors introduced a heterologous glucose kinase from *Zymomonas mobilis* in a *glkA* deletion mutant, and although the kinase activity was similar to that in wild-type *S. coelicolor*, it again did not restore glucose repression. This argues against a role for the glucose flux through glycolysis in carbon catabolite repression. A better explanation would be that a signal is generated in the presence of a repressing carbon source, which introduces a conformational change in glucose kinase (*glkA* gene), analogous to the response of the yeast hexokinase to binding of ATP or glucose (4, 9, 13). These putative effectors must be molecules whose concentrations can vary considerably depending on the carbon source and which have affinity for glucose kinase (*glkA* gene). Candidates are therefore glycolytic intermediates such as glucose, glucose-6-phosphate, fructose-1,6-bisphosphate, and nucleotides involved in maintenance of the energy balance (ATP, ADP, AMP). Repression could be achieved by direct binding of the modified glucose kinase to promoter regions of operons which are sensitive to catabolite repression. Excess glucose kinase could compete with the modified glucose kinase for the DNA-binding sites, thereby relieving repression. Another possibility is that the modified form of glucose kinase can form a repressor complex by binding a second protein. The loss of repression by overexpression of glucose kinase could then be explained by competition for this factor with unmodified glucose kinase, which does not form a repressor complex. This repressor complex can in turn directly interact with enzymes like glycerol kinase, thereby inhibiting its function, or it can bind to regulatory DNA sequences and function at the level of transcription. The protein sequence of glucose kinase (2) shows homology to those of the repressors of the *nag* operon of *E. coli* (NagC) (14) and of the *xyl* operon of *B. subtilis* (XylR)

(12). The N-terminal extensions of these proteins, which contain the DNA-binding motifs, are not present in glucose kinase. The homology probably reflects the sugar-binding capacity of the proteins. The lack of DNA-binding elements argues against the hypothesis that glucose kinase can repress transcription by binding DNA promoter regions. A model of repression via heterodimer formation is still possible, but it requires the assumption that unmodified glucose kinase has a higher affinity for the complexing factor as the repressing form of glucose kinase. In this model, the limiting factor would become the modification of glucose kinase, which could be induced by binding of a glycolytic metabolite.

The model which we propose for catabolite repression via glucose kinase in *S. coelicolor* is at this stage largely hypothetical. There are, however, numerous examples of concentration-dependent regulation of gene expression, and these usually invoke homo- or heterodimerization resulting in activating or repressing complexes. For enteric bacteria it has been shown that the regulatory protein IIA^{Glc}, which is central in catabolite repression, forms stoichiometric complexes with several target proteins (15). Examples in higher eukaryotes are transcriptional regulation by oncogenes in human cells (3, 6) and differential gene expression in the early development of *Drosophila* embryos (17). Carbon catabolite repression also requires a fine-tuned mechanism of gene expression, and in the case of *S. coelicolor* it may turn out that apart from heterodimerization, homodimerization and covalent modifications of glucose kinase also take part in the regulation. The fact that glucose kinase not only plays a role in glucose repression but has a more general role in carbon catabolite repression is a good starting point for further research.

ACKNOWLEDGMENTS

We thank Richard Baltz of Eli Lilly and Company for sending us the plasmid pCZA168 carrying transposon Tn5096, and we thank Sue Angell for sending us the construct pIJ2427. We are also grateful to David Hodgson for the protocol of silica-based plates and to Mervyn Bibb and Karel van Dam for careful reading of the manuscript. Jan den Blaauwen is gratefully acknowledged for photography.

This project was funded by an EEC/BRIDGE program (contract no. BIOT-CT91-0255 [SMA]).

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