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Glucose repression of anthracycline formation in *Streptomyces peucetius* var. *caesius*

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Abstract The effect of glucose on growth and anthracycline production by *Streptomyces peucetius* var. *caesius* was examined in a chemically defined medium. Glucose concentrations above 100 mM inhibited anthracycline synthesis in the original strain without causing significant change in growth and final pH values. This effect was observed when the carbohydrate was added initially or after 24 h fermentation, but not when added during the stationary growth phase. When the microorganism was pregrown in 100 mM glucose and then transferred to a resting cell system with 444 mM glucose, no significant differences in antibiotic production were observed compared to the control without glucose. The negative effect of glucose on antibiotic synthesis was not observed in a mutant (2-dog^R–21) resistant to growth inhibition by 2-deoxyglucose. Glucose consumption by this mutant was approximately 30% of that utilized by the original strain. Compared to the original strain, the mutant 2-dog^R–21 exhibited a reduction of 50% in glucose transport and an 85% decrease in glucose kinase activity. The experimental evidence obtained suggests that glucose represses anthracycline formation in a transitory manner and that this effect is related to glucose transport and phosphorylation.

Introduction

Glucose has been widely used as a substrate for growth and for the fermentative production of a large number of antibiotics and other secondary metabolites (Rose 1979). However, the production of these compounds can be negatively affected by high concentrations of this carbohydrate as well as other carbon sources which are readily assimilated. The negative influence exerted by D-glucose has been well documented for several antibiotics (Demain 1989). Nevertheless, except for a few examples such as erythromycin biosynthesis by *Saccharopolyspora erythraea* or actinomycin D formation in *Streptomyces antibioticus* (Jones 1985; Cortes et al. 1986), the biochemical and molecular bases for this repression have not been clearly established and the regulatory processes involved still require elucidation (Champness and Chater 1994). *Streptomyces peucetius* var. *caesius* is a mutant isolated from *Streptomyces peucetius* by its ability to produce doxorubicin (Arcamone et al. 1969). This strain produces a family of polyketide antibiotics known as anthracyclines. Among these, daunorubicin and doxorubicin are of clinical importance due to their antineoplastic activity (Strauss 1978). Dekleva and Strohl (1987) have observed that the production of daunorubicin in *Streptomyces peucetius* is negatively affected by glucose. They have suggested that this inhibitory effect is due to the acidity of the fermentative medium caused by excretion of pyruvate and 2-oxoglutarate following hexose metabolism. In this work, we examine the effect of glucose on growth and anthracycline production by *Streptomyces peucetius* var. *caesius* in a chemically defined medium with $CaCO_3$ as buffer. Experimental evidence suggests that this carbohydrate temporarily represses the formation of anthracyclines and that this repression is determined by glucose transport and phosphorylation.

Materials and methods

Bacterial strains and cultivation

Streptomyces peucetius var. *caesius* NRRL B-5337 was kindly provided by the Agriculture Research Service culture collection, US Department of Agriculture, Peoria, Ill., USA. The 2-dog^R–21 strain is a spontaneous mutant previously isolated in this laboratory

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through its insensitivity to growth inhibition by 2-deoxyglucose (DOG) (Segura et al. 1996). This analogue is incorporated by the streptomycete-mycelia and converted into 2-DOG 6-phosphate (I. Ramos et al., unpublished data) which, like glucose 6-phosphate, has been suggested to owe its toxicity to a destructive interaction with both proteins and DNA (Angell et al. 1994). The mutants are deposited at the UNAM-Biomedicas Culture Collection, Mexico, DF. Spores from these microorganisms were obtained and maintained as previously reported (Segura et al. 1996). For glucose kinase activity and anthracycline production assays, 2.5 ml of a seed culture (Segura et al. 1996) previously washed and resuspended with sterile distilled water were used to inoculate a 250-ml Erlenmeyer flask containing 50 ml of a chemically defined (CD) medium. The CD medium contained 0.5 g NaCl, 0.03 g K_2HPO_4, 0.133 g $(NH_4)_2SO_4$, 0.05 g $MgSO_4$, 0.002 g $FeSO_4$, 0.005 g $ZnSO_4$, 0.0005 g $MnCl_2$, 0.0005 g $CoCl_2$ and 0.3 g $CaCO_3$ per 100 ml distilled water (pH 7.2). The medium was supplemented with the desired lactose and/or glucose concentration (autoclaved separately and added before inoculation). Other compounds used (monosaccharides and disaccharides) were sterilized separately and added at the required concentrations for each experiment. Glucose kinase activity was determined in cultures grown for 48 h in CD medium supplemented with 100 mM D-glucose. For uptake experiments, 50 ml seed culture previously washed and resuspended in sterile distilled water was used to inoculate a 2.8-l Fernbach flask containing 500 ml uptake medium (UM). This medium contains: 0.5 g NaCl, 0.03 g K_2HPO_4, 2 g MOPS (3-[*N*-morpholino]propanesulfonic acid), 0.05 g $MgSO_4 \cdot 7H_2O$, 0.002 g $FeSO_4 \cdot H_2O$, 0.005 g $ZnSO_4 \cdot H_2O$, 0.0001 g $MnCl_2$, and 0.0001 g $CoCl_2 \cdot 6H_2O$ per 100 ml distilled water (pH 7.2). The medium was supplemented with 10 mM $(NH_4)_2SO_4$ and 100 mM D-glucose. Cultures were grown at 29 °C on a rotary shaker at 180 rpm.

Assay for anthracyclines and protein determination

At specified intervals, anthracyclines were extracted from harvested mycelia using acetone and 0.05 M sulfuric acid (4:1) according to Arcamone et al. (1969). Total anthracyclines were quantitated at 495 nm with an absorption coefficient of 220 (1%, 1 cm). For protein determination, samples were processed as previously reported (Segura et al. 1997) and assayed by the Lowry method, using bovine serum albumin as standard. For some fermentation experiments, doxorubicin and daunorubicin found in the extracts were separated and identified by thin layer chromatography (Vigevani and Williamson 1980) and quantitated against specific standards using a CAMAG TLC-Scanner II densitometer.

Glucose kinase assay

Glucose kinase activity was measured spectrophotometrically at 48 h by monitoring the reduction of NADP in a glucose 6-phosphate dehydrogenase-coupled reaction at pH 7.2 and at 25 °C, as previously reported (Segura et al. 1996). Glucose kinase activity was expressed as units per milligram of total cell protein. One unit (U) is defined as the amount of enzyme that produces 1 nmol NADPH per minute, under given conditions. Previous studies in our laboratory have shown that maximal glucose kinase activity occurs after 48 h growth in both the original and the mutant strain (Segura et al. 1996).

Uptake experiments

Each strain was grown for 36 h in supplemented UM medium, harvested, and washed with distilled water. Mycelia (250 mg wet weight) were resuspended in a vial containing 4.5 ml saline. The suspension was incubated for 10 min under agitation (250 rpm) at room temperature and the transport started by the addition of 0.5 ml D-[U-^{14}C]glucose (3.88 Bq mmol^{-1}) (Amersham). At desired times, a 1-ml sample was withdrawn, filtered (Millipore, type HA), washed with 10 ml cold distilled water, and dried at room temperature for 10 min. Radioactivity was determined by soaking the filter in 4 ml toluene-based scintillation solution containing 0.4% PPO (2,5-diphenyloxazole) and 0.04% POPOP (1,4-*bis*[5-phenyl-2-oxazolyl]benzene;2,2′-*p*-phenylene-*bis*[5-phenyloxazole]) (w/v).

Glucose determination

Glucose concentration was estimated directly from the fermentation medium by the enzymatic method reported by Trinder (Lott and Turner 1975).

Resting cell studies

Two-hundred-fifty-milliliter Erlenmeyer flasks containing 50 ml CD medium with 100 mM D-glucose were inoculated with 2.5 ml of a seed culture (Segura et al. 1996). Cells were grown for 60 h at 29 °C. The mycelia was harvested, washed with saline solution, and resuspended in 25 ml resting medium (RM). This medium is made in the same manner as CD medium but without $(NH_4)_2SO_4$ and $CaCO_3$. The RM medium was supplemented with 50 μg/ml chloramphenicol. Under these conditions, the mycelium produced anthracyclines for 20 h without any change in total protein concentration. This system allows measurement of antibiotic synthetase activities after these enzymes have been produced under established fermentation conditions (Demain and Kennel 1978).

Reproducibility of results

All experiments were repeated at least once (two independent experiments) in triplicate and the results are the mean values. Observed variations were consistently less than 10%.

Results

Effect of D-glucose on anthracycline biosynthesis

Streptomyces peucetius var. *caesius* was able to grow and produce anthracyclines in CD medium supplemented with 100 mM D-glucose as sole carbon source (Fig. 1). Under these conditions, antibiotic production initiated after 24 h and continued to increase almost linearly up to 120 h incubation. Glucose was completely consumed after 72 h fermentation. At glucose concentrations higher than 100 mM, the specific anthracycline production decreased between 30% and 50% (Fig. 1). Protein concentration also decreased at sugar concentrations higher than 194 mM. There were no significant differences in the evolution of pH in cells grown at the various glucose concentrations, suggesting that the changes in pH are not responsible for the reduction in antibiotic production and implicating a repressive effect of glucose on anthracycline biosynthesis. At glucose concentrations above 100 mM, the carbohydrate was only partially consumed, but the rate of consumption was similar for all concentrations tested. Chromatographic studies from extracts of mycelia grown under inhibitory glucose concentrations show a generalized reduction in production of the whole family of anthracyclines including daunorubicin and doxorubicin (data not shown). Elevated concentrations of other carbohydrates (monosaccharides

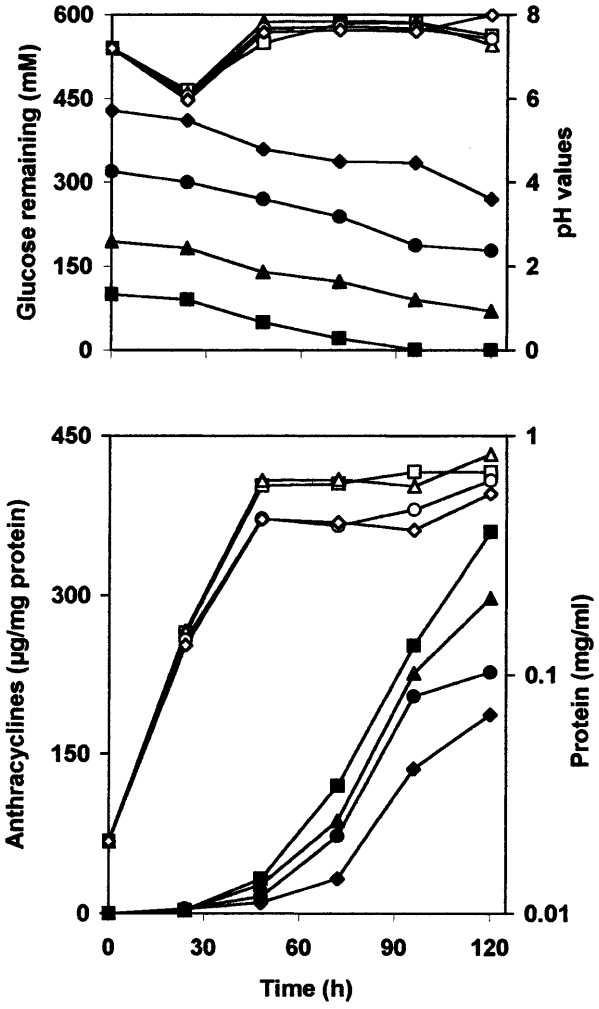

Fig. 1 Time course of *Streptomyces peucetius* var. *caesius* protein concentration and pH values (□, △, ○, ◇), anthracycline formation, and glucose consumption (●, ▲, ■, ◆) in CD medium supplemented with 100 (■, □), 194 (▲, △), 333 (●, ○) and 444 mM (◆, ◇) D-glucose. Fermentations were performed at 29 °C and 180 rpm in 250-ml Erlenmeyer flasks containing 50 ml culture medium

and disaccharides) showed a similar suppressive effect on antibiotic synthesis (Table 1), xylose and sucrose being the most effective, reducing anthracycline production by 62% and 65%, respectively.

Effect of glucose concentration

In order to further elucidate the effect of glucose on anthracycline production and cell protein, these parameters were assayed in the original strain (*Streptomyces peucetius* var. *caesius*) and a 2-DOG-resistant mutant (2-dog^R–21) in the presence of different glucose concentrations. As shown in Fig. 2, glucose concentrations above 167 mM negatively affected growth and anthracycline synthesis in the original strain without generating significant changes in final pH. Conversely, the mutant strain showed stimulated growth and a strong increase in antibiotic production. That is, the 2-dog^R–21 strain almost doubled the maximum production of anthracyclines compared to the original strain, showing a release from the inhibitory effect of glucose. Although the final pH values were only slightly different, we found that glucose consumption by the mutant strain was approximately 36% of that consumed by the original (Fig. 2). Moreover, elevated glucose concentrations (above 100 mM) inhibited doxorubicin biosynthesis in the original strain but not in the mutant 2-dog^R–21 (not shown). In the absence of high concentrations of glucose, we have seen no sensitivity of growth and antibiotic production to pH values in the range of 6–8.

Glucose additions

In order to further elucidate the effect of glucose on anthracycline biosynthesis, we added carbohydrate at different times during the fermentation process. As can be observed in Fig. 3, addition of a high concentration

Table 1 Effect of different carbohydrate concentrations on growth and anthracycline formation[a]

Carbohydrate	Carbohydrate concentration (mM)	Growth (mg/ml)	Anthracyclines	
			(µg/ml)	(µg/mg protein)
Monosaccharides				
D-Fructose	110	0.80 ± 0.02	199.80 ± 5.00	249.7
	333	0.94 ± 0.03	159.57 ± 14.75	169.7
D-Mannose	110	0.96 ± 0.02	319.13 ± 22.12	332.4
	333	0.92 ± 0.03	221.26 ± 4.01	240.4
D-Xylose	133	0.98 ± 0.08	231.97 ± 20.77	236.7
	399	1.05 ± 0.06	95.20 ± 1.35	90.6
Disaccharides				
Lactose	58	0.29 ± 0.03	35.90 ± 7.30	123.8
	175	0.43 ± 0.00	25.26 ± 7.76	58.7
Maltose	58	0.10 ± 0.01	13.14 ± 0.96	131.2
	175	0.13 ± 0.01	9.22 ± 1.15	65.8
Sucrose	58	0.72 ± 0.02	79.72 ± 16.49	110.7
	175	0.45 ± 0.02	17.46 ± 0.27	38.8

[a] Fermentations were performed for 120 h at 29 °C and 180 rpm as described in Fig. 1

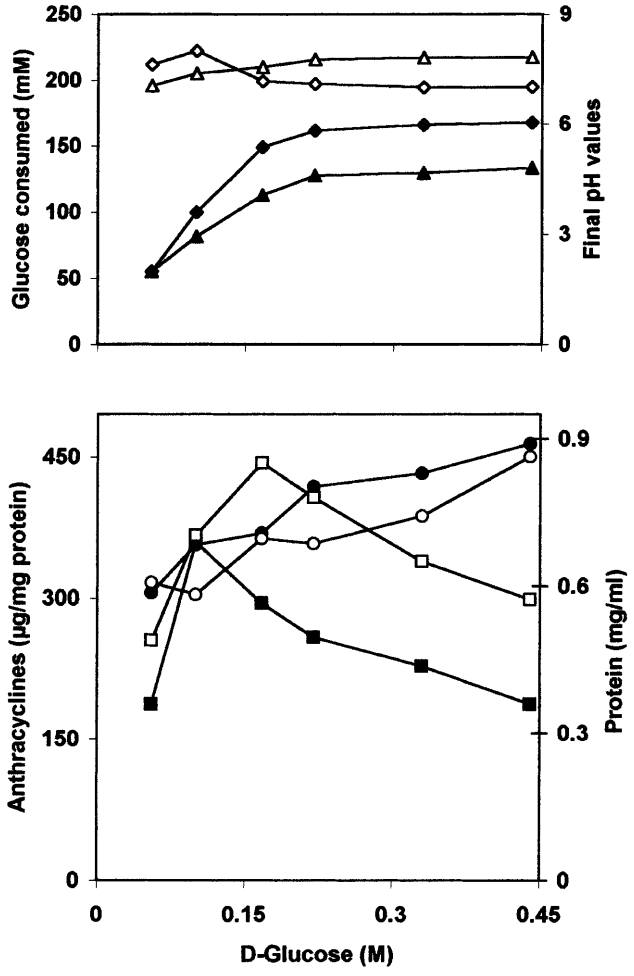

Fig. 2 Effect of D-glucose concentrations on maximum growth (○, □), anthracycline formation (●, ■), glucose consumption (◆, ▲), and final pH values (◇, △) of the original (■, □, ◆, ◇) and 2-dog^R–21 (○, ●, △, ▲) strains. Fermentations were performed as described in Fig. 1

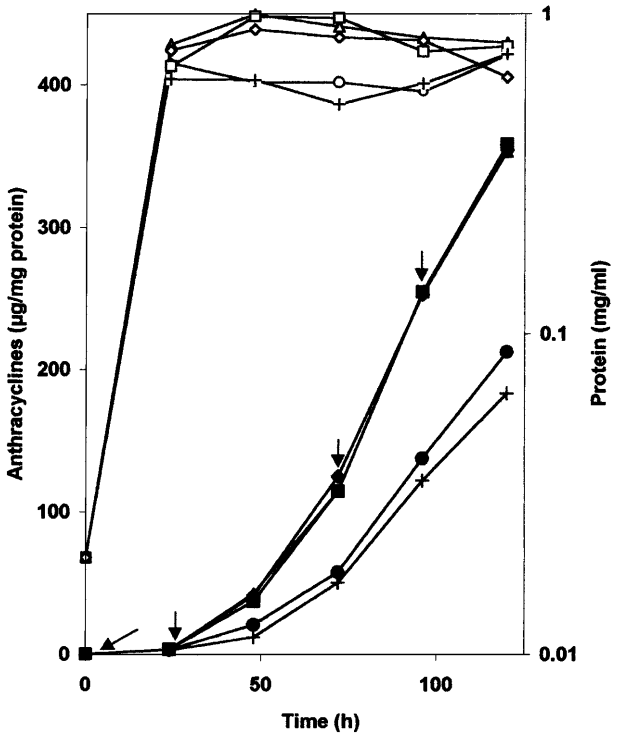

Fig. 3 Effect of the time of D-glucose addition on growth (*open symbols*) and anthracyclines production (*filled symbols*). The cultures were grown in 100 mM D-glucose. Further D-glucose feeding (444 mM) was performed at 0 (+), 24 (●), 72 (■), and 96 h (▲) fermentation. Control (◆) without further feeding

of carbohydrate (444 mM) at the beginning or after 24 h fermentation temporarily delayed and reduced antibiotic production, compared to a control with 100 mM glucose. However, at 72 and 96 h of fermentation, when the culture had reached the stationary growth phase, addition of glucose no longer delayed anthracycline production. Moreover, chloramphenicol (50 µg/ml) interrupted antibiotic biosynthesis when added at the beginning or after 24 h fermentation but lacked any effect when added at 72 h and 96 h. A similar effect was observed with the addition of the glucose analogue 2-DOG (60 mM) (not shown). Thus, suppression was observed only when these compounds were added either at the beginning of fermentation or during the exponential growth phase. In each case the relationship between production and growth was modified.

Resting cell system studies

To distinguish whether the negative effect of glucose was due to inhibition of synthesis or activity of the enzymes involved in anthracycline formation, we used a resting cell system in the presence of chloramphenicol (Demain and Kennel 1978). As shown in Fig. 4, cells pregrown in 100 mM glucose and transferred to a resting cell system in the absence of glucose generated moderate anthracycline production. In addition, when the microorganism was transferred to a resting cell system with 444 mM glucose, there were no significant differences in the evolution of the antibiotic in relation to the control without glucose. In the presence of 444 mM D-glucose there was a slight decrease in the medium pH, probably due to the absence of $CaCO_3$ in the medium.

Similar results were observed when the cells were pregrown in 166 mM and 444 mM glucose, but the initial anthracycline concentrations were reduced by approximately 20% and 60%, respectively (data not shown). This suggests a transitory effect of high glucose concentrations on anthracycline biosynthesis.

Glucose kinase activity and glucose uptake

Glucose transport and glucose kinase activity in the original strain and in the mutant 2-dog^R–21 were determined in order to elucidate the relationship between the repressive effect of glucose and its assimilation by the cells. Glucose uptake was determined after 36 h growth where both strains are in logarithmic growth phase.

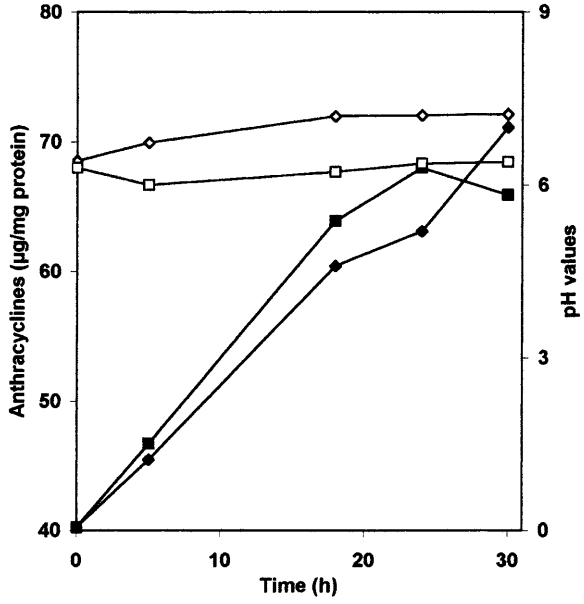

Fig. 4 Time course of anthracycline production in a resting cell system prepared from mycelia pregrown for 60 h in CD medium with 100 mM D-glucose. Antibiotic production in resting cells without glucose (■) and with 444 mM D-glucose (◆). The pH values for each are shown with open symbols (□, ◇, respectively)

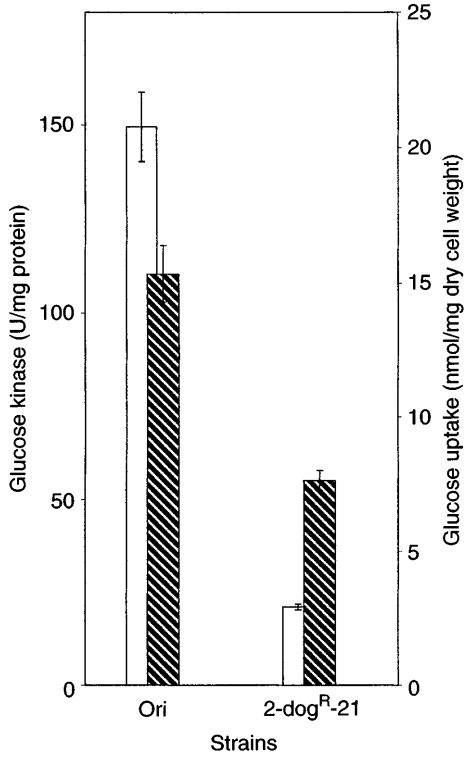

Fig. 5 Glucose kinase (□) and glucose uptake (▨) levels of the original and 2-dog^R–21 strains. Cultures were grown for 48 and 36 h, respectively, in CD medium supplemented with 100 mM D-glucose, under the conditions mentioned in Fig. 1

Compared to the original strain, the 2-dog^R–21 mutant showed a 50% reduction in glucose transport. The maximal glucose kinase activity has been previously reported at 48 h for both strains (Segura et al. 1997); at this time we found an 85% decrease in glucose kinase activity in the mutant strain compared to the original (Fig. 5).

Discussion

Glucose concentrations above 100 mM negatively affect the synthesis of anthracyclines (including doxorubicin), in *Streptomyces peucetius* var. *caesius*. Dekleva and Strohl (1987) observed similar results in daunorubicin production in *Streptomyces peucetius*. They suggested that this effect is not due to a phenomenon of catabolic repression by glucose but is, instead, an inhibitory effect caused by the reduction in pH brought about by the excretion of organic acids (pyruvate and 2-oxoglutarate) derived from hexose metabolism and excreted into the fermentation medium. More recently, studies using *Streptomyces lividans* demonstrated that the excretion of organic acids produced by glucose metabolism is dependent on the nitrogen source utilized (Madden et al. 1996). Nitrogen sources such as nitrate and amino acids favor excretion of elevated levels of pyruvate and 2-oxoglutarate, respectively. However, addition of ammonium salts completely prevents the formation of organic acids. In this study, by using a chemically defined medium with $(NH_4)_2SO_4$ as nitrogen source and $CaCO_3$ as buffer, the negative effect of glucose was still evident without significant changes in pH, ruling out acidity as responsible for the suppression of anthracycline production and suggesting a direct effect of glucose on antibiotic biosynthesis. In support of a mechanism of repression is the fact that high concentrations of glucose exhibit a negative influence when administered during logarithmic growth phase, presumably before or during the synthesis of enzymes involved in antibiotic production, but not when added during the stationary phase. This suggests that high concentrations of glucose have no inhibitory effect on previously synthesized enzymes, since addition of chloramphenicol during the stationary phase shows very similar results. The anthracycline biosynthetic enzymes seem to be relatively stable during this phase, allowing continued production of anthracyclines. In addition, the profile observed for the differential rate of antibiotic biosynthesis in 444 mM glucose was similar to that reported for the transitory repression of β-galactosidase and erythromycin in *Escherichia coli* and *Streptomyces erythraeus*, respectively (Tyler and Magasanik 1970; Escalante et al. 1982). Further evidence supporting a repressive effect was obtained using a resting cell system in the presence of a protein synthesis inhibitor. This demonstrated that anthracycline formation was not directly affected by high glucose concentrations, excluding inhibition as the mechanism of action. The repression of anthracycline synthesis was transitory, and antibiotic production was reinitiated after a few hours.

Release from this inhibition occurred even when there were still elevated levels of glucose in the fermentation medium. Tyler and Magasanik (1970), when studying the effects of glucose on lactose metabolism in *Escherichia coli*, found that this carbohydrate per se is capable of temporarily repressing β-galactosidase. Subsequently, there is a release from this repression and the enzyme is synthesized again. In our system the cause of this release is as yet unknown; however, it was abrogated when the analogue 2-DOG was added. This suggests that the microorganism may require a product of glucose catabolism (possibly an intermediary of glycolysis) or some other signal induced by the action of these metabolites to carry out the release from glucose repression and thus resume antibiotic biosynthesis. As mentioned before, we have seen that the final destination of 2-DOG in the mycelia is 2-DOG 6-phosphate (I. Ramos et al., unpublished data), suggesting that this intermediate may be involved in the regulatory process. Chromatographic studies demonstrated that by increasing glucose concentration in the medium, total anthracycline production, including doxorubicin and daunorubicin, was reduced proportionately. This suggests that repression by glucose may target the formation of precursors (acetyl CoA, propionyl CoA, and/or methyl malonyl CoA) or suppress the synthesis of antibiotic synthases. The negative effect of high glucose concentrations on growth probably results from the increase in osmotic pressure outside the cells.

A number of groups have reported a relationship between sensitivity to carbon repression and the concentration of an ATP-dependent glucose kinase (Hodgson 1982; Angell et al. 1994; Kwakman and Postma 1994). In accordance with these studies, we found glucose kinase activity was strongly diminished in the mutant 2-dog^R–21, which was insensitive to the negative effect exerted by glucose. Besides having low glucose kinase activity, this mutant incorporated only 50% of the glucose present in the medium. It is likely that a double mechanism (glucose kinase/glucose uptake ratio) insures limited glucose catabolism in the mutant and consequently reduces the concentration of intermediates of glycolysis. In this manner, the 2-dog^R–21 mutant would be freed from regulation by glucose and would insure utilization of alternative carbon sources in the presence of 2-DOG. This suggests the possibility that intermediates of glycolysis would function as metabolic effectors of catabolic repression in this *Streptomyces*. In order to exert their action at the DNA level, these compounds probably need to interact with other macromolecules before binding to operators which may be regulated by their action.

On the other hand, a phosphoenolpyruvate : sugar phosphotransferase system for fructose transport has been reported in several strains of *Streptomyces* (Titgemeyer et al. 1995) presenting specific activities for the enzyme I, Hpr, and enzyme II. In addition, Pope et al. (1998) have cloned a gene coding for the synthesis of a small protein (99 amino acids) capable of restoring morphogenesis, antibiotic production, and carbon utilization in *bld*B mutants of *Streptomyces coelicolor*. These mutants are pleiotropically defective in the initiation of development, the ability to produce antibiotics, the ability to regulate carbon utilization, and the ability to send and/or respond to extracellular signals. Consequently, the possibility that various components of this system may also participate in carbon catabolite repression in *Streptomyces* has been envisaged. Nevertheless, more studies will be required to explain the role of these systems in the carbon catabolite repression process.

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