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Glucokinase-Deficient Mutant of *Penicillium chrysogenum* Is Derepressed in Glucose Catabolite Regulation of Both β-Galactosidase and Penicillin Biosynthesis

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One glucokinase-deficient mutant (*glk1*) of *Penicillium chrysogenum* AS-P-78 was isolated after germ tube-emitting spores were mutated with nitrosoguanidine and selected for growth on lactose-containing medium in the presence of inhibitory concentrations of D-2-deoxyglucose (3 mM). Penicillin biosynthesis was greatly reduced (55%) in D-glucose-grown cultures of the parental strain, but this sugar had no repressive effect on the rate of penicillin biosynthesis in the mutant *glk1*. This mutant was deficient in ATP-dependent glucokinase and showed a greatly reduced uptake of D-glucose. The parental strain *P. chrysogenum* AS-P-78 showed in vitro ATP-dependent phosphorylating activities of D-glucose, D-2-deoxyglucose, and D-galactose. The *glk1* mutant was deficient in the in vitro phosphorylation of D-glucose and D-2-deoxyglucose but retained a normal D-galactose-phosphorylating activity. D-Glucose repressed both β-galactosidase and isopenicillin-*N*-synthase but not acyl coenzyme A:6-aminopenicillanic acid acyltransferase in the parental strain. The glucokinase-deficient mutant was simultaneously derepressed in carbon catabolite regulation of β-galactosidase and isopenicillin-*N*-synthase, suggesting that a common regulatory mechanism is involved in carbon catabolite regulation of both sugar utilization and penicillin biosynthesis.

It is now well established that D-glucose and other rapidly utilized sugars exert a negative regulation on the biosynthesis of many types of antibiotics (17). The biosynthesis of penicillin by *Penicillium chrysogenum* is negatively regulated by high glucose concentrations in the culture medium (27, 28); this phenomenon is similar in many respects to the carbon catabolite regulation of the biosynthesis of cephalosporins (12, 34) and cephamycins (5, 6, 21). Understanding of these regulatory mechanisms would be extremely useful for the selective removal of bottlenecks in the antibiotic biosynthesis pathways (16, 19).

D-Glucose represses the formation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), the first intermediate of the penicillin and cephalosporin biosynthetic pathways (28). The repression of ACV formation by D-glucose is not reversed by the addition of α-aminoadipic acid, cysteine, or valine. D-Glucose also represses isopenicillin-*N*-synthase (IPNS) which converts ACV into isopenicillin-*N*, but does not affect acyl coenzyme A (CoA):6-aminopenicillanic acid (6-APA) acyltransferase (21, 28), the last enzyme of the penicillin biosynthetic pathway (1). A detailed study of the molecular mechanism of carbon catabolite regulation of fungal beta-lactams has been hampered by the lack of molecular genetic techniques in the beta-lactam-producing fungi (18). The recent development of cloning techniques in *P. chrysogenum* (2, 3, 16, 29) has provided the tools for molecular genetic studies of carbon catabolite regulation of the biosynthesis of these antibiotics.

In *Saccharomyces cerevisiae*, D-glucose represses the enzymes that are required for the utilization of exogenous carbon sources, such as maltose (32), sucrose and D-galactose (31), and some glucogenic enzymes (11). The molecular mechanism of carbon catabolite regulation in yeasts differs from that in *Escherichia coli* since it does not involve cyclic AMP (22).

Mutants of *S. cerevisiae* that are resistant to carbon catabolite repression have been isolated (35) to elucidate the mechanism of D-glucose repression. One of these mutants, *hex1*, had reduced hexokinase activity (10) and was shown to be altered in the structural gene for hexokinase isoenzyme PII (7, 9). The unique role of the hexokinase isoenzyme PII in glucose repression has been corroborated by cloning experiments (8).

In order to establish whether the carbon catabolite regulation of penicillin biosynthesis in *P. chrysogenum* also affects the enzymes involved in the catabolism of exogenous carbon sources in this fungus and to elucidate whether the molecular mechanism is similar to that of *S. cerevisiae*, we isolated mutants of *P. chrysogenum* AS-P-78 (by resistance to D-2-deoxyglucose [D-2-DOG]) which were deficient in carbon catabolite repression.

We report here that one of these mutants is deficient in glucokinase and is simultaneously derepressed in carbon catabolite regulation of both β-galactosidase and penicillin biosynthesis.

MATERIALS AND METHODS

Nitrosoguanidine mutation and selection of D-2-DOG-resistant mutants. A suspension of 10^6 spores of *P. chrysogenum* AS-P-78 was placed in 0.1 M Tris maleate buffer (pH 9.0) supplemented with 7.5 mM $(NH_4)_2SO_4$; this buffer was devoid of the carbon source. Spores swelled synchronously for 10 h at 25°C, but they were unable to germinate due to the lack of a carbon source. After this time, 0.16 M D-glucose was added, and the suspension was incubated for 4 h to allow germ tubes to protrude. Under these conditions DNA synthesis began synchronously just before germ tube formation (20). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was

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added at this time (final concentration, 47 μM) and incubated at 25°C for 45 min. Afterward, NTG was neutralized with cysteine (final concentration, 82 mM; cysteine and other reducing agents such as ascorbate can prevent the formation of short-lived, highly reactive electrophilic NTG decomposition products, thus preventing its mutagenic effect); the spore suspension was diluted and plated onto minimal medium containing KH_2PO_4, 7.3 mM; $FeSO_4 \cdot 7H_2O$, 0.18 mM; $MgSO_4 \cdot 7H_2O$, 2 mM; $ZnSO_4 \cdot 7H_2O$, 35 μM; $CuSO_4 \cdot 5H_2O$, 40 μM; $MnSO_4 \cdot 4H_2O$, 60 μM; $CoSO_4 \cdot 7H_2O$, 17 μM; NaCl, 17 μM; $(NH_4)_2SO_4$, 37 mM; agar 30 g/liter, supplemented with lactose (27 mM) and D-2-DOG (3 mM). The parental strain of *P. chrysogenum* (AS-P-78) did not grow on this medium because of carbon catabolite repression by D-2-DOG of the utilization of lactose as a carbon source. D-2-DOG-resistant mutants were isolated on this medium after incubation of the NTG-treated spores at 28°C for 5 days.

Penicillin production. Penicillin biosynthesis by the parental strain (AS-P-78) and the D-2-DOG-resistant mutants was studied on a Pharmamedia (Difco Laboratories, Detroit, Mich.)-based medium containing the following, in grams per liter: Pharmamedia (Difco), 20; $(NH_4)_2SO_4$, 3; $CaCO_3$, 5; and phenylacetic acid, 4; with lactose, 50 (nonrepressing conditions; referred to as PNL), or D-glucose, 50 (repressing conditions; referred to as PNG), as carbon sources. In experiments on the regulation of β-galactosidase by D-glucose, D-glucose (0.14 M, or 50 g/liter) was added to a lactose-based (0.07 M, or 50 g/liter) medium. Penicillin G was determined by bioassay by using *Bacillus subtilis* ATCC 6633 as the test strain, as described previously (12–14).

β-Galactosidase activity. β-Galactosidase activity was measured in cell extracts of *P. chrysogenum* by using the spectrophotometric method based on the hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside (ONPG) (A_{405}) (33). Control reactions without ONPG and without enzyme were always carried out in parallel. D-Glucose was determined by using the D-glucose oxidase-peroxidase method.

Glucokinase activity. Glucokinase activity was determined in 50 mM triethanolamine buffer (pH 7.4) containing $MgCl_2$ (10 mM), NADP (0.5 mM), ATP (1 mM), glucose-6-phosphate dehydrogenase (1.4 U), D-glucose (10 mM), and enzyme (500 μl of cell extract containing 2.5 mg of total protein per ml) in a reaction volume of 2.5 ml (15). Controls lacking D-glucose, ATP, glucose-6-phosphate, or enzyme were included in every determination. The enzyme activity was quantified by taking into account that for each 1 μmol of NADPH formed, there was an increase in the A_{340} of 6.02 (30).

IPNS. IPNS was determined in cell extracts, which were prepared by sonication of mycelium (28), by measuring the conversion of ACV into isopenicillin-*N* by using the optimized conditions described previously (26). The isopenicillin-*N* formed was determined microbiologically by using *Micrococcus luteus* ATCC 9431 as a test strain and by reversed-phase high-pressure liquid chromatography, as reported elsewhere (4, 24).

Phenylacetyl-CoA:6-APA acyltransferase. Phenylacetyl-CoA:6-APA acyltransferase activity was measured in cell extracts of *P. chrysogenum* by reacting 6-APA with phenylacetyl-CoA to form penicillin G, as described before (1).

Uptake of [^{14}C]glucose. The uptake of D-glucose was carried out by using mycelium grown for 48 h in a nitrogen-limited suspension medium (28). Mycelium was collected by filtration and washed 3 times with sterile saline solution (9 g of NaCl per liter). One gram (wet weight) was suspended in 25 ml of nitrogen-limited suspension medium supplemented with 1 mM D-glucose as the carrier. After shaking for 2 h at 25°C, 15 μl of [U-^{14}C]glucose (specific activity, 275 mCi/mM; Amersham Corp., Arlington Heights, Ill.), equivalent to 3 μCi, was added to each flask and incubated under the same conditions (20°C in a rotary water incubator). Samples (200 μl) were taken rapidly every 2.5 min, and the incorporation of the label was stopped by placing the samples in a 0.1 M solution of nonradioactive D-glucose.

In vitro phosphorylation of hexoses. In vitro activities of hexoses were measured in cell extracts obtained by sonication of *P. chrysogenum* mycelium grown in a medium containing D-glucose, D-fructose, or D-galactose (277 mM); sodium citrate (34 mM); acetic acid (41 mM); $(NH_4)_2SO_4$ (30 mM); KH_2PO_4 (7.3 mM), $FeSO_4 \cdot 7H_2O$ (0.18 mM); $MgSO_4 \cdot 7H_2O$ (2 mM); $ZnSO_4 \cdot 7H_2O$ (35 μM); $CuSO_4 \cdot 5H_2O$ (40 μM); $MnSO_4 \cdot 4H_2O$ (60 μM); and NaCl (17 μM); the pH was adjusted to 6.2 with NaOH and $CaCO_3$. The phosphorylation reaction was carried out in triethano-

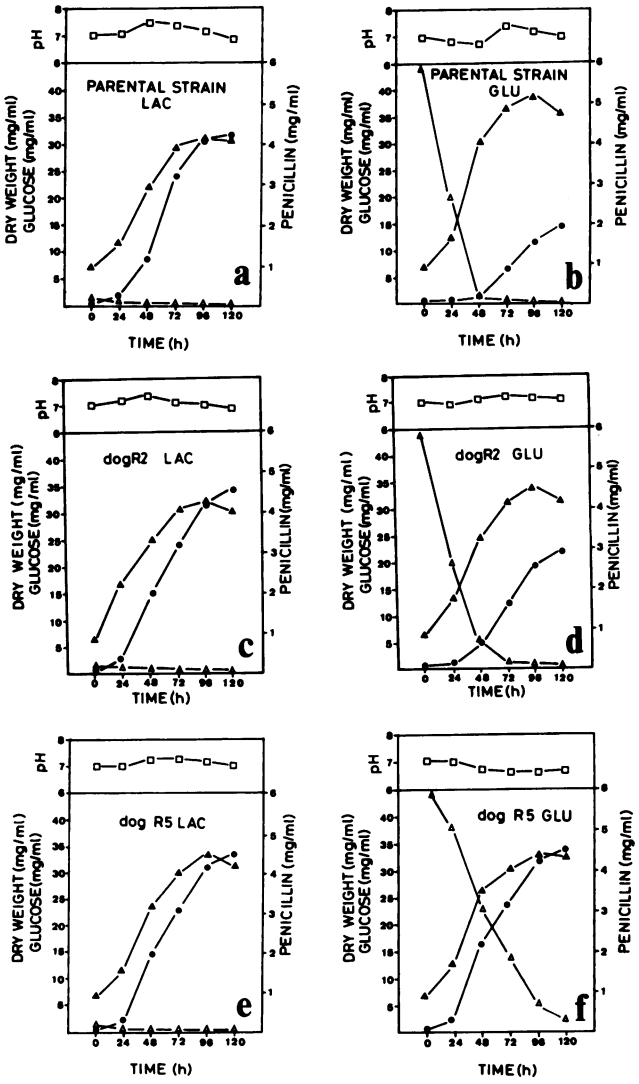


FIG. 1. Growth (▲), penicillin production (●), pH (□), and residual D-glucose levels (△) in cultures of the parental strain (a and b), mutant *dogR2* (c and d), and mutant *dogR5* (e and f) grown in lactose (a, c, and e) or D-glucose (b, d, and f).

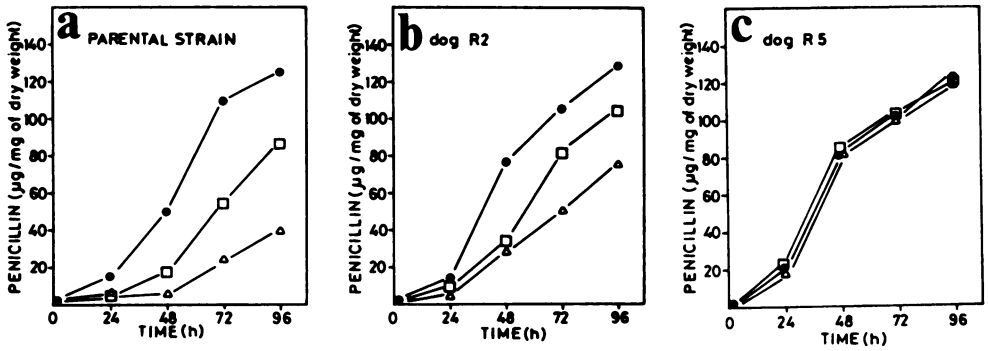
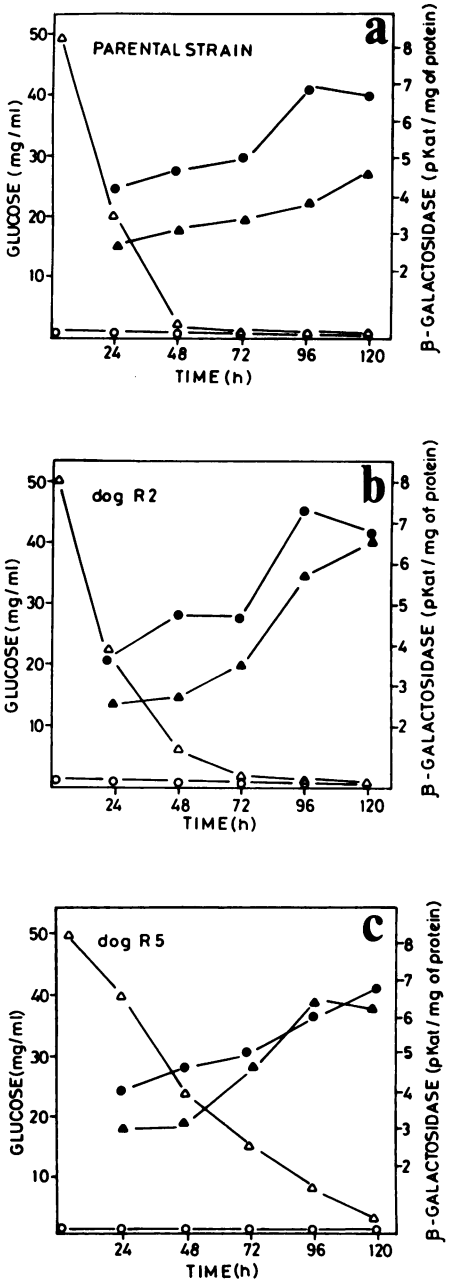


FIG. 2. Effect of D-glucose on the specific rate of penicillin production. Symbols: ●, control lactose-grown (50 g/liter) cultures; □, cultures grown in lactose (25 g/liter) supplemented with D-glucose (25 g/liter) at the time of inoculation; △, D-glucose-grown (50 g/liter) cultures. (a) Parental strain; (b) *dogR2* mutant; (c) *dogR5* mutant.



lamine buffer (50 mM; pH 7.8) containing 10 mM $MgCl_2$, 10 mM ATP, 2 µCi of [^{14}C]hexose, 5 mM carrier hexose, and cell extract (about 300 µg of total protein) at 29°C in a total volume of 100 µl (25). Every 5 min, samples (10 µl) were taken, filtered through Whatman DE-81 filters (Whatman, Inc., Clifton, N.J.), and washed with 40 ml of water. The filters were dried at 80°C, and their radioactivity was measured in a liquid scintillation counter.

RESULTS

Growth, penicillin production, and D-glucose utilization by the D-2-DOG-resistant mutants. Ten mutants selected for resistance to D-2-DOG, as indicated above, were tested for their stability after several transfers in the same D-2-DOG-containing medium. Two of these mutants, *dogR2* and *dogR5* (later designated *glk1*) were selected for further studies.

The kinetics of growth (with the same amount of inoculum), penicillin production, and the level of residual D-glucose in PNL (with lactose as the carbon source) and PNG (with D-glucose as the carbon source) media are shown in Fig. 1. No free D-glucose was detected in the lactose-based medium, and therefore, no D-glucose repression was observed in the parental strain or in any of the mutants (Fig. 1). In cultures of the parental strain grown in D-glucose, utilization of D-glucose was very rapid and no penicillin was produced until D-glucose was almost depleted. Mutant strain *dogR2* also showed a rapid utilization of D-glucose, although some sugar (about 5 mg/ml) remained at 48 h and less growth (32 mg/ml of dry weight) than that in the wild type (39 mg [dry weight]/ml) was observed. Penicillin biosynthesis seemed to be partially derepressed in mutant *dogR2* since about 500 µg of this antibiotic per ml was already synthesized at 48 h and 1,700 µg/ml was produced at 72 h, when D-glucose was depleted.

Mutant *dogR5* was completely deregulated with respect to the effect of D-glucose on penicillin biosynthesis. D-Glucose was utilized slowly by this mutant, and some D-glucose remained at the end of the fermentation (120 h). However, growth of this mutant on this complex medium was normal when compared with that of the parental strain.

FIG. 3. β-Galactosidase activities of cultures of the parental strain (a) and mutants *dogR2* (b) and *dogR5* (c) grown in lactose (●) or D-glucose (▲). Residual levels of D-glucose in lactose-grown (○) and D-glucose-grown (△) cultures are indicated.

The effect of D-glucose on the specific rate of penicillin biosynthesis by the mutants in lactose-based PNL medium was compared with that of the parental strain (Fig. 2). D-Glucose was added at the time of inoculation. For comparison, the levels of penicillin synthesis in the D-glucose-based medium are also shown (Fig. 2). D-Glucose reduced the rate of penicillin biosynthesis in the lactose-based medium by about 50% in the parental strain and by 20 to 25% in mutant *dogR2* (Fig. 2). D-Glucose had no repressive effect on penicillin biosynthesis by mutant *dogR5* (Fig. 2).

β-Galactosidase activity in the wild type and in the mutants. The level of β-galactosidase activity in the parental strain and in mutants *dogR2* and *dogR5* under repressing and nonrepressing conditions is shown in Fig. 3. D-Glucose reduced the level of β-galactosidase activity in *P. chrysogenum* cultures of the parental strain and the *dogR2* mutant but not of the *dogR5* mutant.

Mutant *dogR5* appeared to be altered in carbon catabolite regulation, and therefore, further experiments were carried out only with this strain.

Uptake of glucose. Uptake of [U-^{14}C]glucose by both the parental strain and mutant *dogR5* is shown in Fig. 4. The parental strain showed a linear uptake of D-glucose with time, but the rate of D-glucose uptake in the *dogR5* mutant was very slow.

Glucokinase activity. The specific activities of the glucokinase of the parental strain and the *dogR5* mutant in determinations carried out at increasing reaction times is shown in Fig. 5. The rate of glucose-6-phosphate formation in mutant *dogR5* was very slow when compared with the reaction rate in the parental strain. Since this mutant was deficient in glucokinase activity, it is hereafter designated *glk1*.

Phosphorylation of different hexoses. To establish whether the *glk1* mutant was also deficient in the phosphorylation of other sugars, the presence of different hexose-phosphorylating activities in extracts of *P. chrysogenum* was studied, as described above.

A strong ATP-dependent glucose-phosphorylating activity (glucokinase) was found in extracts of the parental strain. D-2-DOG was also phosphorylated by the same extracts but very inefficiently (4.5% with respect to D-glucose). D-Galactose was phosphorylated poorly, and there was no D-fructose-phosphorylating activity at all in the extracts (Fig. 6). The *glk1* mutant was deficient in D-glucose-phosphorylating activity. It showed only about 10% of the D-glucose-phosphorylating activity of the parental strain. This mutant was also deficient in D-2-DOG phosphorylation but retained a normal D-galactose-phosphorylating activity and did not show D-fructose phosphorylation, as occurred in the wild type (Fig. 7).

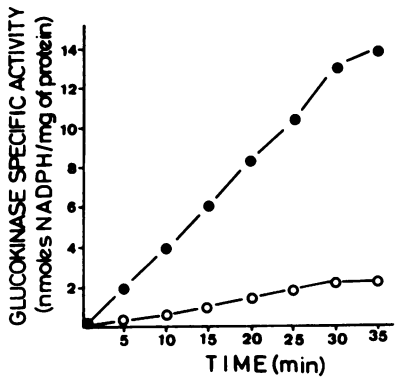


FIG. 5. Glucokinase activity, at increasing reaction times, of the parental strain (●) and the *dogR5* mutant (○) in lactose-grown cultures.

All these results support the conclusion that the *glk1* mutant is deficient in a D-glucose- and D-2-DOG-specific kinase.

D-Glucose regulation of IPNS. D-Glucose catabolism exerts a strong negative regulatory effect on the IPNS of cells taken after 36 and 60 h of incubation (Fig. 8). The level of IPNS activity in D-glucose-grown cells of the parental strain was about 17% of that in the control (lactose-grown) cultures. However, no such repression of IPNS activity was shown in cultures of the *glk1* mutant grown in D-glucose under the same experimental conditions. These results were consistently obtained in four different experiments, and they explain the lack of carbon catabolite regulation of total penicillin production in the mutant described above.

Lack of D-glucose regulation of the 6-APA acyltransferase. The 6-APA acyltransferase activity, the last enzyme complex of the penicillin biosynthetic pathway, was not regulated by D-glucose either in the parental strain or in the *glk1*

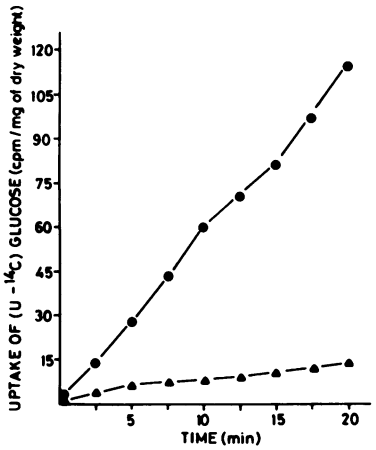


FIG. 4. Uptake of [U-^{14}C]glucose by cultures of the parental strain (●) and the *dogR5* mutant (▲) grown in lactose and suspended in nitrogen-limited suspension medium.

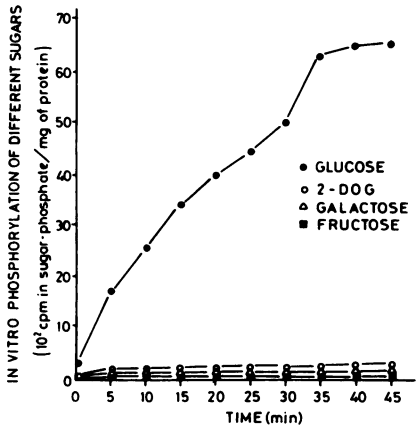


FIG. 6. In vitro ATP-dependent phosphorylation of D-glucose, D-deoxyglucose, D-galactose, and D-fructose by using cell extracts of cultures of the parental strain grown, respectively, in D-glucose (for both the D-glucose and D-deoxyglucose assays), D-galactose, and D-fructose.

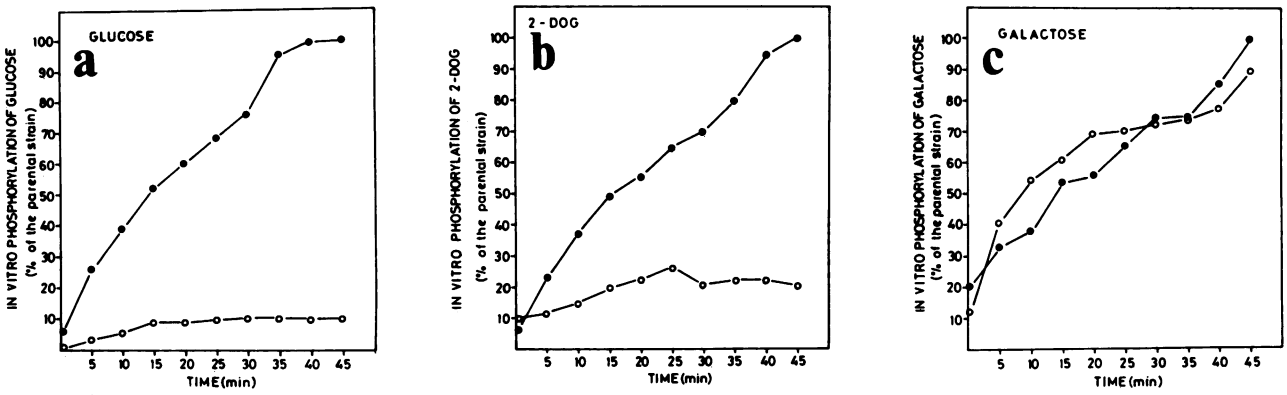


FIG. 7. Relative in vitro ATP-dependent phosphorylation of D-glucose (a), D-2-DOG (b), and D-galactose (c) by cell extracts of the *dogR5* (*glk1*) mutant (●) in comparison with that of the parental strain (○). Cultures were grown as described in the legend to Fig. 6.

mutant. The level of enzyme was similar in D-glucose-grown and control cultures throughout the fermentation (Fig. 8).

DISCUSSION

Two of the mutants of *P. chrysogenum* isolated in this study that were able to utilize lactose in the presence of inhibitory concentrations of D-2-DOG (i.e., they were selected for derepressed β-galactosidase activity) showed an alteration in the control of glucose of penicillin biosynthesis (Fig. 1 and 2). One of the D-2-DOG-resistant mutants studied in detail was deficient in glucokinase activity (*glk1*) and showed a low D-glucose uptake rate. D-Glucose uptake and phosphorylation by glucokinase are deeply interrelated in several microorganisms including the beta-lactam-producing strain *Streptomyces clavuligerus* (M. García-Domínguez and P. Liras, personal communication). Phosphorylation appears to drive the entrance of D-glucose. Therefore, glucokinase-deficient mutants are impaired in their D-glucose transport ability. In *S. cerevisiae*, some D-2-DOG-resistant mutants (35) had reduced hexokinase activities (10). Similarly, an unusual hexose-ATP-kinase with two catalytic sites plays a role in carbon catabolite repression in the yeast *Schwannyomyces occidentalis* (23).

The hexokinase-deficient mutant of *S. cerevisiae* was shown to be altered in the structural gene for the hexokinase isoenzyme PII (7, 9), which phosphorylates glucose and fructose. *P. chrysogenum* AS-P-78 contains two ATP-hexose kinase activities which might be equivalent to the two hexokinase isoenzymes of *S. cerevisiae*. One of them phosphorylates D-glucose in vitro (and most likely D-2-DOG as well), and the second is involved in the phosphorylation of

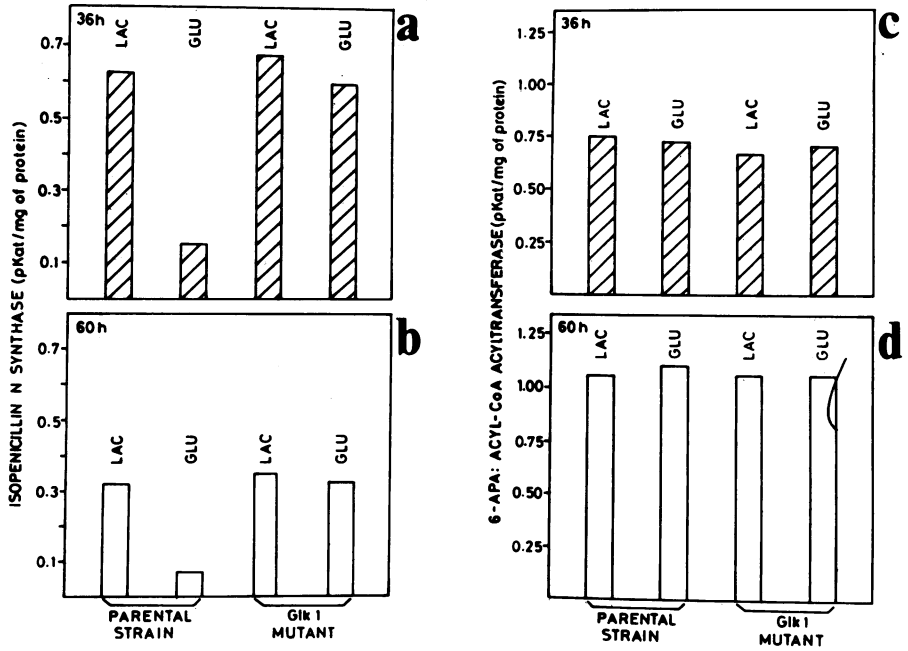


FIG. 8. Effect of D-glucose on IPNS (a and b) and acyl-CoA:6-APA acyltransferase (c and d) activities in cultures of the parental strain and the *glk1* mutant after 30 h (a and c) or 60 h (b and d) of incubation.

D-galactose. The *glk1* mutant of *P. chrysogenum* was deficient in the D-glucose- and D-2-DOG-phosphorylating activities, which suggests that both analogs are phosphorylated by the same glucokinase. However, this mutant retained a normal D-galactose-phosphorylating activity, suggesting that D-galactose is phosphorylated by a different kinase.

The role of hexokinase PII in D-glucose repression in yeasts has been corroborated by cloning and transformation experiments (8). After the transformation system in *P. chrysogenum* was developed, we were then able to introduce the *kex* PII gene of yeasts into *P. chrysogenum*, to study the effect of this gene on the regulation of penicillin biosynthesis by D-glucose.

A very interesting result is the simultaneous alteration in the *glk1* mutant of D-glucose catabolite repression of β-galactosidase and of IPNS and penicillin biosynthesis. These results suggest that there is a common mechanism in *P. chrysogenum* that is involved in D-glucose catabolite repression of (i) catabolic enzymes for sugar utilization and (ii) enzymes of the antibiotic biosynthetic pathways. The molecular mechanism may involve glucokinase as an effector, although the exact role of this enzyme is still unclear.

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