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Penicillin production by glucose-derepressed mutants of *Penicillium chrysogenum*

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SUMMARY

Wild-type strains of *Penicillium chrysogenum* produce lower penicillin V titers in media containing excess glucose. Two mutant strains were isolated and shown to produce normal penicillin V titers in the presence of excess glucose. These strains, designated as glucose-repression insensitive (GRI) mutants, produced higher penicillin V titers than the wild-type strain in media containing lactose as the main carbohydrate source. In lactose-based media, the production of penicillin V was depressed to a much lesser extent by in-cycle additions of glucose with the GRI mutants when compared to the wild-type strain. In short-term biosynthesis experiments using washed cells in a medium containing glucose as the sole carbon source, the GRI mutants produced penicillin V at a faster rate than the wild-type strain. In fed-batch fermentations in 14-liter fermentors, where glucose was fed continuously and pH controlled, both GRI mutants produced more than 10% higher penicillin V titers than the wild-type strain. These results suggest that isolation of GRI mutants is an effective way to select for higher producing strains and that the synthesis of penicillin synthesizing enzymes in GRI mutants may be less repressed by glucose than in wild-type strains.

INTRODUCTION

Enzymes responsible for the synthesis of many secondary metabolites, including antibiotics, are often depressed in the presence of excess glucose. Although glucose is one of the better carbon sources for growth of most antibiotic-producing microorganisms, rapid utilization of glucose often leads to reduced rates of antibiotic production [3]. To circumvent this "glucose repression" or "carbon catabolite repression", industrial production of penicillins or cephalosporins is carried out either by feeding glucose intermittently or by using slowly-utilized carbohydrates such as lactose, sucrose, or starch. In the latter case, lactose or starch is slowly hydrolyzed into glucose, thus avoiding accumulation of high concentrations of glucose in the medium.

The precise molecular mechanisms for glucose repression are not clearly understood at this time. Revilla et al. [7] reported that high glucose levels repress the synthesis of several penicillin synthesizing enzymes, including ACV synthetase and isopenicillin-*N*-synthetase (cyclase). In *Cephalosporium acremonium*, high glucose concentrations repress the synthesis of one of the key enzymes in cephalosporin *C* biosynthesis, deacetoxycephalosporin-*C*-synthetase or expandase [1].

We report in this paper the isolation and characterization of two mutants of *P. chrysogenum* that have been rendered less sensitive to glucose repression with respect to penicillin production. The results indicate that isolation of glucose derepressed mutants is an effective methodology for discovering improved penicillin-producing strains.

MATERIALS AND METHODS

Organisms. The strains of *Penicillium chrysogenum* used in this study were used for the industrial production of penicillin V at the Bristol-Myers Squibb Company. Strain 1901 was derived from a continuous strain improvement program selecting for improved penicillin productivity and low *p*-OH penicillin V titers (< 1% of total penicillin produced).

Culture conditions and media. Penicillium strains were maintained on a sporulation agar (SA) medium having the following composition: (g/l): lactose, 30; peptone, 5; NaCl, 4; $CuSO_4 \cdot 5H_2O$, 0.001; $FeSO_4$, 0.003; $MgSO_4 \cdot 7H_2O$, 0.05; KH_2PO_4, 0.06; corn steep liquor, 3 ml; agar, 30; pH adjusted to 5.5 with 10 N NaOH. Slant cultures were incubated for 7–10 days at 24°C for sporulation. The techniques used for the agar plug method have been reported [2,4]. The medium used for the agar plug

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method for screening of glucose repression-insensitive (GRI) mutants had the following composition (g/l): lactose, 50; Pharmamedia, 50; sodium phenoxyacetate (NaPOAc), 10; $CaCO_3$, 10; $(NH_4)_2SO_4$, 6; agar, 30; pH 6.3 before sterilization. Each agar plug was point-inoculated with spores from a 7–10 day colony and incubated for seven days at 24°C in a moist chamber. Antibiotic activity present in the agar plug was bio-assayed on Nutrient Agar medium (Difco) seeded with spores of *Bacillus subtilis* ATCC 6633. In order to restrict the size of inhibition zone, penicillinase (Difco) was added to the agar medium at a concentration of 100 IU/ml.

For penicillin V fermentations in shake flasks, spores from a 7–10 day slant culture (15 mm × 150 mm test tube) were scraped from the agar surface in 5 ml sterile water. One ml of spore suspension was used to inoculate 25 ml seed medium (SM) contained in 125 ml Erlenmeyer flask. The composition of the seed medium is the same as that published by Lein [5]. After 48 h of incubation at 25°C on a gyrotory shaker operating at 235 rpm (35 mm stroke), 0.75 ml of the seed culture was used to inoculate 15 ml of fermentation medium in 125 ml Erlenmeyer flask. The composition of the fermentation medium is the same as that described by Lein [5], except that the concentrations of Pharmamedia and POAc were increased to 4% and 1.5%, respectively. For short-term biosynthesis of penicillin V using washed cells, mycelia grown in shake flask fermentation medium for four days were harvested by filtration, washed twice in sterile water, and resuspended in a synthetic medium having the following composition (g/l): glucose, 30; $(NH_4)_2SO_4$, 10; NaPOAc, 2; NaH_2PO_4, 20.4; $Na_2HPO_4 \cdot 7H_2O$, 14.2; pH 6.4. The mycelium was incubated at 24°C on a gyrotory shaker operating at 235 rpm for 24 h. Samples were removed for determination of dry cell weights, penicillin V titers, and residual glucose concentrations.

Fed-batch fermentations in 14-liter fermentors. A complex fermentation medium was used for fed-batch fermentations in 14-liter jar fermentors. Two hundred ml of 48 h seed culture was used to inoculate 5 l of fermentation medium contained in a 14-l New Brunswick Microferm fermentor. The pH was controlled with 5.7 N NH_4OH using a pH controller. Initially, the pH was controlled at 6.2. After 48 h, the pH set point was raised to 6.5. The initial agitation rate was 500 rpm and raised to 800 rpm after 48 h. The aeration rate was maintained at 1.2 vvm throughout the fermentation. Glucose was fed according to a predetermined schedule as shown in Fig. 5 using a 60% (w/v) glucose solution.

Mutagenic treatment. Spores collected from a 7–10 day slant culture were washed in saline and adjusted to a density of 2×10^6 spores/ml. Ten ml of spore suspension was placed in a small glass petri dish on a magnetic stirrer under a bank of four 15-watt germicidal lamps (GTE/Sylvania no. G15T8) for UV irradiation. The distance between the surface of the petri dish and the lamp was 50 cm. The UV intensity (UV dose rate) at the surface of spore suspension was 1 erg/mm^2/s. The UV dose was adjusted to achieve a killing rate of greater than 90%.

The irradiated spore suspension was diluted and plated out on a sporulation agar. After 10 days of incubation at 24°C, colonies were transferred to agar plugs for detection of GRI mutants according to the procedure described in the Culture Conditions and Media section.

Analytical procedures. The concentrations of penicillin V, phenoxyacetic acid (POAc), *p*-hydroxypenicillin V (*p*-OH pen V), and *p*-hydroxyphenoxyacetic acid (*p*-OH POAc) in fermentation broths were determined by high performance liquid chromatography (HPLC) using a μ-Bondapak C18 column. The chromatogram was developed with a mobile phase having a composition of 250 ml acetonitrile, 750 ml water, and 1.4 ml phosphoric acid per liter of solution. At a flow rate of 2 ml/min, the following retention times were obtained (min): *p*-OH POAc 1.99; *p*-OH pen V, 3.61; POAc, 4.26; penicillin V, 12.88 (detector: Waters A440 filter detector at 280 nm).

The glucose concentration was determined by the glucose oxidase method using a diagnostic kit purchased from Sigma (Kit No. 510-DA).

RESULTS

Isolation of glucose repression-insensitive (GRI) mutants

When a cylindrical agar piece (agar plug) was inoculated with spores of a wild-type colony and incubated at 24°C for seven days, the antibiotic activity produced by the mold colony can be detected by a simple bioassay. A typical inhibition zone having a diameter ranging from 25–28 mm was observed with the wild-type strain, 1901, using *B. subtilis* as the assay bacterium. When glucose (3–5%) was included in the agar medium, a smaller (16–22 mm) zone of inhibition was obtained with the wild-type strain (Fig. 1A). Such reduction in zone

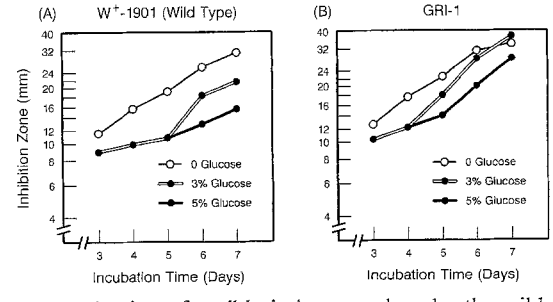

Fig. 1. Production of antibiotic in agar plugs by the wild-type strain and GRI-1 mutant: time course and the effect of glucose.

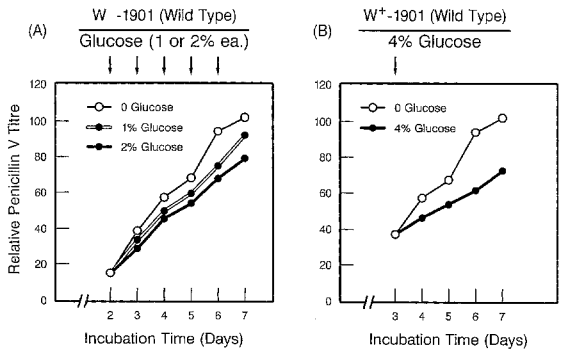

Fig. 2. Effect of glucose addition on penicillin V production in the wild-type strain in shake flasks.

diameter has provided for a rapid test for apparent glucose repression sensitivity.

Two methods have been used for the isolation of glucose insensitive mutants. The first method involved the isolation of mutants resistant to 2-deoxyglucose (DOG) [8]. Over 500 DOG-resistant mutants were isolated from strain 1901 following UV mutagenesis and plating on DOG-containing agar medium (0.2 mg/ml). None of the DOG-resistant mutants were found to be insensitive to glucose repression using the agar plug test.

Another isolation method involved the direct screening of survivors of the mutagenic treatment for sensitivity to glucose repression. Colonies producing the largest inhibition zones (>24 mm) when grown in the presence of 3–5% glucose were retained and tested in shake flasks for penicillin production in the presence of glucose. Two mutants, designated GRI-1 and GRI-2, that produced normal penicillin V titers in the presence of 3% glucose, were obtained after screening over 6000 mutated colonies. Both mutants exhibited similar inhibition zones (25–27 mm) in media containing either glucose (3%) or lacking glucose (control medium) (Fig. 1B).

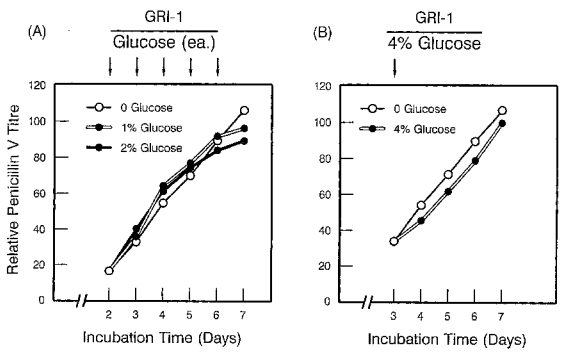

Fig. 3. Effect of glucose addition on penicillin V production in the GRI-1 mutant in shake flasks.

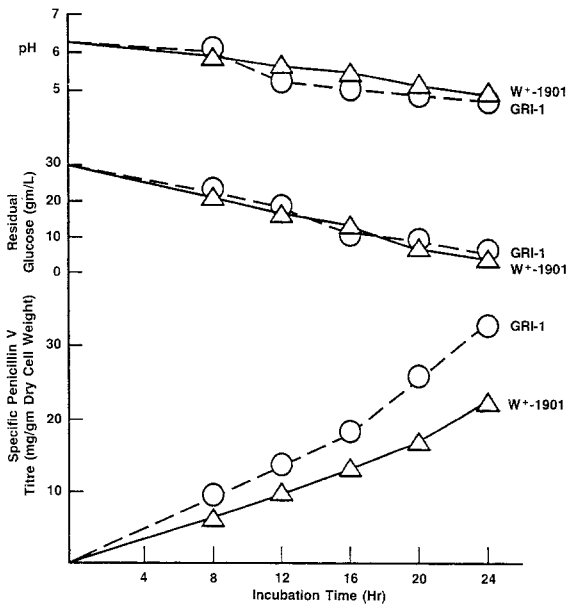

Fig. 4. Penicillin V biosyntheses using washed cells of the GRI-1 mutant and the wild-type strain.

Characterization of GRI mutants

Both mutants, GRI-1 and GRI-2, produced slightly higher penicillin V titers in shake flask media containing lactose as the sole carbon source when compared to the wild-type strain, 1901 (Fig. 2 and 3). When glucose was added daily at 1% or 2% concentrations after 48 h or a single addition of 4% at 72 h, penicillin V production by the wild-type strain was severely inhibited (Fig. 2A,B), whereas production by mutants GRI-1 and GRI-2 was affected to a lesser degree (Fig. 3A,B). These results are consistent with those obtained on the preliminary agar plug test.

The two GRI mutants and wild-type strain were also compared for their ability to produce penicillin V in media containing other carbohydrate sources. All ingredients in the media were kept constant except lactose, which was replaced with another carbohydrate at the same concentration. The results showed that, even though GRI-1 and GRI-2 produced higher penicillin V titers than the wild-type in a medium containing lactose, maltose, or starch, neither the GRI mutants nor the wild-type strain produced significant penicillin V titers in media containing other carbohydrate sources (Table 1).

Penicillin V biosynthesis by washed cells

To study short-term effects of glucose on penicillin V biosynthesis with both GRI mutants and the wild-type strain, cells grown in a lactose-based medium for 96 h were harvested, washed, resuspended, and incubated for 24 h in medium containing glucose as the sole source of

TABLE 1

Penicillin V production by the wild-type strain and GRI-1 mutant in media containing different carbohydrate sources

Strain	Carbohydrate sources[a]	Final pH[b]	Dry cell weight (G/L)	Peak relative penicillin V titer
W+/−1901	Lactose (Control)	6.6	49	100.0
	Glucose	4.7	57	0.6
	Galactose	4.9	60	2.8
	Fructose	4.7	59	0.6
	Sucrose	4.4	65	0.6
	Maltose	7.5	72	20.7
	Starch	7.8	70	30.8
GRI-1	Lactose (Control)	6.5	48	125.0
	Glucose	4.7	58	0.6
	Galactose	5.5	56	1.1
	Fructose	4.8	59	1.7
	Sucrose	4.6	58	0.6
	Maltose	7.5	70	27.5
	Starch	7.9	68	27.4

[a] Each carbohydrate was added to the fermentation medium at 12%.

[b] Broth pH at 7th day. The optium pH range for penicillin production was 6.2–6.7.

carbon. The data showed that the GRI-1 mutant produced penicillin V at a faster rate than the wild-type strain, even though both strains utilize glucose at the same rate (Fig. 4).

Fed-batch fermentation studies in 14-liter fermentors

In fed-batch fermentations where glucose was fed according to a fixed schedule and the pH was controlled by NH_4OH solution, the GRI-1 mutant produced more than 10% higher penicillin V titers when compared with the wild-type strain under conditions of nearly equal sugar utilization by both strains (Fig. 5).

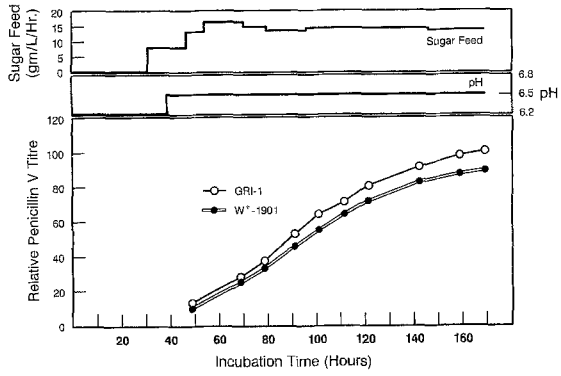

Fig. 5. Comparison of the GRI-1 mutant and the wild-type strain in fed-batch fermentation in 14-l fermentors.

DISCUSSION

The regulation of antibiotic production either by different carbon sources or by carbon limitation via intermittent feeding is well known in the antibiotic fermentation industry. Different sugars exert varying effects on growth as well as on antibiotic production. Even though glucose is a readily utilizable sugar for growth (trophophase), rapid utilization of glucose during the production phase (idiophase) often leads to decreased antibiotic productivity via glucose repression mechanisms. Thus, various sugar feed regimens have been designed to maximize antibiotic production at minimum sugar concentration.

In modern penicillin fermentation process technology, sugar is usually fed intermittently according to a designated rate regimen to avoid sugar accumulation [6]. However, localized accumulation of sugar may occur in a fermentor immediately following the addition of sugar due to stratification in a highly viscous fermentation medium. This localized accumulation of sugar can result in temporary, albeit brief, repression of antibiotic production. One can theorize that due to the constant oscillation of sugar concentrations in a fermentor, the antibiotic biosynthetic machinery thereby fluctuates between a fully derepressed state and a partially repressed state, resulting in significant variation in antibiotic production rate. Hence, a mutant which is less sensitive to glucose repression may prove to be less sensitive to fluctuation in

sugar concentrations. Such strains could also produce penicillin at a fully derepressed rate.

Our efforts to isolate such mutants using random screening of colonies for reduced sensitivity to glucose repression have resulted in the isolation of two mutants, GRI-1 and GRI-2. Both mutants produced greater penicillin V titers than the wild-type strain in a medium containing lactose as the major carbohydrate source. In media containing other carbohydrates such as glucose, galactose, or sucrose, neither GRI mutants nor the wild-type strain produced significant quantities of penicillin V. Both GRI mutants and the wild-type strain 1901 exhibited similar growth patterns and pH profiles in all media tested.

In lactose-based media, the productivity of penicillin V by the GRI mutants was depressed to a much lesser degree by the in-cycle additions of glucose compared to the wild-type strain. In short-term experiments using washed cells in a medium containing glucose as the main source of carbon, the GRI mutants produced penicillin V at a faster rate than the wild-type strain. In fed-batch fermentation in 14-l fermentors where glucose was fed continuously, the GRI mutants produced 10% more penicillin V than the wild-type strain. These results suggest that the penicillin-synthesizing machinery in the GRI mutants was less sensitive to glucose repression than the wild-type strain.

One possible effect of GRI mutation may involve the derepression of one or more of the penicillin-synthesizing enzymes in the presence of glucose, e.g., ACV synthetase and isopenicillin-*N*-synthetase (IPNS), which have been reported to be repressed by high glucose concentrations [7]. Work is in progress to assay both the ACV synthetase and IPNS enzyme activities from the two GRI mutants and the wild-type progenitor strain in order to compare their relative sensitivity to glucose.

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