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Carbon catabolite regulation of rebeccamycin production in *Saccharothrix aerocolonigenes*

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SUMMARY

A new antitumor antibiotic named rebeccamycin was isolated from fermentations of an actinomycete, *Saccharothrix aerocolonigenes*. A defined medium was developed to study the regulation of synthesis of rebeccamycin by *S. aerocolonigenes*. In glucose medium formation of rebeccamycin was detected only after glucose was depleted. Examination of eleven different carbon sources revealed that carbon catabolite regulation is a major control mechanism for rebeccamycin production.

INTRODUCTION

Rebeccamycin is a novel antitumor secondary metabolite isolated from cultures of the actinomycete *Saccharothrix aerocolonigenes* [2]. Rebeccamycin represents a novel antitumor antibiotic chemotype with a broad spectrum of activity in tumor-bearing murine models such as P388 leukemia, L1210 leukemia and B16 melanoma, and it inhibits the growth of human lung adenocarcinoma cells in vitro [2]. Its structure, consisting of a halogenated indolocarbazole chromophore N-glycosylated with 4-*O*-methylglucose, was elucidated using a combination of spectroscopic methods [10] and confirmed by X-rays [10] and total synthesis [4]. This novel chemotype shows good potential to yield a candidate for development toward clinical trials. Therefore, optimal fermentation conditions need to be developed for production of rebeccamycin-type compounds.

The effects of carbon catabolites on the regulation of secondary metabolism are well documented [9]. Studies on the regulatory mechanisms of antibiotic biosynthesis often provide valuable information toward the design of improved medium and fermentation conditions for the production of a giv-

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en antibiotic. In this paper we present evidence for carbon catabolite regulation as a control mechanism for rebeccamycin production in *S. aerocolonigenes*.

MATERIALS AND METHODS

Microorganism

The rebeccamycin-producing culture, *S. aerocolonigenes* [2], had been deposited with the American Type Culture Collection with the accession number ATCC 39243.

Media and culture conditions

Strain ATCC 39243 was grown on slants of yeast-malt extract agar. This medium consisted of glucose 4 g, yeast extract 4 g, malt extract 10 g and agar 20 g in 1 liter of distilled water (pH 7.6). To prepare an inoculum for the shake flask culture, surface growth from a 5-day-old slant culture was transferred to a 500-ml Erlenmyer flask containing 100 ml of medium consisting of glucose 30 g, soy flour 10 g, cottonseed embryo meal 10 g and calcium carbonate 3 g in 1 liter of distilled water (pH 7.5).

This vegetative culture was incubated at 28°C for 48 h on a Gyrotary shaker (Model G53, 5 cm stroke, New Brunswick Scientific) set at 250 rpm. Two milliliters of vegetative culture were transferred to a 500-ml Erlenmyer flask containing 100 ml of production medium. The production medium contained a carbon source such as glucose or other carbohydrate 10 g, ammonium sulfate 2 g, potassium phosphate 2 g, magnesium sulfate 1 g, and calcium carbonate 2 g, in 1 liter of distilled water with no pH adjustment. The production culture was incubated at 28°C and 250 rpm on the same shaker.

Analytical methods

The analysis of rebeccamycin in fermentation extracts was performed by HPLC using a C-18 reverse phase column (μ-Bondapak, 3.9 × 300 mm, Water Associates). The solvent system was ammonium acetate (0.1 M)/methanol/acetonitrile (4:3:3, v/v) at a flow rate of 1.5 ml/min and the detector wavelength set at 313 nm. The fermentation extract was processed by centrifuging 10 ml of culture broth for 20 min at 1500 × g. The supernatant was decanted and the mycelium was extracted with 3 ml of acetone. Fifty microliters of the extract were used for HPLC analysis.

Dry cell weight (DCW) was determined by centrifuging 10 ml of culture broth in tared tubes for 20 min at 1500 × g, discarding the supernatant and drying overnight at 100°C and weighing the samples.

Residual glucose in the medium was measured by the glucose oxidase procedure [11] using Glucostat reagents supplied by the Sigma Chemical Company (St. Louis, MO).

RESULTS AND DISCUSSION

We have examined the production of rebeccamycin by *S. aerocolonigenes* in a defined medium (DF-1) which contained glucose (10 g/l) and ammonium sulfate (2 g/l) as the sole carbon and nitrogen source respectively (Fig. 1). The medium was supplemented with $MgSO_4$ (1 g/l), KH_2PO_4 (2 g/l) and $CaCO_3$ (2 g/l). The titer of rebeccamycin in the DF-1 medium was 1.3 μg/ml. Examining the pattern of glucose consumption and rebeccamycin production (Fig. 1), it is evident that carbon catabolite

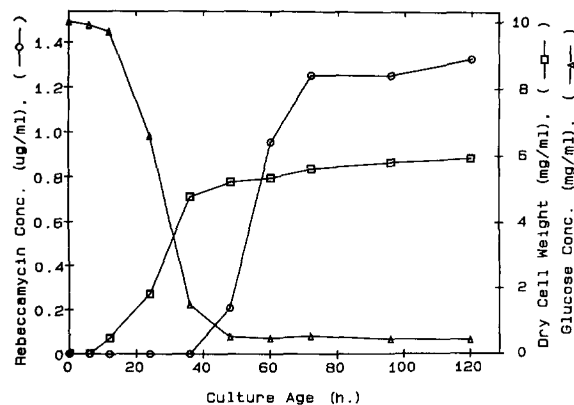

Fig. 1. Time course of rebeccamycin production by *S. aerocolonigenes* in DF-1 medium. O: rebeccamycin; △: glucose; □: DCW.

Table 1

Effect of carbon sources on growth and rebeccamycin production[a]

Carbon source	pH	DCW (mg/ml)	Growth rate (mg/ml per h)	Volumetric productivity (μg/ml)	Specific productivity (μg/mg DCW)
Mannitol	6.3	6.97	0.301	0.72	0.10
Fructose	6.3	6.20	0.268	1.2	0.19
Glucose	6.6	6.20	0.243	1.5	0.24
Galactose	6.2	4.83	0.183	0.53	0.11
Sucrose	5.9	6.33	0.235	1.7	0.27
Maltose	6.2	5.96	0.224	2.1	0.35
Melibiose	6.3	5.78	0.177	2.0	0.35
Lactose	6.2	6.05	0.192	5.1	0.84
Raffinose	6.7	3.81	0.096	3.0	0.79
Melezitose	6.7	3.10	0.079	2.4	0.77
Starch	6.6	5.20	0.161	18.4	3.54

[a] All data except for growth rate reported in this table represent values determined on fermentation samples harvested at 144 h. The growth rate was determined by measuring the slope of the active growth phase of the growth curve (DCW versus time).

regulation may be present, since rebeccamycin production did not start until 95% of the glucose was consumed in the medium. To further investigate the effect of carbon catabolite regulation in the fermentation of rebeccamycin, we have examined the production of rebeccamycin in the presence of 11 different carbon sources. Table 1 summarizes the relationship between pH, maximum dry cell weight, growth rate and production of rebeccamycin in these media. The monosaccharides, except for galactose, supported very good growth and high growth rate, while the production of rebeccamycin was poor. Mannitol supported the highest DCW (6.97 mg/ml) and highest growth rate (0.301 mg/ml per h). Both volumetric productivity (0.72 μg/ml) and specific productivity (0.10 μg/mg DCW) were the lowest among the carbon sources that were tested. Fructose-containing medium supported a higher growth rate (0.268 mg/ml per h) than the glucose medium (0.243 mg/ml per h) even though the two media yielded the same DCW (6.2 mg/ml). The production of rebeccamycin in the fructose medium was lower than that of the glucose medium. The disaccharide media supported about the same DCW as the monosaccharide media but the growth rates were lower in the disaccharide media than in the monosaccharide media. The production levels of rebeccamycin were higher in the disaccharide media than the monosaccharide media. Lactose medium had about the same DCW as the glucose medium but a lower growth rate. The lactose medium supported higher volumetric productivity (5.1 μg/ml) and specific productivity (0.84 μg/mg DCW) than those of the glucose medium. The trisaccharides, raffinose and melezitose, both have lower growth rate than lactose but did not improve on the specific productivity of rebeccamycin over lactose. These two carbon sources yielded very poor levels of biomass. Since rebeccamycin is an intracellular metabolite, obtaining a high level of cell mass may be important for rebeccamycin production. When starch is used as the carbon source, the growth rate of the organism was 0.161 mg/ml per h and the highest volumetric and specific productivity, 18.4 μg/ml and 3.45 μg/mg DCW respectively, were observed.

These results indicate that the production of rebeccamycin is regulated by carbon catabolism, as is the case with many other antibiotic fermentations, such as actinomycin [3], penicillin [14], cephalosporin C [5], cephamycin [1], chloramphenicol [13], neomycin [8], puromycin [12], and novobiocin [6]. Rap-

idly utilized carbon sources such as mannitol, fructose and glucose yielded low titers of rebeccamycin, while yielding high growth rates and DCW. The high titers and high specific productivity obtained when starch was used as a carbon source with relatively low growth rate and DCW may be due to the slow hydrolysis of starch generating a situation of carbon limitation and releasing the culture from carbon source regulation.

Our first application of this defined medium, using starch as the carbon source, was the incorporation of ^{13}C- and ^{14}C-labeled precursors into rebeccamycin to determine the biosynthetic origins of this antibiotic. Specific incorporation of labeled precursors into rebeccamycin in the DF-1 starch-based medium was much higher than in complex medium [7]. In other applications, production of rebeccamycin in fermentors does benefit from slow feeding of carbon sources into the culture in order to overcome carbon catabolite regulation.

Further studies on the regulation of rebeccamycin production, such as phosphate and nitrogen effects, are ongoing.

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