

## CARBON CATABOLITE REGULATION OF GENTAMICIN FORMATION

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The effect of utilizable carbon sources on the production of gentamicin by *Micromonospora purpurea* has been studied. High D-glucose and D-xylose concentrations (40 mg/ml), exerted a strong and permanent negative action on antibiotic formation. On the other hand, similar concentrations of D-fructose, D-mannose, maltose and starch caused no effect. The glucose action is seen only if added during the logarithmic growth phase; moreover, the sugar needs to be metabolized to show its negative effect.

Gentamicin is a complex of aminocyclitol compounds produced under aerobic conditions by some species of *Micromonospora*. The complex consists of three major compounds referred to as C1, C1a and C2, highly active against Gram-negative bacteria and *Mycobacterium tuberculosis*<sup>1</sup>. As with other aminocyclitol antibiotics, gentamicin is synthesized from 2-deoxystreptamine<sup>2</sup>, probably through a branched biosynthetic pathway, as suggested from biotransformation experiments using idiotrophic mutants of *Micromonospora purpurea*<sup>3</sup>. Little information is available with respect to the fermentative production of gentamicin by *M. purpurea* and the factors and conditions that control its biosynthesis.

The negative influence exerted by the type and concentration of the carbon source on the fermentative production of secondary metabolites, has been well documented for several antibiotics<sup>4</sup>. Although the pattern of carbohydrate utilization by *M. purpurea* has been established<sup>5</sup>, there is no information concerning the influence of the carbon source on antibiotic formation. In this paper we report of the effect of utilizable carbon sources on growth and gentamicin biosynthesis in this actinomycete.

### Materials and Methods

#### Microorganism and Cultivation

*Micromonospora purpurea* NRRL-2953 was kindly supplied by the ARS Culture Collection, U.S. Department of Agriculture, Peoria, IL., U.S.A.

Spores of this microorganism were obtained from slants containing 0.5 g yeast extract, 0.5 g casein hydrolysate, 0.1 g calcium carbonate, 2.0 g starch, 1.0 g glucose (pH 7.6) and 1.5 g agar per 100 ml distilled water. They were maintained at  $-20^{\circ}\text{C}$  in 2 ml vials containing 1 ml of a mixture consisting of 40% glycerol, 1% malt extract and 0.4% yeast extract. For seed culture, approximately  $10^6$  *M. purpurea* spores (from a 5-day old slant) were inoculated into a 50-ml Erlenmeyer flask containing 10 ml of the following medium: 0.3 g beef extract, 0.5 g Tryptone, 0.5 g yeast extract, 0.4 g calcium carbonate, 0.1 g glucose and 2.4 g starch (pH 7.6) per 100 ml distilled water. Incubations were carried out at  $29^{\circ}\text{C}$  for 60 hours on a rotary shaker (175 rpm). For antibiotic production, 2 ml of the above culture were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of the chemically defined medium reported by CARVAJAL (U.S. Patent 2,808,364, 1957) supplemented with 0.004 mM  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ . After preparation, the medium was adjusted to pH 7.0 and sterilized. Carbohydrates were sterilized separately and added before inoculation. Fermentations were carried out at  $29^{\circ}\text{C}$  for 7 days on a rotary shaker at 175 rpm.

#### Assay of Gentamicin

At specified intervals, the production of antibiotic was determined by an agar disk technique using

*Bacillus subtilis* ATCC 6633 as the assay organism<sup>6</sup>). After incubation (36 hours at 29°C) in a bioassay medium containing 0.5 g yeast extract, 1.0 g Tryptone, 0.5 g NaCl and 1.0 g agar per 100 ml distilled water, pH 7.0, zones of inhibition were measured and the amount of gentamicin estimated using gentamicin sulfate (Sigma Chemical Co., St. Louis, MO) as standard.

#### Growth Determination

Samples of mycelia were harvested, washed with 2 vol distilled water and placed in 2 ml of 0.3 M trichloroacetic acid. After centrifugation, the pellet was resuspended in 1 ml of 0.4 N NaOH, and its protein concentration determined by the Lowry method using bovine serum albumin as standard<sup>7</sup>.

#### Reproducibility of Results

The experiments reported were repeated at least twice (two independent experiments) and the results reported are the mean values. The observed variations were consistently less than 10%.

### Results

#### Effect of Glucose Concentration on Fermentations

*M. purpurea* was able to utilize glucose as the only carbon source for growth and gentamicin formation. Fig. 1 shows maximum growth and specific antibiotic production of this microorganism in fermentations with glucose concentrations ranging from 1 to 50 mg/ml. As seen in the figure, growth of the culture increases in proportion to the sugar concentration added to the fermentation medium (up to 15 mg/ml). Plots of growth versus time gave profiles showing an increase in the extent of growth but not in the culture growth rate (not shown). In regard to specific gentamicin formation, an inverse correlation was found between the maximum antibiotic produced and the initial carbohydrate concentration added to the fermentation medium, with a maximum effect seen at 50 mg/ml (Fig. 1). As shown, only slight differences were observed in the medium pH at all sugar concentrations tested, excluding this possibility as the cause of gentamicin repression.

#### Differential Rate of Antibiotic Synthesis

To gain more information about the carbohydrate effect, *M. purpurea* was grown with low (10 mg/ml) and high (40 mg/ml) glucose concentrations, and growth was plotted versus antibiotic production at different times during the fermentation. As can be seen in Fig. 2, all differential antibiotic rate values in the high sugar fermentations were reduced approximately by one half in comparison to those obtained with the lower glucose concentration. In other words, the use of high glucose resulted in an increase of growth but a reduction in the slope of antibiotic production.

Fig. 1. Effect of different D-glucose concentrations on the maximum growth ( $\Delta$ ), gentamicin formation ( $\square$ ), final pH of the medium ( $\bullet$ ), and specific gentamicin formation ( $\circ$ ).

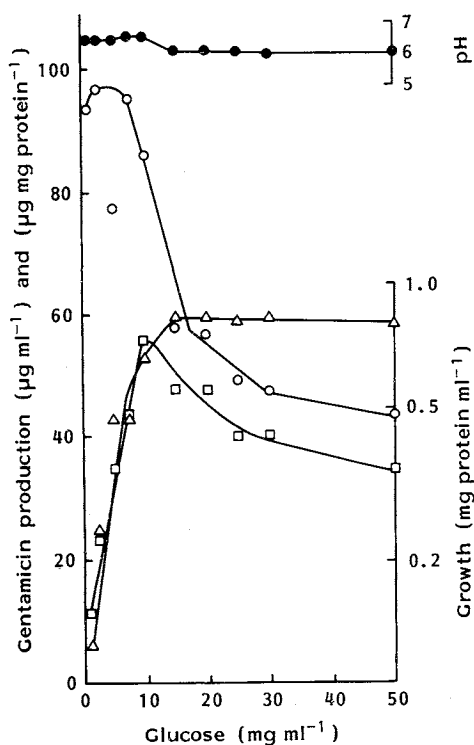


Fig. 2. Effect of D-glucose on differential rate of gentamicin formation.

Cultures were grown in fermentation medium with D-glucose 10 (○) or 40 (△) mg/ml.

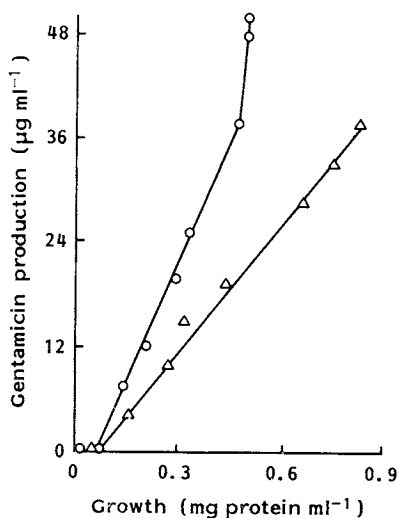


Fig. 3. Effect of the time of D-glucose addition on gentamicin production.

The carbohydrate was added at 0 (●), 24 (△), 48 (▲), 60 (□) and 96 hours (■) fermentations. Control (○) without further feeding.

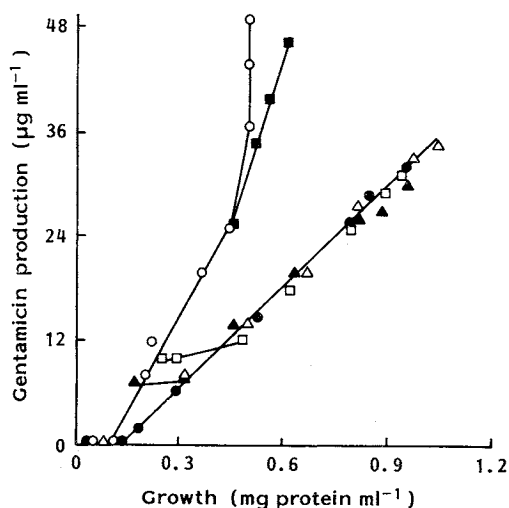


Table 1. Effect of 2-deoxyglucose on maximum growth and gentamicin formation.

Condition	Time of addition (hours)	Growth (mg/ml)	Antibiotic	
			µg/ml	µg/mg protein
Control		0.5	48	96
D-Glucose	48	0.96	30	31
2-Deoxyglucose	48	0.16	16	100
D-Glucose	72	0.8	22	27
2-Deoxyglucose	72	0.35	28	80

Cells were grown with 1% glucose and at specified intervals 3% glucose or 2-deoxyglucose was added to the fermentation. Cultures were incubated for 168 hours at 29°C.

Table 2. Effect of different carbohydrates on gentamicin formation.

Carbon source	Specific antibiotic formation (µg/ml protein)			
	Carbohydrate concentration (mg/ml)			
	5.0	10.0	20.0	40.0
D-Glucose	97.5	74.5	50.5	34.0
D-Xylose	107.5	86.5	34.0	0.0
D-Fructose	182.5	190.0	147.0	151.5
D-Maltose	31.0	34.0	24.0	36.0
D-Mannose	44.5	40.5	46.0	46.0
Sucrose	103.5	103.0	63.0	69.5
Starch	47.0	38.0	36.0	48.0

After 96 hours incubation, gentamicin was determined in the fermentation broths.

#### Addition of D-Glucose during the Fermentation

In order to characterize the glucose effect, the carbohydrate was added at different times during fermentation, to cultures growing with a low glucose concentration (10 mg/ml). As seen in Fig. 3, in contrast to the control without additional glucose, the addition of 40 mg/ml D-glucose at the beginning of the fermentation, or during the first 6 hours of the growth phase, reduced by approximately one half the differential rate of antibiotic biosynthesis. On the other hand, no significant changes were found when the carbohydrate was added to cultures at 96 hours.

#### Effect of a Glucose Analogue on Antibiotic Formation

To establish whether or not glucose *per se* or a metabolite was responsible for this effect, a

non-metabolizable analogue (2-deoxyglucose), was supplied to cultures grown with a low sugar concentration<sup>8</sup>). As revealed in Table 1, although growth was severely affected by the analogue addition, only minor changes were observed on specific antibiotic formation, as compared to cultures without it. On the other hand, 40 mg/ml D-glucose reduced specific gentamicin formation by 70%. Similar observations were obtained from plots of this data, showing the differential rate of antibiotic production *i.e.* that analogue affected growth but not antibiotic production.

#### Effect of Other Carbohydrates on Antibiotic Formation

In addition to D-glucose, D-xylose also exerted a negative effect on gentamicin biosynthesis (Table 2). On the other hand, fructose, maltose, mannose and starch had no influence whereas sucrose showed only a slight one.

#### Addition of Precursors of the Antibiotic Pathway

In order to define the step in antibiotic formation that is affected by glucose, we treated repressed cultures with several precursors and intermediates of the gentamicin pathway in an attempt to reverse the negative action. L-Glutamate, L-glutamine, D-glucosamine and 2-deoxystreptamine (ranging from 1 to 10 mM) were employed for this purpose. However, although these compounds were efficiently taken up by the cells, none of these was able to overcome the negative action of the high glucose concentration when added during or at the end of the logarithmic growth phase.

### Discussion

The effect of utilizable carbon sources on the production of gentamicin by *M. purpurea* has been studied. An inverse correlation was found between the maximum antibiotic produced and the initial glucose and xylose concentration added to the fermentation medium. Other carbohydrates such as fructose, maltose, mannose and starch caused no repression. Considering that D-xylose is a precursor of the gentamicin moiety<sup>9</sup>, its negative influence on antibiotic biosynthesis is unexpected. Plots of growth versus antibiotic production in low and high glucose concentrations gave profiles comparable to the catabolite repression of  $\beta$ -galactosidase elicited by glucose in *Escherichia coli*<sup>10</sup>. As pointed out by these authors, such an effect is characterized by a strong and permanent negative action on the formation of a given metabolite. Therefore, the possibility of a catabolite repression exerted by D-glucose was considered as the cause of its negative action on antibiotic formation. In agreement with this hypothesis it was found that: a) the carbohydrate influences antibiotic formation only if added during the stage of active protein synthesis; b) is characterized by a strong and permanent negative effect on the synthesis of gentamicin and c) D-glucose needs to be metabolized to exhibit its negative action on antibiotic formation. Finally, the regulation observed seems to take place at a step beyond the intermediate 2-deoxystreptamine.

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