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Carbon Catabolite Regulation of Cephalosporin Production in *Streptomyces clavuligerus*

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Cephalosporin production by *Streptomyces clavuligerus* is regulated by some type of carbon catabolite control. Increasing concentrations of preferred carbon sources, such as glycerol and maltose, decreased production of the antibiotics. Poorer carbon sources, such as α-ketoglutarate and succinate, led to high specific production of cephalosporins and shifted the dynamics of fermentation to a greater degree of association with growth. The results support the concept that the phase in which a product is made by a microorganism is not a function of the particular molecule produced, but rather of the nutritional environment presented to the organism.

The biosynthetic pathways leading to the production of secondary metabolites such as antibiotics are often connected to and influenced by the pathways of primary metabolism (4). For example, an end product or an intermediate of a primary pathway frequently serves as a precursor for the antibiotic molecule. Such intermediates or end products could be part of a biosynthetic, amphibolic, or catabolic pathway. Catabolic pathways involving energy metabolism of the organism are under strict control in microorganisms. When a favored carbon/energy source is used, the cell is usually prevented from producing enzymes which catabolize other carbon compounds. It is possible that such a catabolic regulatory mechanism could also control the production of secondary metabolites. The strong influence of carbon sources on the production of antibiotics has been reported in several cases (3).

Our interest has been focused on *Streptomyces clavuligerus*, which was first described by Higgens and Kastner (6). This strain produces four β-lactam antibiotics (2, 7, 11): two of these are cephalosporins [7-(5-amino-5-carboxyvaleramido)-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid and 7-(5-amino-5-carboxyvaleramido)-7-methoxy-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (also known as cephamycin C)]; penicillin N and clavulanic acid are also produced. We have developed a chemically defined medium for growth and antibiotic production, and have described phosphate control of cephalosporin production in this strain (1). We now report on carbon source regulation of the formation of these antibiotics.

MATERIALS AND METHODS

Organism. *S. clavuligerus* NRRL 3585 (ATCC 27064) (6) was used throughout this study.

Media. Our basal chemically defined fermentation medium contained 0.06% $MgSO_4$, 0.35% K_2HPO_4, and 1 ml of trace salts stock solution (containing 100 mg of $FeSO_4 \cdot 7H_2O$, 100 mg of $MnCl_2 \cdot 4H_2O$, 100 mg of $ZnSO_4 \cdot H_2O$, and 100 mg of $CaCl_2$ per 100 ml of water) per liter of 0.1 M 3-(*N*-morpholino) propane sulfonic acid (MOPS) buffer (Sigma Chemical Co., St. Louis, Mo.). Carbon and nitrogen sources added to the basal medium are described in the text. Inoculum medium contained, in addition to the basal medium, 1% glycerol (autoclaved separately), 0.2% L-asparagine, 0.1% yeast extract, and 0.1% NH_4Cl. Yeast extract-malt extract agar (13) was used for the preparation of spores. The initial pH of all media was adjusted to 6.8 with KOH or HCl as required.

Culture conditions. All liquid cultures were conducted in 250-ml Erlenmeyer flasks containing 50 ml of medium at 30°C on a rotary shaker at 250 rpm. About 10^8 spores were inoculated into 50 ml of inoculum medium and incubated as above for 1 to 2 days. A sample of 1 ml of the resulting seed culture was inoculated into the desired medium and incubated for up to 7 days. At various times, samples were taken for pH, biomass, and antibiotic analysis. Growth was measured by absorbance using the Klett-Summerson colorimeter (Klett Manufacturing Co., New York, N.Y.) with a red filter. A cell dry weight of 1 g/liter is equivalent to 260 Klett units. All suspensions were mixed on a Vortex mixer and diluted to 50 to 150 Klett units before measurements were made; absorbance was linearly related to dry cell weight.

We assayed the production of cephalosporins by the standard disk-agar plate technique, using as the assay organism an *Escherichia coli* strain that is supersen-

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sitive to β-lactam antibiotics. Inoculum of *E. coli* Ess was grown overnight in nutrient broth (Difco Laboratories, Detroit, Mich.). Each petri dish (diameter 87 mm) contained 10 ml of the assay medium (nutrient broth solidified with 1% agar). The diameter of the paper disk was 6.35 mm. Because of its commercial availability, cephalosporin C was used as the standard, even though it is not produced by *S. clavuligerus* NRRL 3585. 7-(5-Amino-5-carboxyvaleramido)-3-carbamoyloxy-methyl-3-cephem-4-carboxylic acid has the same activity as cephalosporin C against the assay organism, whereas cephamycin C is about three times more active. Our assay detected only the cephalosporins because (i) clavulanic acid is a weak antibiotic, having only 5 to 10% the activity of cephalosporin C in this assay, and (ii) penicillin N is produced only in small amounts under our conditions. The latter was shown by the inability of penicillinase (Difco) to decrease the zone size obtained with fermentation broth. The amount of penicillinase used (500 U/ml of agar) was sufficient to destroy completely the zone produced by a disk dipped into 100 μg of penicillin N per ml even when the penicillin N sample included 100 μg of the penicillinase inhibitor, clavulanic acid, per ml.

RESULTS

Utilization of amino acids. Preliminary experiments showed that NH_4^+ was a poor nitrogen source for cephalosporin production. Before studies on carbon sources could begin in earnest, a nitrogen source compatible with cephalosporin production was needed. A high ratio of nitrogen (0.4% nitrogen source) to carbon (0.1% glycerol) was used to eliminate those nitrogen sources that would suppress antibiotic production. Table 1 shows that many amino acids can serve as nitrogen sources in the presence of 0.1% glycerol for the growth of *S. clavuligerus*. From the level of biomass obtained in many cases (above 0.5 g/liter), it is obvious that the amino acid was used not only as a nitrogen source but as a carbon source as well. In all cases when antibiotic was produced, this secondary process continued after growth had ceased and often during lysis. On the basis of rapid growth and subsequent antibiotic production, L-asparagine was chosen as a nitrogen source for the experiments on carbon source utilization.

Utilization of carbohydrates as major carbon source. Using 0.2% L-asparagine as a nitrogen source, carbohydrates were screened as possible carbon sources. We found starch, maltose, and glycerol to be substrates (Table 2); of these, starch appeared to be most favorable for cephalosporin production.

Utilization of organic acids as major carbon source. Organic acids were also tested as carbon sources. As shown in Table 3, succinate, α-ketoglutarate, and fumarate supported some growth. Of these, α-ketoglutarate was best for cephalosporin production. Unlike growth on carbohydrates, growth on salts of organic acids was accompanied by a marked rise in pH.

Effect of increasing glycerol concentrations. *S. clavuligerus* was grown in defined medium containing 0.4% L-asparagine as a nitrogen source and increasing concentrations of glycerol as a carbon source. In the absence of glycerol, asparagine served as both carbon and nitrogen source. The addition of glycerol caused an increase in the rate and extent of growth (Fig. 1), which indicated that the carbohydrate was the factor limiting growth. A glycerol concentration of 0.2% stimulated the growth rate. Higher levels had no effect on growth rate, but did further increase the extent of growth. The culture

TABLE 1. *Effect of amino acids on growth and antibiotic production*[a]

Nitrogen source	48 h		96 h		144 h		
	DCW (mg/ml)	Cephalosporins (μg/ml)	DCW (mg/ml)	Cephalosporins (μg/ml)	DCW (mg/ml)	Cephalosporins (μg/ml)	pH
L-Alanine	0.81	12	0.90	20	0.54	55	7.4
L-Arginine	0.03	0	0.10	0	0.27	15	7.1
L-Asparagine	1.07	0	1.33	35	0.64	65	7.7
L-Aspartic acid	1.43	12	0.59	25	0.46	30	7.2
L-Glutamic acid	1.45	0	0.94	12	0.88	20	7.3
L-Glutamine	0.68	22	0.70	50	0.59	55	7.7
L-Histidine	0.98	0	1.05	10	0.78	35	7.2
L-Isoleucine	0.03	0	0.05	0	0.10	0	6.9
L-Leucine	0.03	0	0.05	0	0.11	0	6.9
L-Lysine	0.16	0	0.39	0	0.41	12	7.0
L-Phenylalanine	0.05	0	0.24	0	0.70	0	6.9
L-Proline	0.10	0	0.20	0	0.20	0	6.9
L-Threonine	0.51	0	0.90	25	0.68	68	7.4

[a] Cells were grown in basal chemically defined medium using 0.1% glycerol as a carbon source. The amino acids were used at 0.4%. DCW, Dry cell weight.

TABLE 2. *Effect of carbohydrates as major carbon sources on growth and antibiotic production*[a]

Carbon source	48 h		96 h			
	DCW (mg/ml)	Cephalosporins (μg/ml)	DCW (mg/ml)	Cephalosporins (μg/ml)	Cephalosporins (μg/mg DCW)	pH
Cellulose	0.08	0	0.08	0		6.9
D-Galactose	0.09	0	0.09	11		7.0
D-Glucose	0.09	0	0.12	12		7.1
Glycerol	0.23	0	1.62	120	74	6.8
Lactose	0.01	0	0.15	10		7.1
Maltose	0.20	0	1.20	130	108	6.3
D-Ribose	0.08	0	0.18	10		7.0
Soluble starch	0.18	10	0.90	180	200	7.1

[a] Cells were grown in basal chemically defined medium using 0.2% L-asparagine as the nitrogen source and the specified carbon source at 0.5%. The small amount of growth in the presence of lactose, galactose, glucose, ribose, or cellulose is due to the utilization of the asparagine as the carbon source. DCW, Dry cell weight.

TABLE 3. *Effect of organic acids as major carbon source on growth and antibiotic production*[a]

Carbon source	48 h		96 h			
	DCW (mg/ml)	Cephalosporins (μg/ml)	DCW (mg/ml)	Cephalosporins (μg/ml)	Cephalosporins (μg/mg DCW)	pH
Sodium acetate	0.15	0	0.23	10		7.2
Sodium α-ketoglutarate	0.15	7	0.32	110	344	8.4
Sodium citrate	0.05	0	0.06	0		6.9
Sodium fumarate	0.17	0	0.35	30	86	7.5
Sodium malate	0.10	0	0.13	7		7.0
Sodium pyruvate	0.17	0	0.21	10		7.1
Sodium succinate	0.18	12	0.55	90	164	7.9
Sodium tartarate	0.11	0	0.13	13		7.1

[a] Cells were grown in basal chemically defined medium using 0.2% L-asparagine as the nitrogen source and the specified carbon source at 0.5%. DCW, Dry cell weight.

reached stationary growth phase after 70 to 80 h of growth, and antibiotic production continued until 120 h.

When the maximum values for biomass, volumetric cephalosporin titer, and specific cephalosporin titer are plotted against glycerol concentration (Fig. 2), it can be seen that the extent of growth increased linearly with increasing glycerol concentration. Whereas volumetric production of cephalosporins was optimal at 0.4% glycerol, specific cephalosporin production of the antibiotic was inversely proportional to glycerol concentration. These data show that growth on glycerol inhibits or represses cephalosporin production. To achieve the highest volumetric titer, it is not sufficient to provide conditions for high specific production; a critical level of biomass is also needed. Glycerol at 0.4% provided the critical degree of biomass development in the present experiments.

Effect of carbon source on the relationship between trophophase and idiophase. Figure 3 represents a differential plot of the data presented in Fig. 1. Increasing glycerol concentration not only affected the volumetric titer but also the relationship between the growth and production phases. At low glycerol concentrations, production appeared to be associated with growth; however, at concentrations above 0.2%, we observed a distinct separation between growth and secondary metabolism. Similar results were obtained when maltose was used as a major carbon source.

When growth and production on glycerol, starch, α-ketoglutarate, and succinate are compared on a differential plot (Fig. 4), it can be seen that, whereas glycerol utilization led to a typical biphasic relationship, starch, α-ketoglutarate, and succinate fermentations were characterized by a lack of separation between trophophase and idiophase.

DISCUSSION

S. clavuligerus was found to grow on a variety of carbon sources including starch, maltose (al-

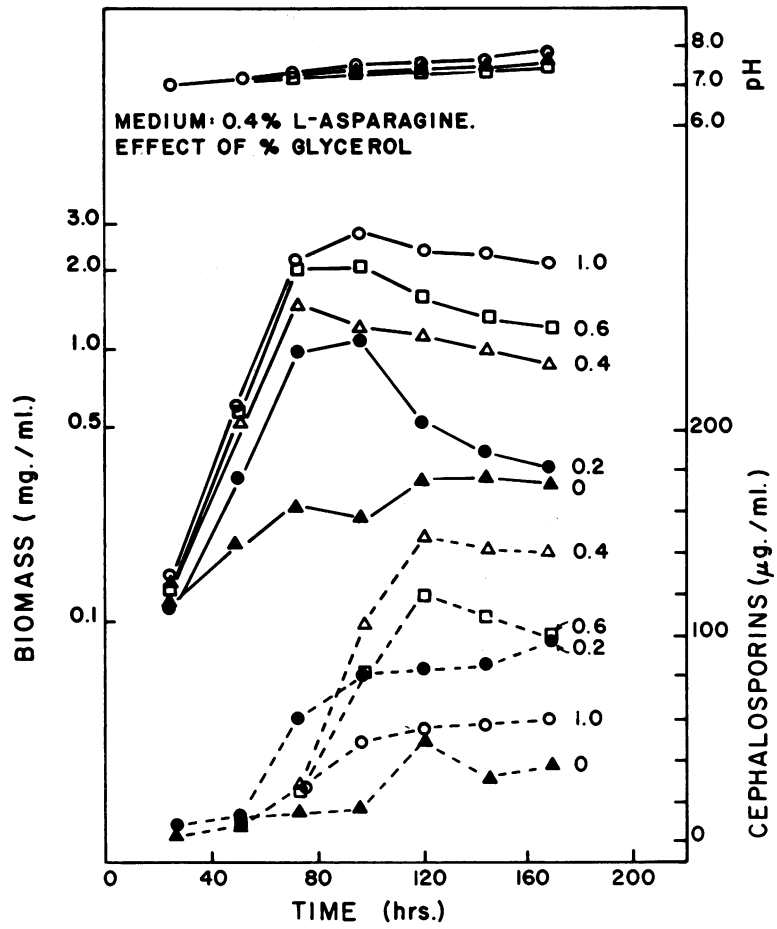

FIG. 1. *Growth, cephalosporin production, and pH as a function of time in the presence of different glycerol concentrations and 0.4% L-asparagine. The pH curves for 0.2% and 0.4% glycerol were not drawn for the sake of clarity.*

though not glucose), glycerol, organic acids, and amino acids. Growth on glycerol and maltose led to high biomass levels, but the specific production of cephalosporins decreased as carbohydrate concentration was increased. Hence, maximal product concentrations were not achieved under conditions supporting the highest biomass level. Our results strongly suggest some type of carbon catabolite control of cephalosporin production in *S. clavuligerus*. Whether the regulation is due to carbon catabolite repression, inhibition, or inactivation is not yet known. However, because pH was relatively constant (1), and because the negative effect occurred at low biomass concentrations where oxygen could not have been limiting, it is clear that the negative effect of preferred carbon sources is not due to trivial effects such as acid production or oxygen limitation; indeed, we are dealing with a true regulatory effect.

When starch was used as a carbon source, production was much more closely associated with growth than was the case with glycerol or maltose. We postulate that this is due to the slow hydrolysis of starch creating a situation of carbon limitation and releasing the culture from carbon source regulation. When *S. clavuligerus* was grown on organic acids such as α-ketoglutarate or succinate, which are relatively poor carbon sources, antibiotic production resembled that on starch, i.e., specific production was high and associated with growth.

It has been shown in several systems (see references 3 and 4 for citations) that production of antibiotics and other secondary metabolites is affected negatively when a rapidly used carbon source is utilized for growth; however, when the carbon source is slowly fed to the culture, the secondary metabolite is produced effectively. Although carbon source regulation of antibiotic

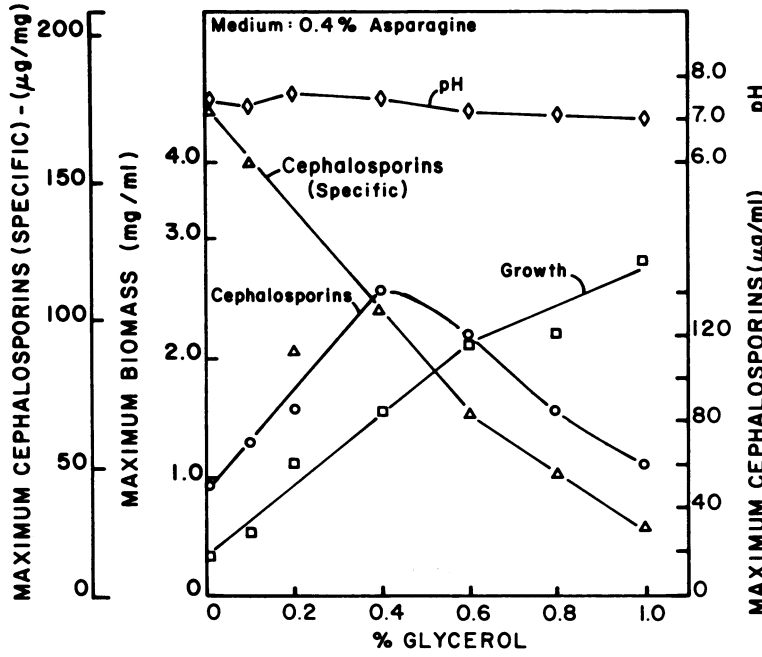

FIG. 2. *Effect of glycerol concentration on maximum values of biomass, cephalosporin titer, specific cephalosporin titer, and pH in the presence of 0.4% L-asparagine. Maximum specific cephalosporin titer is the maximum cephalosporin titer per milliliter (in micrograms) divided by the maximum biomass per milliliter (in micrograms).*

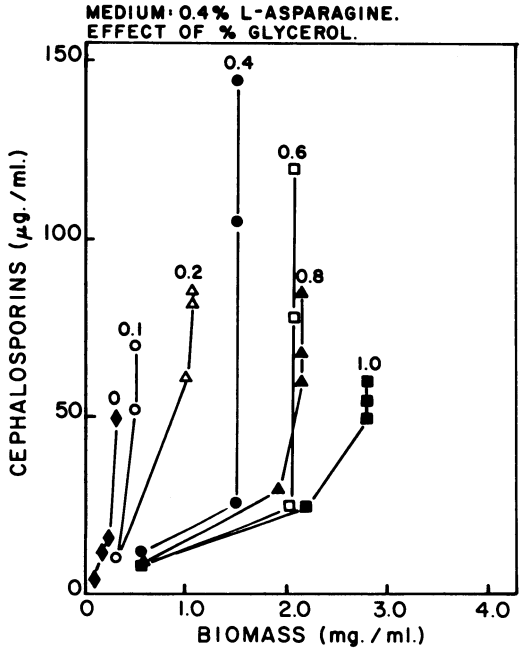

FIG. 3. *Effect of glycerol concentration on the differential rate of cephalosporin production. The volumetric titer of cephalosporin is plotted against the highest biomass reached up to that particular point, i.e., lysis is ignored in this plot.*

biosynthesis has been recognized since the 1950s (9, 14), the mechanisms involved are known in only a few cases. For example, in the production of actinomycin (5), carbon catabolite repression of an enzyme of secondary metabolism is involved. On the other hand, production of β-lactam antibiotics by *Cephalosporium acremonium* seems to be affected by some type of catabolite inhibition (Y. M. Kennel, Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, Mass., 1977).

The data presented in this paper support the concept that the definition of a secondary metabolite should not include the stage in the growth cycle in which it is produced. It is particularly confusing to see secondary metabolites defined as products formed after growth has ceased or in the absence of growth (although we have no argument with those who state that secondary metabolites are often produced after growth in batch culture). Of course, those who define secondary metabolites by stage of growth have long ignored the production of antibiotics by steady-state (exponentially growing) cultures in the chemostat (12), the production of primary metabolites such as glutamic acid, citric acid, and vitamin B_{12} during the stationary phase of their respective cultures, and the findings that antibiotic production is often associated with

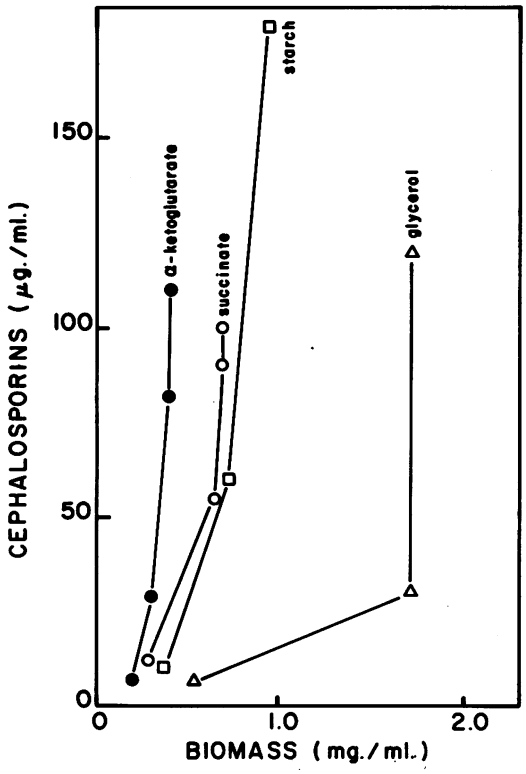

FIG. 4. *Effect of different carbon sources on the differential rate of cephalosporin production. The concentrations of glycerol, starch, succinate, and α-ketoglutarate were 0.5%. The volumetric titer of cephalosporin is plotted against the highest biomass reached up to that particular point, i.e., lysis is ignored in this plot.*

growth in one medium but produced after growth in another medium (8, 10, 12). It is important to realize that trophophase-idiophase dynamics are a function of nutrition, or, more precisely, of nutritional regulation, and not a function of the type of molecule being produced. In our opinion, the only valid definition for a secondary metabolite is that it is a molecule not required for the growth of the organism which produces it.

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