BIOSYNTHESIS OF TETRACYCLINES

II. Simple, Defined Media for Growth of *Streptomyces aureofaciens* and Elaboration of 7-Chlorotetracycline

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Received for publication September 25, 1958

A number of nutrient media have been described by Goodman (1954), Katagiri (1954), Niedercorn (1952), Petty *et al.* (1953), Van Dyke and De Somer (1952), and others that allow *Streptomyces aureofaciens* Duggar to grow in aerated, liquid culture and to accumulate substantial quantities of 7-chlorotetracycline.[1] The concentrations of 7-chlorotetracycline accumulated on these different media vary from about 0.1 to 2.5 g per L. These media are, in general, complex in composition, making it difficult to define the requirements of the organism for growth and to determine the origins of the carbon and the nitrogen appearing in the accumulated 7-chlorotetracycline.[2] This paper describes a series of simple, chemically defined nutrient media suitable for growth and 7-chlorotetracycline production by *S. aureofaciens* strain BC-41, a strain which is characteristic of this species. It is a descendant, through a series of mutation treatments and selections, of the original *S. aureofaciens* strain A-377 soil isolate of Duggar. The data show that spores of strain BC-41 can germinate and form mycelium, which can produce 7-chlorotetracycline, all in a medium composed of very simple materials.

EXPERIMENTAL METHODS

All fermentations were conducted at 25 C in 250-ml Erlenmeyer flasks containing 25 ml of medium and agitated on a Gump rotary shaker. Washed vegetative inoculum, where used, was prepared by transferring *S. aureofaciens* strain BC-41 spores to sterile nutrient medium, allowing a 24-hr growth period, centrifuging, washing twice with the original volume of sterile distilled water, and making up to the original volume with sterile distilled water. Four per cent of such inoculum was used to initiate the fermentation, which lasted 5 days. In fermentations inoculated directly with spores (table 3), the spore usage rate was approximately one million spores per ml.

The corn steep liquor-sucrose inoculum medium (Niedercorn, 1952) contains per L: corn steep liquor, 20 g; sucrose, 30 g; $(NH_4)_2SO_4$, 2.0 g; $CaCO_3$, 7.0 g. The MB-9A medium is a basal, inorganic salt composition based on corn steep liquor media previously reported by Goodman (1954). It contains per L: $CaCO_3$, 9.0 g; $(NH_4)_2SO_4$, 5.0 g; NH_4Cl, 1.5 g; $MgCl_2 \cdot 6H_2O$, 2.0 g; KCl, 1.3 g; H_3PO_4, 0.40 g; $FeSO_4 \cdot 7H_2O$, 0.060 g; $ZnSO_4 \cdot 7H_2O$, 0.10 g; $MnSO_4 \cdot 4H_2O$, 0.050 g; $CoCl_2 \cdot 6H_2O$, 0.0050 g. This basal mineral salts medium was supplemented with carbohydrate (either starch or sucrose at 55 g/L), amino acids (0.80 g/L of L-histidine and 0.80 g/L of L-methionine), and lipid (either lard oil or glyceryl trioleate at 20 ml/L). The starch was a commercial grade of acid-treated corn starch.

The concentration of 7-chlorotetracycline was determined by fluorometric assay as described by Feldman *et al.* (1957). The values reported are the averages from fermentations run in triplicate.

RESULTS AND DISCUSSION

The first experiments of this series were carried out in the MB-9A basal mineral salts medium, supplemented with starch and lard oil and inoculated with washed, vegetative inoculum grown on the corn steep liquor-sucrose medium. Under these conditions, about 1.2 to 1.5 g of 7-chlorotetracycline per L was normally produced. A number of substances were added, singly over a range of 0.2 to 2.0 g per L, to this basal medium,

[1] The trademark of the American Cyanamid Company for 7-chlorotetracycline is Aureomycin.

[2] The origin of the chlorine in the accumulated 7-chlorotetracycline has been discussed previously (Doerschuk *et al.*, 1956). A more detailed description is in press (Doerschuk *et al.*, 1959).

TABLE 1

Effects of graded additions of L-methionine and L-histidine on 7-chlorotetracycline accumulation by Streptomyces aureofaciens strain BC-41 in medium MB-9A supplemented with starch and lard oil†*

L-Methionine (g/L)	L-Histidine (g/L)				
	0.00	0.20	0.40	0.80	1.60
	7-Chlorotetracycline accumulated (g/L)				
0.00	1.40	1.65	1.65	1.90	1.90
0.20	1.95	2.55	2.60	2.45	2.60
0.40	2.15	2.65	2.75	2.40	3.00
0.80	2.50	3.40	3.70	4.30	3.75
1.60	1.75	2.20	2.30	2.60	3.10

* Washed vegetative inoculum grown on corn steep liquor-sucrose medium (see text).

† Starch at 55 g per L; lard oil at 20 ml per L.

in an effort to increase the concentration of 7-chlorotetracycline produced. Substances showing no significant enhancement of 7-chlorotetracycline production under the conditions tested were: L-alanine, β-alanine, allantoin, α-aminoadipic acid, *p*-aminobenzoic acid, anthranilic acid, DL-arginine, L-arginine, L-asparagine, L-aspartic acid, butyrin, L-cystine, dipropionin, gluconic acid, DL-glutamic acid, L(+)-glutamine, glutathione, glycine, L-hydroxyproline, L-isoleucine, L-leucine, L-lysine, maleic acid, L-malic acid, malonic acid, DL-mandelic acid, methyl-α-D-glucoside, monoacetin, monopropionin, L-phenylalanine, L-proline, salicylamide, L-serine, shikimic acid, sodium succinate, L-sorbose, L-threonine, L-tryptophan, L-tryosine, urea, and L-valine. Only two substances resulted in reproducible stimulation of the quantity of 7-chlorotetracycline accumulated; these were L-histidine and L-methionine.[3]

Accordingly, in a second series of experiments, graded quantities of both L-histidine and L-methionine were added to the MB-9A-starch-lard oil medium. The results presented in table 1

[3] Previously reported work with C^{14}-labeled compounds (Miller *et al.* (1956)) has shown that some of the above substances (glycine-2-C^{14}, D,L-serine-3-C^{14}, and L-methionine-CH_3-C^{14}) can contribute carbon to 7-chlorotetracycline synthesized by this strain. The present work is concerned only with net increase in synthesis by added substances, whether by incorporation or by other favorable effects.

TABLE 2

Effects of inoculum and medium composition on 7-chlorotetracycline accumulation by Streptomyces aureofaciens strain BC-41*

Washed Vegetative Inoculum Grown in	Fermentation Medium MB-9A Supplemented with			7-Chlorotetracycline (g/L)
	Carbohydrate	Amino acid	Lipid	
CSL-Su	St	—	LO	1.30
MB-9A, Su, Hi, Me	St	—	LO	0.90
CSL-Su	St	—	GTO	1.35
MB-9A, Su, Hi, Me	St	—	GTO	1.35
CSL-Su	Su	—	LO	0.35
MB-9A, Su, Hi, Me	Su	—	LO	0.50
CSL-Su	Su	—	GTO	0.95
MB-9A, Su, Hi, Me	Su	—	GTO	0.85
CSL-Su	St	Hi, Me	LO	3.35
MB-9A, Su, Hi, Me	St	Hi, Me	LO	1.10
CSL-Su	St	Hi, Me	GTO	3.75
MB-9A, Su, Hi, Me	St	Hi, Me	GTO	2.05
CSL-Su	Su	Hi, Me	LO	1.00
MB-9A, Su, Hi, Me	Su	Hi, Me	LO	0.80
CSL-Su	Su	Hi, Me	GTO	1.30
MB-9A, Su, Hi, Me	Su	Hi, Me	GTO	0.70

* CSL-Su = corn steep liquor-sucrose inoculum medium (see text); MB-9A = MB-9A medium (see text); St = starch at 55 g per L; Su = sucrose at 55 g per L; LO = lard oil at 20 ml per L; GTO = glyceryl trioleate at 20 ml per L; Hi = L-histidine at 0.80 g per L; Me = L-methionine at 0.80 g per L.

TABLE 3

7-Chlorotetracycline production by Streptomyces aureofaciens strain BC-41 on a completely defined medium with glycerol as the sole carbon source*

Glycerol Conc (g/L)	7-Chlorotetracycline Conc (g/L)	
	Spore inoclum	Washed vegetative inoculum grown in corn steep liquor-sucrose inoculum medium
0.0	0.00	0.00
2.5	0.02	0.02
5.0	0.10	0.06
10.0	0.24	0.18
20.0	0.22	0.49
40.0	0.02	0.52

* MB-9A basal mineral medium, supplemented with glycerol.

show that these two amino acids exert additive effects on the level of 7-chlorotetracycline accumulated. The optimal concentration under these conditions was about 0.80 g of each amino acid per L, and these levels were adopted for further work.

Subsequent experiments took the form of stepwise changes in the direction of more rigorously defined media: (a) washed vegetative inoculum grown on the corn steep liquor-sucrose medium was replaced by washed vegetative inoculum grown on the MB-9A-sucrose-histidine-methionine medium; (b) starch was replaced by sucrose; (c) the amino acids, L-histidine and L-methionine, were omitted; and (d) lard oil was replaced by an equal weight of glyceryl trioleate. The results of these systematic changes are presented in table 2. Even with all these simplifications, a significant amount (0.85 g/L) of 7-chlorotetracycline was produced. In general, the first three changes reduced the amount of product. Lard oil could, however, be replaced by the purified glyceryl trioleate with equivalent or better results, showing the absence of other essential substances in the crude lard oil.

A further extension of the above work took the form of devising a medium containing only a single, simple organic compound as a source of energy and carbon,[4] all the remaining requirements, including those for nitrogen and sulfur, being satisfied by inorganic salts. Table 3 presents a glycerol-inorganic salt medium allowing mycelium formation from spores and permitting 7-chlorotetracycline biosynthesis.

SUMMARY

Streptomyces aureofaciens strain BC-41 can germinate from spores, form mycelium, and accumulate up to 2 g of 7-chlorotetracycline per L on media of simple, defined composition. The use of a washed vegetative cell inoculum previously grown on a corn steep liquor medium allows a yield of up to 4 g of 7-chlorotetracycline per L on media of simple defined composition. In addition, strain BC-41 can germinate from spores, form mycelium, and synthesize 7-chlorotetracycline on a medium containing glycerol as the sole carbon source and ammonium ion as the sole nitrogen source, although, under these conditions, the 7-chlorotetracycline concentrations attained are reduced. The requirements for all the other elements, particularly sulfur, phosphorus, and chlorine, are satisfied by inorganic salts.

[4] It has been previously shown by Miller *et al.* (1956) that carbon added as $Na_2C^{14}O_3$ is not significantly incorporated into 7-chlorotetracycline. It is therefore assumed that CO_2 from the $CaCO_3$ is similarly not incorporated.

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