SHORT COMMUNICATIONS

Growth of *Arthrobacter citreus* in a Chemically Defined Medium and its Requirement for Chelating Agents

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(Accepted for publication 14 November 1969)

Previous studies on the physiology of *Arthrobacter citreus* have been made with either complex or partially defined media. To further our studies on the roles played by cations following the observation that certain metallic ions promote growth of the organism at elevated temperatures (Wong, Chan & Page, 1966), it was necessary to determine the exact nutritional requirements of *A. citreus* NCB8915. We report the formulation of a chemically defined medium suitable for the growth of this organism.

The basal medium used was described by Chan (1964); it was adjusted to pH 6.8 before autoclaving at 121° for 15 min. Glucose was autoclaved separately as a 50 % (w/v) solution and was added aseptically to the sterile medium to a final concentration of 1 % (w/v). Cultures were grown in 50 ml. volumes in 250 ml. nephelo-culture flasks (Bellco Glass, Inc., Vineland, N.J., U.S.A.) and incubated on a rotary shaker (Pscrotherm Incubator Shaker, New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 25°.

Cultures used for inocula were grown on Trypticase Soy Agar (Baltimore Biological Laboratory, Baltimore, Maryland, U.S.A.) slopes at 25° for 24 hr. Bacteria were then suspended in basal medium salt solution, washed three times by centrifugation at $7710 \times g$ and suspended to a density of 100 Klett units. Two-tenths ml. of this suspension was used to inoculate 50 ml. of medium. Growth was followed turbidimetrically with a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., New York) with a no. 42 blue filter (400 to 450 nm.).

All chemicals used were of purest grade available commercially. Inorganic salts and chelating agents (other than sideramines) were from Fisher Scientific Co., Montreal, Canada, with the exception of 2,3-dihydroxybenzoic acid from Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A. Casamino Acids and vitamin-free Casamino Acids were from Difco Laboratories, Detroit, Michigan, U.S.A.; vitamins, purines, pyrimidines and amino acids from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Samples of sideramines were from Dr J. B. Neilands, University of California at Berkeley (ferrichrome), Dr Hans Bickel, Ciba Ltd., Basel, Switzerland (ferricrocin, ferrirubin); Dr I. H. Zähler, Universität Tübingen, Tübingen, Germany (ferrinoxamine); Dr J. Gelzer, Ciba Pharmaceutical Co., Summit, N.J., U.S.A. (desferal), and Dr G. A. Snow, I.C.I. Ltd., Alderley Park, Macclesfield, Cheshire (mycobactin P, mycobactin S).

Preliminary experiments indicated that *Arthrobacter citreus* grew in the basal medium + 1 % (w/v) Casamino Acids + thiamine-HCl (0.2 µg./ml.), but only after a lag phase

of 3 to 4 days. No decrease of the lag nor stimulation of growth was observed on adding purine and pyrimidine mixtures, both separately and together, or with the ash of Trypticase Soy Broth.

Lankford, Kustoff & Sergeant (1957) reported that several compounds having only one property in common, i.e. ability to chelate metal ions, were effective in initiating and maintaining exponential growth of a strain of *Bacillus subtilis*, and suggested that these compounds performed a function in common with a natural bacterial metabolite. We tested several chelating agents for capacity to initiate growth of *Arthrobacter*

Table 1. *Effect of chelating agents on the growth of Arthrobacter citreus*

Compound tested	Concentration* ($\mu\text{g./ml. medium}$)	Reference used	Bacterial concn. in Klett units Time (hr)		
			0	48	96
Control†	—	—	0	0	52
Disodium EDTA	8	<i>a</i>	0	20	595
Oxalic acid	1000	<i>a</i>	0	36	750
2,3-Dihydroxybenzoic acid	50	<i>a</i>	0	10	410
Sodium citrate	2000	<i>a</i>	0	0	0
Sodium salicylate	150	<i>a</i>	0	0	11
Salicylaldehyde	4	<i>b</i>	0	16	483
Acetylacetone	50	<i>b</i>	0	10	175
8-Hydroxyquinoline	1	<i>b</i>	0	19	335

* The concentrations used were found to be biologically active in the references cited.

† Basal medium + 1 % (w/v) Casamino Acids + 0.2 $\mu\text{g./ml.}$ thiamine-HCl.

(*a*) Denotes Arceneaux & Lankford (1966); (*b*) denotes Morrison, Antoine & Dewbrey (1965).

citreus. The results (Table 1), indicated that all the compounds tested were active in initiating and maintaining exponential growth, with the exception of sodium citrate and sodium salicylate, which were inhibitory in the concentrations used. Similar positive results were obtained with various sideramines, a group of microbially synthesized ferric hydroxamates with chelating and growth-factor activity (Bickel *et al.* 1960). These were ferrichrome and ferricrocin (tested at 1 and 1000 $\text{m}\mu\text{g./ml. medium}$); ferrirubin (20 and 1000 $\text{m}\mu\text{g./ml.}$); ferrioxamine (0.5 and 1000 $\text{m}\mu\text{g./ml.}$); desferal, the iron-free derivative of ferrioxamine (50 $\text{m}\mu\text{g./ml.}$); mycobactin P and mycobactin S (at 1500 $\text{m}\mu\text{g./ml.}$).

To establish a chemically defined medium from a partially defined one for growth of *Arthrobacter citreus*, the Casamino Acids had to be replaced. The medium did not support growth when either vitamin-free Casamino Acids or an amino acid mixture equivalent to casein hydrolysate replaced Casamino Acids suggesting a requirement for some vitamins. This was confirmed by replacing the Casamino Acids with vitamin mixtures + amino acid mixture. The specific amino acid requirements were determined by using an amino acid block procedure. The essential ones were found to be tyrosine, cystine and methionine. In the amino acid experiments, vitamin mixtures had been included in the medium in addition to thiamine-HCl. In the presence of thiamine, the vitamin mixtures could be replaced by nicotinic acid + biotin. The optimal concentrations of biotin and nicotinic acid were 1 $\text{m}\mu\text{g./ml.}$ and 0.2 $\mu\text{g./ml.}$, respectively, and the vitamin requirements were confirmed by failure of the organism to grow when any one of the three vitamins, thiamine, biotin, nicotinic acid, was deleted from the medium.

Thus the basal medium supplemented with a chelator, tyrosine, cystine, methionine,

thiamine-HCl, biotin, nicotinic acid, and sodium chloride was able to support growth of *Arthrobacter citreus*. However, the amount of growth obtained was far less than that obtained with the amino acid mixture; another amino acid, while not essential, appeared to be required to obtain maximum growth. Since *A. citreus* is reported to be unable to utilize nitrates as a N source (Sacks, 1954), KNO_3 was removed from the medium and replaced by monosodium glutamate. (Monosodium glutamate cannot support growth of *A. citreus* without the essential amino acids so far determined, and, although probably purer, glutamic acid is as effective as monosodium glutamate in replacing nitrate.) The composition of the medium as finally constituted is shown in Table 2.

Table 2. *Composition of a chemically defined medium supporting good growth of Arthrobacter citreus*

	Quantities/l.
Inorganic salts, Solution A	5 ml.
Solution B	5 ml.
Sodium chloride	5.85 g.
Glucose	10 g.
Desferal*	50 μg .
L-Monosodium glutamate	1 g.
L-Tyrosine	0.1 g.
L-Cystine	0.1 g.
L-Methionine	0.1 g.
Thiamine-HCl	0.2 mg.
Biotin	0.001 mg.
Nicotinic acid	0.2 mg.

* May be replaced by any other effective chelator mentioned in text.

Solution A: K_2HPO_4 , 25 g.; KH_2PO_4 , 25 g.; distilled water to 250 ml.

Solution B: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.5 g.; distilled water to 250 ml.

Three other species, *Arthrobacter terregens*, *A. flavescens* and *Arthrobacter* sp. JG-9, require sideramines for growth (Lochhead & Burton, 1953; Lochhead, 1958; Burnham & Neilands, 1961, respectively); our results indicate that *A. citreus* is sideramine-dependent. We found that the sideramine requirement of *A. citreus* can be replaced by other chelating agents; similar observations have been made by other workers for a strain of *Bacillus megaterium* (Arceneaux & Lankford, 1966) and for *A. terregens* (Morrison, Antoine & Dewbrey, 1965).

At present, it is assumed that sideramines act as iron transfer agents (Neilands, 1967; Koser, 1968), and there is evidence to support the hypothesis that at least one sideramine, ferrichrome, is responsible for the insertion of iron into protoporphyrin (Burnham & Neilands, 1961; Burnham, 1962, 1963). Since ferrichrome binds Fe^{3+} tightly but shows only a weak affinity for Fe^{2+} , and since Fe^{2+} is the valence state more readily inserted into protoporphyrin (Lascelles, 1964), the iron bound by ferrichrome could be released upon its reduction to the ferrous form.

This project was supported by a grant from the National Research Council of Canada. The authors gratefully acknowledge the gifts of chemicals from Drs J. B. Neilands, Hans Bickel, I. H. Zöhner, J. Gelzer and G. A. Snow. We thank Dr C. E. Lankford (University of Texas) for his interest in this work, and Miss Maria Nytko for skilled technical assistance.

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