JOURNAL OF BACTERIOLOGY
Vol. 87, No. 4, p. 924-932 April, 1964
Copyright © 1964 American Society for Microbiology
Printed in U.S.A.

NUTRITIONAL CONTROL OF MORPHOGENESIS IN *ARTHROBACTER CRYSTALLOPOIETES*

JERALD C. ENSIGN AND R. S. WOLFE

Department of Microbiology, University of Illinois, Urbana, Illinois

Received for publication 7 October 1963

ABSTRACT

ENSIGN, JERALD C. (University of Illinois, Urbana), AND R. S. WOLFE. Nutritional control of morphogenesis in *Arthrobacter crystallopoietes*. J. Bacteriol. 87:924-932. 1964.—*Arthrobacter crystallopoietes* exhibits the cyclic, morphological variation which is a characteristic of this genus. A simple chemically defined medium was developed in which this organism is restricted to growth and division entirely in the coccoid form. Addition singly to this medium of L-arginine, L-phenylalanine, L-asparagine, L-lysine, succinate, malate, fumarate, lactate, or butyrate results in the formation of the rod-shaped stage. A large number of other compounds either increase, have no effect on, or inhibit growth without inducing morphological change in the organisms.

Cellular differentiation as exhibited by members of the genus *Arthrobacter* has been described by a number of investigators, and was especially well illustrated by Kuhn and Starr (1960) for *A. atrocyaneus* and by Veldkamp, van den Berg, and Zevenhuizen (1963) for *A. globiformis*. Organisms in the stationary phase of growth are characteristically spherical, and appear similar to a culture of micrococci. Upon inoculation of coccoid organisms into fresh medium, the cells gradually elongate into pleomorphic rods. Cell division occurs during the rod stage and continues until the rods fragment into smaller entities which gradually shorten into the typical coccoid cells. The entire cycle is completed in 1 to 3 days, depending on the organism and medium employed.

The ease of cultivation of many of the *Arthrobacter* species and the marked morphological variations accompanying the growth cycle make these organisms particularly suited to a study of the physiological basis of the regulatory mechanisms involved in cellular morphogenesis. We present in this paper the results of our studies concerning the nutritional control of cellular differentiation in *A. crystallopoietes*.

MATERIALS AND METHODS

A. crystallopoietes, isolated and described by Ensign (1963) and Ensign and Rittenberg (1963), was used throughout this investigation.

For the most part, the organisms were cultivated in a defined medium (medium A) containing the following constituents (w/v): 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% glucose, 1.0% (v/v) of a trace salts solution (Wolin, Wolin, and Wolfe, 1963), and 0.025 M potassium phosphate buffer (pH 7.0). Glucose was sterilized by filtration as a 20% solution. Magnesium sulfate and trace salts were sterilized separately. Amino acids used to supplement the glucose-salts medium in certain experiments were filter-sterilized.

Each experiment was carried out in 50 ml of medium in a 300-ml Nephelo flask (Bellco Glass Inc., Vineland, N.J.). The flasks were shaken at 30 C. The inoculum for each experiment was prepared from a 48-hr glucose-salts culture (medium A). The cells were centrifuged aseptically, and resuspended in a volume of distilled water such that 1 ml, when inoculated into an experimental flask, resulted in an initial population of approximately 10^7 cells per ml. Cells prepared in this manner were invariably in the spherical phase of growth.

Turbidity was measured on a Klett-Summerson colorimeter at 660 mμ, and the readings were converted into optical density units. Cell populations were measured by the direct-count technique by use of a Petroff-Hausser counting chamber. The tendency of the organism to remain in small clusters of two to five cells, which were not separated by vigorous shaking, made the use of plate counts impractical.

The growth and multiplication of individual cells were recorded photographically in slide cultures. A thin layer of the appropriate medium solidified with 1.0% Ionagar (Oxoid) was prepared by pipetting 5 ml of the melted medium into a petri plate. The bottom of the petri plate

was warmed with a bunsen burner to allow the medium to cover the entire surface before hardening. A few drops of a washed and diluted suspension of cells were placed upon the solidified medium, and the plate was placed at a 45° angle, allowing the inoculum to run down the surface. A block of agar approximately 1 cm^2 was cut with a sterile scalpel and placed upon a sterile cover slip (25 by 60 mm) so that the organisms were sandwiched between the cover slip and the agar block. The cover slip was then placed upon a sterile slide, and the edges were sealed with melted Vaspar (a 1:1 mixture of paraffin wax and mineral oil). Prior to sealing the chamber, two small drops of sterile water were placed under each end of the cover slip with a Pasteur pipette. During incubation at 24 C, the slide culture was observed under phase contrast by use of a Zeiss photomicroscope. Photographs were recorded on Adox KB-14 film.

Amino acids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, as the L or DL isomers, with the exception of DL-$\alpha\epsilon$-diaminopimelic acid which was purchased from Mann Research Laboratories, Inc., New York, N.Y.

RESULTS

The growth of *A. crystallopoietes* in a variety of complex media and in a synthetic medium is illustrated in Fig. 1. In each of the complex media, the normal sphere-to-rod-to-sphere cycle was evident. During the rod stage, the organisms growing on yeast extract were noticeably more pleomorphic, often appearing branched, and were larger than those observed with other media.

The morphological changes accompanying growth upon peptone agar are illustrated by the photographs of a microcolony developing from a single cell (Fig. 2). The spherical organism enlarges and gradually develops into a rod, the time for this transition being approximately 5 hr. Cell division takes place during the rod stage. At first the daughter cells enlarge between divisions, but toward the end of the logarithmic phase they become progressively smaller as the large rods fragment into smaller rods. Finally, the rods gradually become rounded into coccoid cells characteristic of the stationary phase of growth.

Also shown in Fig. 1 is the growth response in a strictly synthetic medium composed of glucose-salts (medium A). Although the inoculum for all

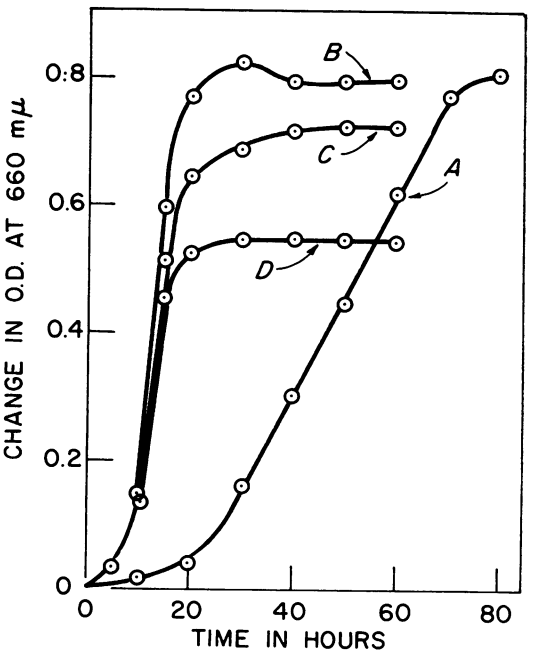

FIG. 1. *Growth of Arthrobacter crystallopoietes in several complex media: (A) glucose-salts (see Materials and Methods), (B) 0.5% yeast extract, (C) 0.5% Casamino Acids (Difco), (D) 0.5% peptone (Difco).*

flasks was taken from this same medium, there was a much longer lag period and slower rate of growth in the synthetic medium as compared with the complex medium. The population level reached was comparable to or greater than that achieved in the complex media. More surprising was the observation that at no time during growth upon the synthetic medium did the organisms develop into the rod stage. Instead, they grew and divided as spheres. This is illustrated in the slide-culture photographs of organisms growing on an agar block of the glucose-salts medium A (Fig. 3). The organisms multiplied entirely as spheres

The failure of the organisms growing on the synthetic medium to undergo the typical developmental cycle stimulated an attempt to define the nutritional conditions required for morphogenesis. The addition to the synthetic medium of a relatively complete mixture of vitamins (Wolin et al., 1963) neither stimulated the growth rate nor induced the rod stage. Alterations in the composition of the salts medium yielded the information that high phosphate concentrations

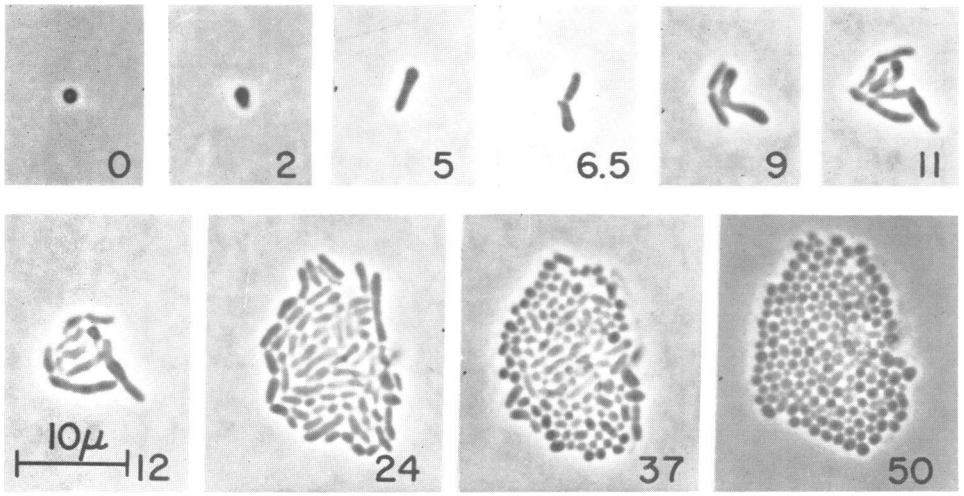

FIG. 2. *Phase-contrast photomicrographs of the development of a microcolony of Arthrobacter crystallopoietes on an agar block containing 0.5% peptone as substrate. Numerals refer to time in hours.*

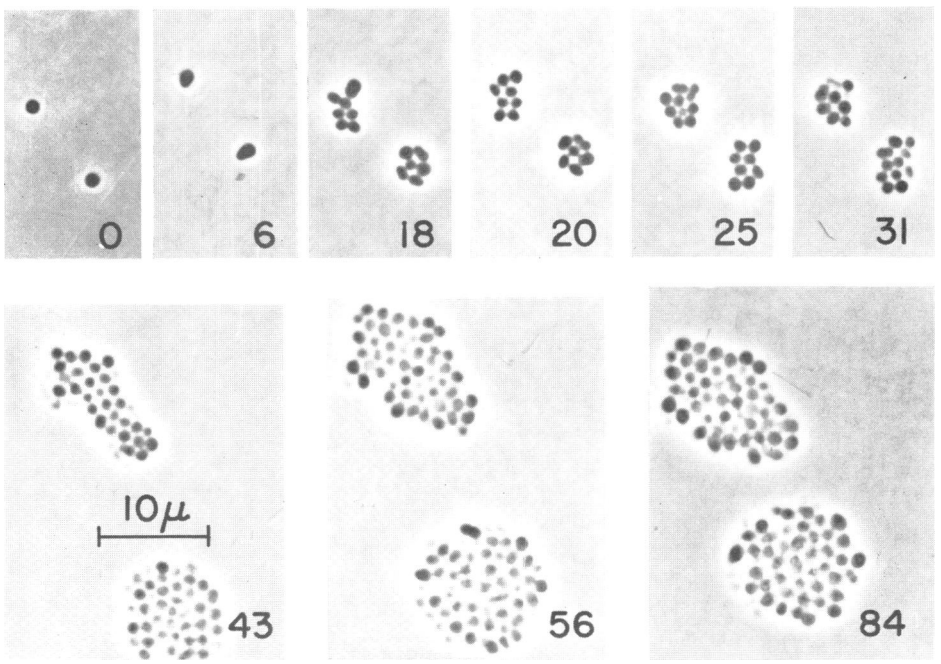

FIG. 3. *Phase-contrast photomicrographs of Arthrobacter crystallopoietes growing on an agar block containing glucose-salts medium A. Numerals refer to time in hours.*

(0.05 to 0.1 M) inhibited growth slightly as compared with lower concentrations (0.01 to 0.025 M) and that optimal growth resulted with the addition of 0.025% $MgSO_4$. These alterations did not, however, affect the growth of the organisms as spheres.

Since the typical life cycle of *Arthrobacter* is exhibited in protein digests or hydrolysates, the

addition of amino acids to the synthetic medium was a logical approach to follow. Thus, the addition of 1.0% of an amino acid mixture (No. 602; Microbiological Associates Inc., Bethesda, Md.) to the glucose salts medium resulted in a greatly increased growth rate and development of the rod stage. The results obtained from the addition of 500 μmoles of each nitrogen compound tested to a separate portion the glucose-salts medium A are presented in Table 1 and fall into four categories. One group, D, inhibited growth either completely or almost completely. The largest group, C, had no effect on the growth rate or division as spheres. A third group, B, yielded a significant increase in the growth rate over the control without resulting in an induction of the rod stage. The fourth group, A, composed of L-phenylalanine, L-asparagine, L-lysine, and L-arginine markedly increased the growth rate with the formation of the typical growth cycle.

The effect upon growth by one of the cycle-inducing compounds, asparagine, is illustrated in Fig. 4. For this experiment, the glucose-salts medium A was supplemented with 0.2% L-asparagine. Growth upon the same concentration of asparagine as sole carbon source and upon unsupplemented glucose medium is shown also. A marked decrease in the lag period resulted in the presence of asparagine. The lag observed by the turbidimetric measurements is somewhat deceiving, in that turbidity increases during the first 6 hr without a concomitant increase in cell population. During this period, the cells increase greatly in size while developing into rods. The organisms divide as rods for several generations until, as evidenced by the cessation of growth in the medium containing only asparagine, the substrate is exhausted. At this time the cells ensphere, resulting in a transient decrease in turbidity. In the flask containing only asparagine as carbon source, the cell count remains constant, indicating that this turbidity drop is a result of the alteration of cell morphology from rods to spheres. In the asparagine-glucose medium, the cell count continued to increase without lag but changed abruptly to the rate characteristic of growth on glucose alone. The inflection in the turbidity curve of the asparagine-glucose flask at the point when asparagine is depleted, 15 hr, coincides with the alteration in cell morphology from rods to spheres. The organisms then continue to divide as spheres until the maximal stationary phase is reached.

TABLE 1. *Effect on growth rate and morphogenesis of addition of single nitrogen compounds* to a glucose-salts medium*

Group	Effect†	Compound
A	Induce morphogenesis, stimulate growth rate	L-Lysine
		L-Asparagine
		L-Arginine
		L-Phenylalanine
B	No morphogenesis, stimulate growth rate	L-Alanine
		D-Alanine
		Glycine
		L-Proline
		L-Glutamic acid
		L-Serine
		L-Tryptophan
C	No morphogenesis, no stimulation of growth rate	L-Ornithine
		L-Methionine
		L-Hydroxyproline
		L-Glutamine
		DL-αε-Diaminopimelic
		L-Leucine
		L-Histidine
		L-Aspartic acid
		L-Cystine
D	No morphogenesis, inhibition of growth rate	L-Threonine (marked)
		L-Valine (slight)
		L-Isoleucine (slight)
		L-Citrulline (slight)
		L-Norvaline (marked)

* The final concentration of each compound was 10 μmoles/ml.

† Growth rate is compared with that obtained in glucose-salts medium without amino acid added.

The morphological changes occurring during growth in a slide culture upon asparagine-glucose agar (same composition as used in Fig. 4 but solidified with agar) are shown in Fig. 5. The spherical organism develops into a rod followed by cell division, the process being similar to that obtained with peptone (Fig. 2). After 18 hr, the organisms become progressively shorter, finally reverting to the spherical form and continuing to divide as such until the maximal stationary phase is reached.

The growth response to the cycle-inducing amino acids, phenylalanine, arginine, and lysine, are essentially identical to the results presented for asparagine. When the concentration of the

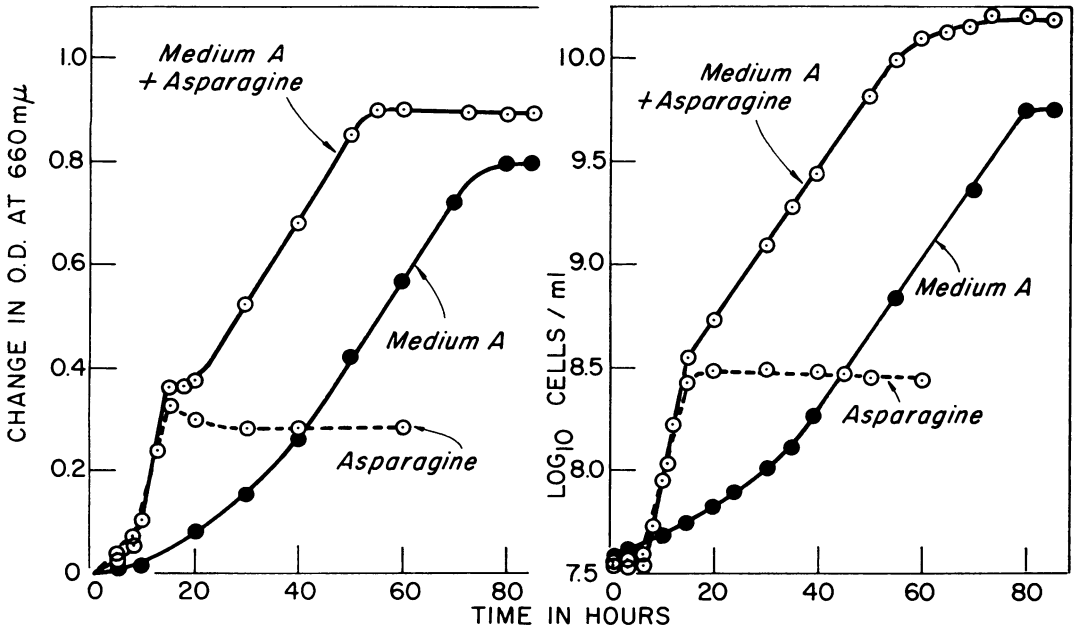

FIG. 4. *Growth of Arthrobacter crystallopoietes in glucose-salts medium A, asparagine-salts medium, and medium containing both glucose and asparagine. Turbidity curves are on the left; direct microscopic counts are on the right.*

cycle-inducing compounds was varied from 0.2 to 10.0 μmoles/ml in the glucose medium, it was observed that the rod stage developed at concentrations of 1.0 μmole/ml and higher but not at 0.5 or 0.2 μmole/ml. At all concentrations, each of these compounds produced a shorter lag period and more rapid initial growth rate than was observed for the unsupplemented medium A.

A variety of organic compounds were tested individually in the glucose-salts medium A for their ability to induce morphogenesis (Table 2). Results obtained for a number of sugars as sole carbon source are also presented (Table 2). All of the tricarboxylic acid cycle intermediates tested, with the exception of citrate, markedly increased the growth rate as compared with the glucose control, and resulted in the development of the typical rod stage. Addition of citrate to the glucose medium resulted in the formation of large clumps of atypical globular cells. In addition to the tricarboxylic acid cycle compounds, lactate and butyrate caused increased growth and typical morphogenesis. In view of these cycle-inducing compounds, it is interesting that pyruvate, acetate, propionate, and β-hydroxybutyrate stimulated growth but did not result in the formation of the rod stage. In the case of acetate, propionate, and pyruvate, the cells increased to approximately two times the normal size and became definitely oval in shape. Increasing the concentration of these compounds to 0.5% in the glucose medium and using them as sole carbon source allowed luxuriant growth with no change in morphology other than that described. The organisms are able to grow at the expense of a number of mono- and disaccharides; in each case, the spherical form is maintained. The rate of growth with each was essentially the same as with glucose alone. Growth upon gluconate at a rate similar to that on glucose occurred after a lag period of 30 hr.

The pattern of growth and morphogenesis with these cycle-inducing compounds was essentially identical to that obtained with the similarly active amino acids. This is illustrated for succinate and butyrate as sole carbon sources in slide-culture photographs (Fig. 6 and 7) and for butyrate in growth-curve data (Fig. 8). The organisms developed into rods with an increase in turbidity but not cell count, divided as rods, and then gradually ensphered with no change in cell count but with a drop in turbidity.

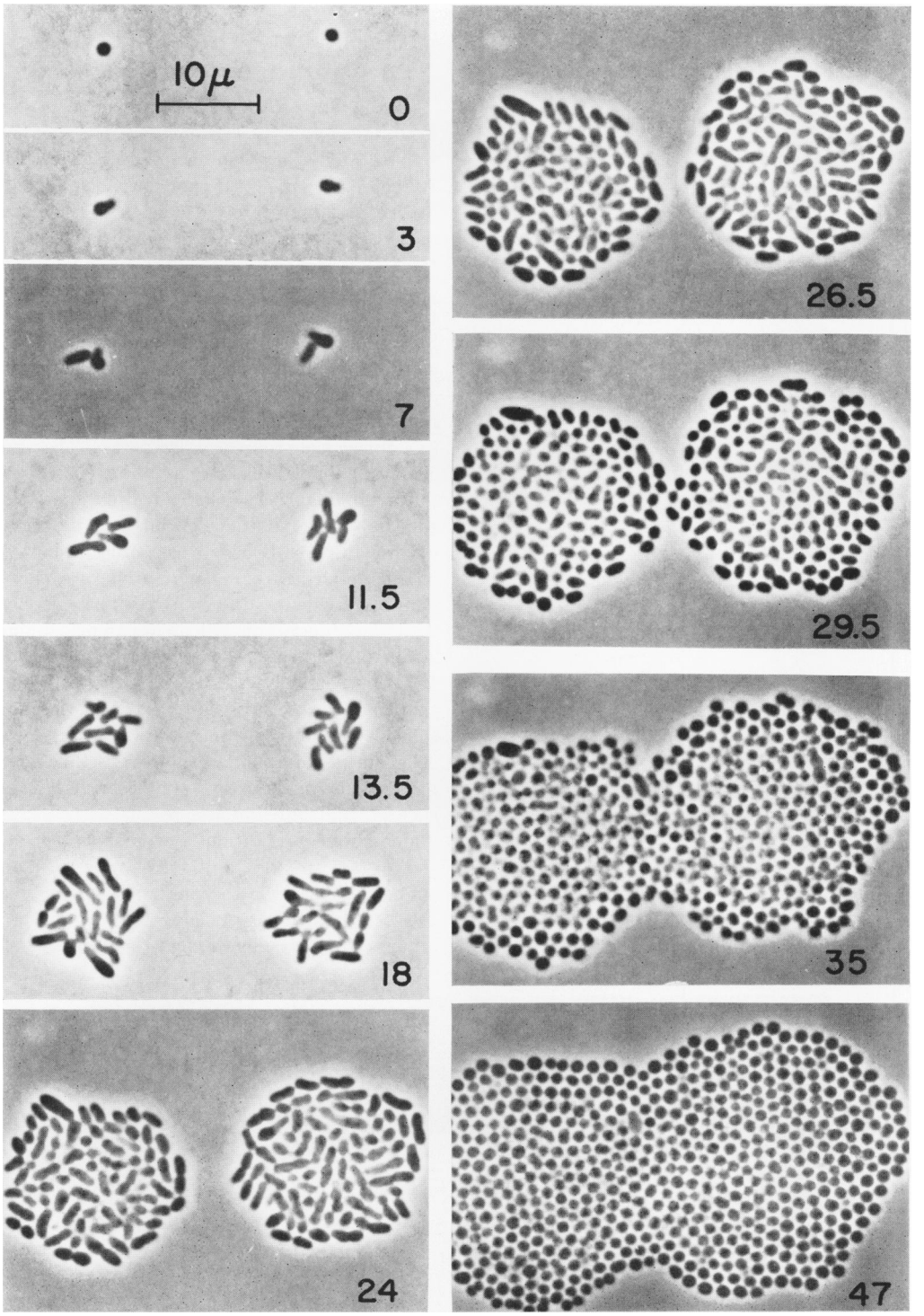

FIG. 5. *Phase-contrast photomicrographs illustrating the normal life cycle of Arthrobacter crystallopoietes resulting from the addition of 0.2% L-asparagine to an agar block of the glucose-salts medium A. Numerals refer to time in hours.*

TABLE 2. *Effect of growth rate and morphogenesis of various carbon compounds* added to glucose-salts medium*

Group†	Effect‡	Compound
A	Induce morphogenesis, stimulate growth rate	Butyrate
		Succinate
		Malate
		Fumarate
		Lactate
B	No morphogenesis, stimulate growth rate	Acetate
		Propionate
		Valerate
		β-Hydroxybutyrate
		Pyruvate
C	No morphogenesis, no stimulation of growth rate	Sodium formate
		Sodium isobutyrate
		Sodium isovalerate
		Ethyl alcohol
		n-Propyl alcohol
		n-Butyl alcohol
		Glycerol
		Sodium citrate
		Mannose
		Ribose
		Potassium gluconate
		Sucrose
		Fructose
D	No morphogenesis, no growth	Galactose
		Lactose
		Glucosamine

* Final concentration of each compound, 0.2%.

† Groups A and B were sodium salts.

‡ Growth rate is compared with that obtained in glucose-salts medium.

DISCUSSION

The ability to grow and divide as spheres exhibited by *A. crystallopoietes* has not been reported for other members of this genus. Most studies of *Arthrobacter* have been conducted in media containing protein digests, yeast extract, or soil extract. Thus, if the conditions found in this study for coccoid division (i.e., a simple growth medium) are a characteristic of the genus, these complex media would mask this event. Stevenson (1962) studied the growth of *A. globiformis*, and reported that the organisms growing in a complex medium increased eight to ten times and in simple medium two times in size during the predivision lag period. The photographs of stained cells taken by Stevenson (1962) during this lag period indicate that the cells in the simple medium develop into very short rods prior to the first division. It is not clear, however, whether during subsequent divisions the organisms divided as the short rods or as spheres. A similar observation with an apparently different strain of this species was reported recently by Veldkamp et al. (1963). These authors stipulated that in a glucose-salts-biotin medium the organisms increased to two times the size of the coccoid cells during division as short rods. We are at present making a survey of other *Arthrobacter* species, and preliminary results indicate that under proper conditions several of the other species also are able to divide as spheres.

Speculation as to the mechanism of action of the morphogenesis-inducing compounds (L-arginine, L-asparagine, L-phenylalanine, L-lysine,

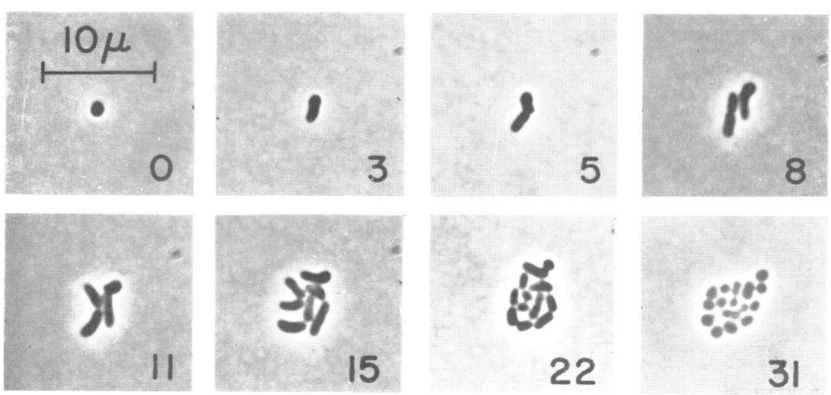

FIG. 6. *Phase-contrast photomicrographs illustrating the growth response of Arthrobacter crystallopoietes to a salts agar medium (see text) containing 0.2% sodium succinate as sole carbon source. Numerals refer to time in hours.*

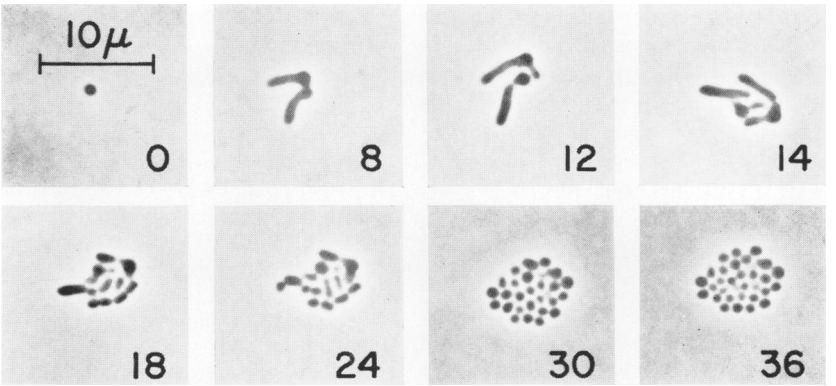

FIG. 7. *Phase-contrast photomicrographs illustrating the growth response of Arthrobacter crystallopoietes to a salts-agar medium (see text) containing 0.2% potassium butyrate as sole carbon source. Numerals refer to time in hours.*

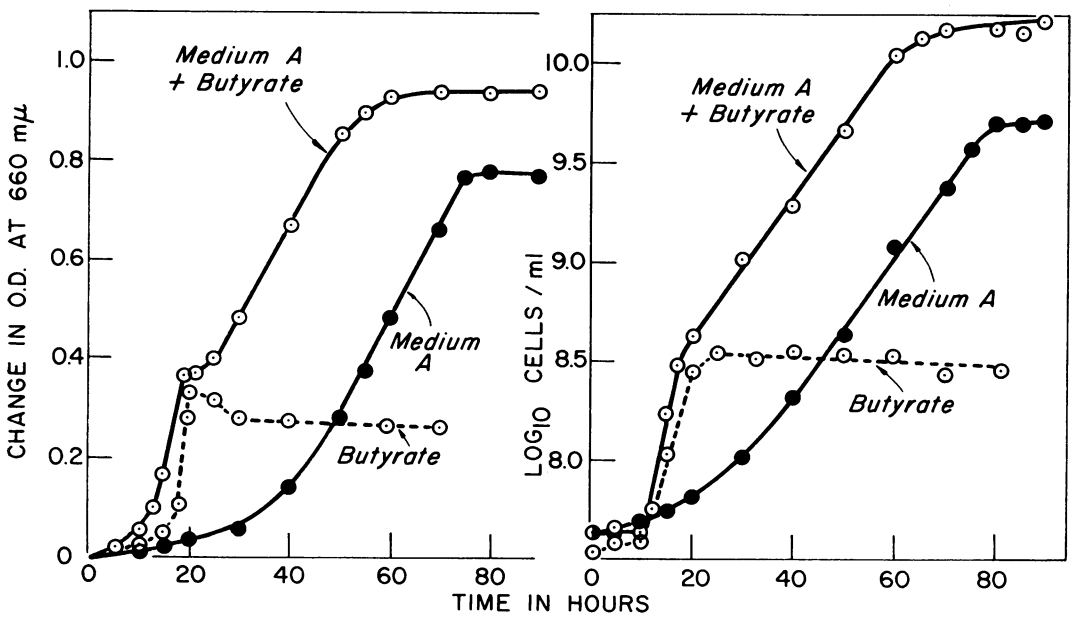

FIG. 8. *Growth of Arthrobacter crystallopoietes in glucose-salts medium A, butyrate-salts medium, and salts medium containing both glucose and butyrate (see text for details). Turbidity curves on the left; direct microscopic counts are on the right.*

succinate, malate, fumarate, lactate, and butyrate) could be attempted if these were closely related. However, it is difficult to envision a unitary mechanism for the heterogeneous chemicals found to be active. Moreover, the problem is further complicated by the failure of compounds closely related to the inducers to affect the growth cycle.

In developing from a spherical to a rod-shaped organism, a major reaction occurring would be an increased synthesis of cell wall, possibly of different chemical composition. In two recent papers, Gillespie (1963*a*, *b*) reported no qualitative difference in the composition of coccoid and pleomorphic (rod)-stage cell walls from *A. globiformis*. Quantitative variations between the

two stages in the wall content of polysaccharide and amino sugars were reported, however. The absolute dependence of *A. crystallopoietes* upon specific compounds for morphogenesis suggests the possibility that these organisms are deficient in the synthesis of some precursor(s) of the rod-stage cell wall. Lysine fits well into such a hypothesis in that this amino acid was found in the walls of five species of *Arthrobacter* by Cummins and Harris (1959). It is difficult, however, to relate the other cycle inducers to known cell-wall components or precursors thereof. It might be particularly significant in relation to this hypothesis that precursors to many of the inducing compounds, i.e., diaminopimelic acid for lysine, aspartic acid for asparagine, and citrulline and ornithine for arginine, are not themselves cycle inducers. A study of the metabolic fate of the morphogenesis-inducing compounds should provide valuable information concerning their mode of action. Such an investigation is underway.

ACKNOWLEDGMENTS

This study was supported in part by grants from the U.S. Public Health Service and the National Science Foundation.

The senior author is a U.S. Public Health Service Postdoctoral Fellow.

ADDENDUM IN PROOF

Mulder and Antheunisse (1963) recently noted that a few cell divisions of *A. globiformis* may occur as the coccus form in an undefined medium consisting of 0.5% agar, soil extract, and 0.05% yeast extract.

LITERATURE CITED

CUMMINS, C. S., AND H. HARRIS. 1959. Taxonomic position of Arthrobacter. Nature **811**:831–833.

ENSIGN, J. C. 1963. The bacterial metabolism of pyridine, 2-hydroxypyridine and nicotinic acid. Ph.D. Thesis, University of Southern California, Los Angeles.

ENSIGN, J. C., AND S. C. RITTENBERG. 1963. A crystalline pigment produced from 2-hydroxypridine by Arthrobacter crystallopoietes n. sp. Arch. Mikrobiol. **47**:137–153.

GILLESPIE, D. C. 1963*a*. Cell wall carbohydrates of *Arthrobacter globiformis*. Can. J. Microbiol. **9**:509–514.

GILLESPIE, D. C. 1963*b*. Composition of cell wall mucopeptide from *Arthrobacter globiformis*. Can. J. Microbiol. **9**:515–521.

KUHN, D. A., AND M. P. STARR. 1960. *Arthrobacter atrocyaneus*, n. sp., and its blue pigment. Arch. Mikrobiol. **36**:175–181.

MULDER, E. G., AND J. ANTHEUNISSE. 1963. Morphologie, physiologie, et ecologie des Arthrobacter. Ann. Inst. Pasteur **105**:46–74.

STEVENSON, I. L. 1962. Growth studies on *Arthrobacter globiformis*. II. Changes in macromolecular levels during growth. Can. J. Microbiol. **8**:655–661.

VELDKAMP, H., G. VAN DEN BERG, AND L. P. T. M. ZEVENHUIZEN. 1963. Glutamic acid production by *Arthrobacter globiformis*. Antonie van Leeuwenhoek J. Microbiol. Serol. **29**:35–51.

WOLIN, E. A., M. J. WOLIN, AND R. S. WOLFE. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.