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Growth and Morphology of *Asticcacaulis biprosthecum* in Defined Media

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Abstract. The growth and morphology of cells of *Asticcacaulis biprosthecum* were studied in defined media to determine the effects of various compounds on the growth rate and on the expression of morphological events of the life cycle.

The length of prosthecae could not be controlled by varying the concentration of inorganic phosphate as has been shown for other caulobacters.

In defined media, growth was inhibited during conditions favoring rapid metabolism, apparently due to an absolute requirement for cells to complete all stages of the life cycle before cell division could occur. The morphology of cells grown under these conditions was aberrant, *i.e.*, cells appeared elongated and branched and few prosthecae or swarmer cells were produced.

Growth of a related bacterium, *Asticcacaulis* strain S-3, was not inhibited by conditions favoring rapid metabolism. During rapid growth, cell division in this organism occurs in the swarmer stage and prosthecae are not produced. Cell division in S-3 is not obligately coupled to completion of all stages in the complex life cycle, and morphogenesis can be controlled by cultural conditions.

Key words: Morphogenesis – Defined media – Growth – Morphology – *Asticcacaulis*.

Asticcacaulis biprosthecum is a gram-negative heterotrophic bacterium which undergoes a dimorphic life cycle characteristic of other bacteria in the caulobacter group (Pate *et al.*, 1973; Poindexter, 1964). Each cell produces two cellular appendages, called prosthecae, perpendicular to the long axis of the cell. The prosthecate cell gives rise to a motile swarmer cell with a single polar flagellum which is eventually lost as the swarmer cell produces a polar holdfast followed by differentiation of prosthecae to complete the life cycle. Prosthecae of *A. biprosthecum* have been shown by Pate *et al.* (1973) to be structurally homologous with prosthecae of other caulobacters.

A. biprosthecum, like other caulobacters, is commonly found in aqueous environments with very low concentrations of organic material. The ability of caulobacters to outgrow most other types of bacteria in dilute environments is the basis for the dilute-peptone (0.01%) enrichment procedure developed by Houwink (1951). The production of prosthecae and the dimorphic life cycle are presumed to provide a definite selective advantage for survival in dilute environments. Caulobacters in general appear to be quite specialized for survival in dilute environments and are unable to grown in rich media.

It was the purpose of this study to develop a defined growth medium for *A. biprosthecum* and to establish the minimum nutritional requirements and optimal conditions for growth. The effects of various nutrients and conditions of growth on the morphological events of the life cycle were also studied to determine: 1) if specific stages of the life cycle could be induced or repressed, 2) if the length of prosthecae could be nutritionally controlled, and 3) the reason for the inability of *A. biprosthecum* to grow in rich media.

Minimal growth requirements of *A. biprosthecum* were satisfied by a mineral salts medium containing glucose, ammonia, and biotin. Certain other sugars could be used in place of glucose. In defined media, growth is inhibited by conditions favoring rapid metabolism. The inability of cells of *A. biprosthecum* to grow in rich media appears to be due to unbalanced growth rather than to the presence of inhibitors, and no conditions were found which would allow cells to grow while bypassing a specific stage of the life cycle.

Materials and Methods

Organism

Asticcacaulis biprosthecum C-19 (ATCC 27554) isolated by Pate and Ordal (1965) was used throughout this study. *Asticcacaulis* strain S-3 (Pate and Ordal, 1965) was used where indicated.

Growth Media

(i) Complex Medium. The dilute peptone-yeast extract medium (PYE) described by Poindexter (1964) was used where

indicated. The pH was adjusted to 7.2 (Pate *et al.*, 1973). When a solid medium was desired, Difco agar was added to the PYE medium to a level of 1.5%. Cells were maintained on refrigerated PYE slants and carried in liquid PYE culture for growth studies.

(ii) Synthetic Medium. 1) Development: Single-omission experiments using 0.1% salt-free HY Casamino acids (Sheffield Chemical Co., Norwich, N.Y.) as a nitrogen source and a mixture of nine filter-sterilized vitamins in various concentrations in a basal salts medium were used to establish the growth factor requirement. The composition and final concentration of the basal salts mixture was as follows: 0.02% $MgSO_4 \cdot 7\ H_2O$, 1.0 mM KH_2PO_4, 1.0 mM Na_2HPO_4. 2 ml of a PYE culture in the early to middle logarithmic phase of growth were inoculated into a flask containing 100 ml of media which was incubated on a rotary platform shaker (approximately 350 rpm) at 30° C. For reasons discussed later, D-fructose, at a concentration of 0.1%, was the only sugar capable of supporting good growth under these conditions. After the growth factor requirements were established, single-omission experiments with a mixture of 22 amino acids in various concentrations were used to find any required amino acids necessary for growth. In all experiments, the failure of the organism to grow in a medium lacking a particular vitamin or amino acid was considered tentative evidence of a requirement for that particular factor. The absolute requirement of that factor was considered established only if a constant growth rate was maintained through successive subcultures in a medium supplemented only with the factor (or factors) in question. All aqueous solutions in experiments with defined media were made with double-distilled water.

2) Composition and formulation: The composition of the mineral salts-biotin medium (MS-B) along with the final, optimal concentrations of the individual components was as follows: 0.01% $MgSO_4 \cdot 7\ H_2O$, 0.5 mM $NaH_2PO_4 \cdot H_2O$, 0.5 mM KH_2PO_4, 10^{-3} dilution of trace salts mixture, 0.01% Na citrate, 0.004 µg/ml d-biotin. The composition of the trace salts mixture was as follows: $CaCl_2 \cdot H_2O$, 1.0 g; $CuSO_4 \cdot 5\ H_2O$, 0.1 g; $CoCl_2 \cdot 6\ H_2O$, 0.1 g; $FeSO_4 \cdot 7\ H_2O$, 1.0 g; $K_2B_4O_7 \cdot 4\ H_2O$, 0.1 g; MoO_3, 0.1 g; $MnSO_4 \cdot H_2O$, 1.0 g; $ZnSO_4 \cdot 7\ H_2O$, 1.0 g; conc. HCl, 5.0 ml; double-distilled water, 95 ml. Na citrate was used as a chelating agent since other common chelators including ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) proved to be toxic and inhibitory to growth at levels necessary for adequate chelation. MS-B was prepared at 10X concentration as follows: 0.5 ml of 1.0 M $NaH_2PO_4 \cdot H_2O$ and 0.5 ml of 1.0 M KH_2PO_4 were added to 88 ml of double-distilled water containing 0.1 g Na citrate. 1 ml from the trace salts stock solution and 0.1 ml from a 40 µg/ml biotin solution were also added to the mixture, and the pH was adjusted to about 7.3 with 10 N NaOH. 10 ml of a 1% $MgSO_4 \cdot 7\ H_2O$ solution were then added to bring the volume to 100 ml and the pH dropped to about 7.2. The solution was filter-sterilized through a 47 mm Swinnex filter holder fitted with a 0.45 µm membrane filter (Millipore Corp., Bedford, Mass.) and stored refrigerated in dark bottles. Any other method of formulation of MS-B resulted in precipitation, presumably of magnesium phosphate. The concentration of citrate could not be increased to improve its chelating activity and prevent precipitation since it became toxic at higher levels. The following nitrogen sources were filter-sterilized and stored in dark bottles under refrigeration: L-alanine (ala), L-proline (pro), DL-serine (ser), L-aspartic acid (asp), L-glutamic acid (glu), and methylamine hydrochloride ($CH_3NH_2 \cdot HCl$). Carbon sources, including D-glucose, maltose, D-galactose, lactose, D-fructose, D-xylose, sodium citrate and casamino acids (CAA), were initially filter-sterilized but later autoclaved, along with all ammonia salts, at 120° C for 20 min when it was found that this did not prevent growth on these substrates. Pyruvic acid and DL-lactic acid were filter-sterilized. All acidic and basic substances were neutralized to pH 7.2 before sterilization if neutral salts of these compounds could not be obtained.

Growth Measurements

(i) Turbidity. In liquid suspension, cells of *Asticcacaulis* sp. were evenly dispersed throughout the medium. For growth experiments, liquid cultures were incubated at 30° C in 250 ml or 500 ml flasks fitted with sidearms. The cultures were either aerated on rotary platform shakers or allowed to remain standing without aeration. Turbidity of cultures was measured in Klett units with a Klett-Summerson colorimeter fitted with a blue no. 42 filter (approximately 420 nm). One Klett unit equals 0.002 O.D.

(ii) Viable Counts. Liquid cultures were diluted in cold PYE medium and spread in quadruplicate on PYE plates. These were inverted and incubated at 30° C for approximately 4 days before counting. In liquid culture, the determination of cell number by viable counts can be complicated by the occurrence of rosettes of several cells as the population density increases. The problem was largely overcome by sampling at relatively low optical densities (*i.e.*, early in the logarithmic phase of growth) where the cells were dispersed enough to prevent excessive aggregation.

Preparation of Cell Extracts

During the following operations the temperature was maintained below 4° C. Approximately 100 ml of cells were centrifuged at 8000 × g for 15 min and washed once with 100 ml of 0.1 M sodium phosphate buffer, pH 7.4, or 0.02% $MgSO_4 \cdot 7\ H_2O$. They were resuspended in 5 ml of the buffer and placed in a French pressure cell. Cells were broken at a pressure or about 20000 psi and the suspension was centrifuged at 35000 × g for 45 min. The resulting supernatant solution or "soluble fraction" was slightly cloudy and was used immediately for enzyme and protein assays.

Analytical Procedures

(i) Sugar Assays. 5-ml samples of cells suspended in growth media were filtered through 25 mm Swinnex filter holders fitted with 0.45 µm membrane filters. The supernatant solution was analyzed colorimetrically for D-glucose by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). Total sugar was determined by the method of Dubois *et al.* (1956) and assays were performed at 490 nm.

(ii) Protein Assays. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as the protein standard.

(iii) Dissolved Oxygen Measurements. 5-ml samples of cells suspended in growth media were placed in 15 ml beakers and aerated at a constant rate with a micromagnetic stirrer. The level of dissolved oxygen (O_2) maintained in the cultures after equilibration was measured with a model 53 YSI Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) after the instrument was blanked with 5.0 ml of sterile, air-saturated medium. For experiments where

constant dissolved O_2 levels were required, 5 l of cells were grown in a fermenter with a dissolved O_2 controller (New Brunswick Scientific Co., New Brunswick, N.J.).

Enzyme Assays

β-Galactosidase (lactase) (EC 3.2.1.23) was determined by the method of Paigen (1963) with the following modifications. 3 ml of cell extract were added to 3.0 ml of 0.01 M *o*-nitrophenyl-β-D-galactoside (ONPG) in 0.02 M sodium phosphate buffer, pH 7.5, and incubated at 30° C. At 20 min intervals, 2.0 ml samples were withdrawn and pipetted into tubes containing 1.0 ml of 1.0 M Na_2CO_3; the final pH of the mixture was 10.8. The samples were immediately assayed at 420 mm on a Gilford 300 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) with a light path of 1 cm, using on ONPG blank incubated with the samples. Specific activities were calculated using a molar extinction coefficient of 21.3×10^3. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by the method of Demoss (1955) by following the increase in absorbance of NADPH at 340 nm with a Cary 15 recording spectrophotometer (Applied Physics Corp., Monrovia, Ca.) using a light path of 1 cm. Specific activities were calculated using a molar extinction coefficient of 6.22×10^3. Enzymes were assayed at different protein concentrations to insure linearity between protein content and enzyme activity.

Electron Microscopy

A drop of cell suspension was placed on a 200-mesh copper grid with Parlodion and carbon. After 45 sec, the excess liquid was removed by blotting with filter paper and the preparation was shadowed at an angle of 30° with platinum-carbon in a Kinney vacuum evaporator. The preparations were examined in a Zeiss EM9S-2 electron microscope with an accelerating voltage of 60 kV.

Reagents

All chemicals were of the highest quality commercially available.

Results

Effect of Aeration on Growth with Glucose

In early experiments, it was not possible to grow cells of *Asticcacaulis biprosthecum* with glucose as a source of carbon and energy and ammonia or amino acids as sources of nitrogen. Little growth (less than 40 Klett units) was obtained on MS-B plus 0.1% glucose and 100 µg/ml each ala, pro, ser, glu, asp (MS-B-AA) or on MS-B plus 0.1% glucose and 0.05% $(NH_4)_2HPO_4$ (MS-B-NH_4). Cells were not viable and could not be subcultured to fresh media. They appeared elongated and branched (misshapen) and few prosthecae or swarmer cells were produced (Fig. 1 d). The same results were obtained when maltose, galactose or xylose were used in place of glucose. Growth could be obtained with 0.1% fructose in place of glucose, but the growth rate was much lower than control

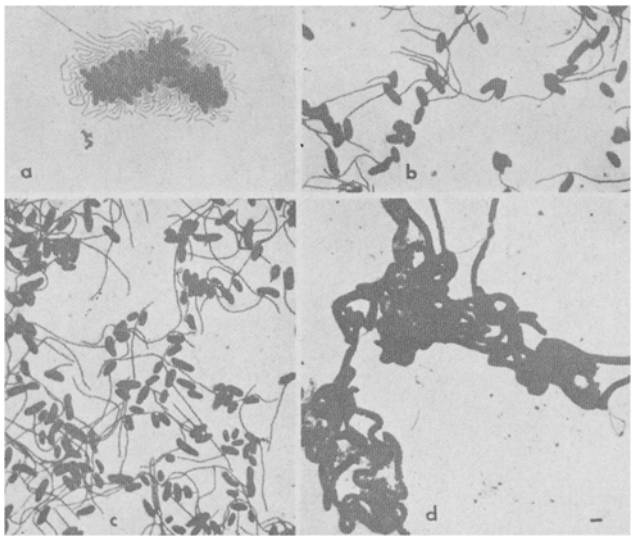

Fig. 1 a–d. Electron micrographs of *Asticcacaulis biprosthecum* shadowed with platinum-carbon comparing the morphology of cells grown on defined and complex media. The cultures were agitated immediately after inoculation. (a) Complex PYE media. (b) Lactose-amino acids. (c) Fructose-amino acids. (d) Glucose-amino acids. Bar 1 µm

cultures grown on PYE. Cells grown on complex media or fructose (Fig. 1 a and c, respectively) were not elongated or branched and both prosthecate and swarmer cell types were produced. In all of these experiments, cultures were vigorously aerated on a rotary platform shaker immediately after inoculation. It was subsequently discovered that MS-B-AA plus glucose would support growth of *A. biprosthecum* if the cultures were incubated without aeration (static growth). Therefore, the effects of aeration were taken into account in all further attempts to define the growth requirements for this organism.

Growth and Morphology with Good Carbon Sources

The static growth of cells of *A. biprosthecum* in MS-B AA plus 0.1% glucose, maltose, galactose or xylose is shown in Fig. 2. If static growth was allowed for 24 hrs before vigorous aeration on a shaker, the results shown in Fig. 3 were obtained. Similar results were obtained using the same carbon sources plus 0.05% $(NH_4)_2HPO_4$ as the sole nitrogen source.

The morphology of cells grown on MS-B-AA plus glucose is shown in Fig. 4 a. Cells were allowed a 24-hr period of static growth before they were aerated by vigorous agitation. Similar results were observed when maltose, galactose or xylose were used in place of glucose, or when 0.05% $(NH_4)_2HPO_4$ was used as the nitrogen source in place of amino acids. Even though the cells appear somewhat misshapen, high final optical densities were obtained, both prosthecate

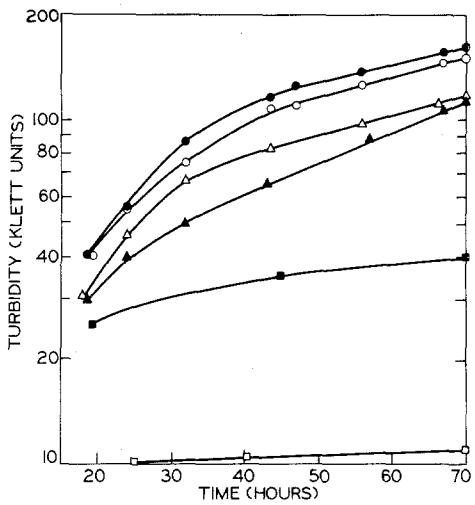

Fig. 2. Static growth of cells of *A. biprosthecum* grown on MS-B plus ala, pro, ser, glu, asp (100 µg/ml each) and the following carbon sources at a concentration of 0.1%: ● maltose; ○ glucose; △ galactose; ▲ xylose; ■ cells were grown on the above carbon sources but were shaken immediately after inoculation; □ cells were grown on MS-B without carbon or nitrogen sources

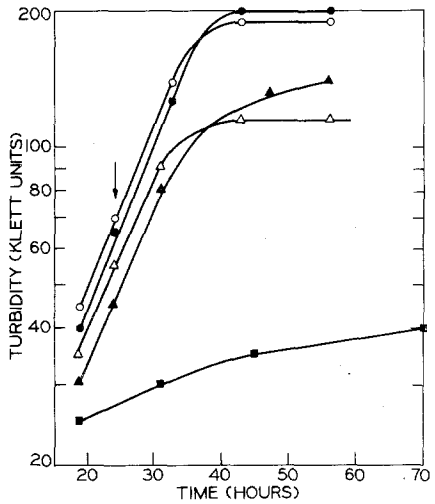

Fig. 3. Growth of *A. biprosthecum* on MS-B plus ala, pro, ser, glu, asp (100 µg/ml each) and various carbon sources at a concentration of 0.1%. Cells remained standing for a period of 24 hrs (indicated by arrow) before they were aerated by vigorous agitation. ● maltose; ○ glucose; ▲ xylose; △ galactose; ■ cells grown with the above carbon sources which were agitated immediately after inoculation

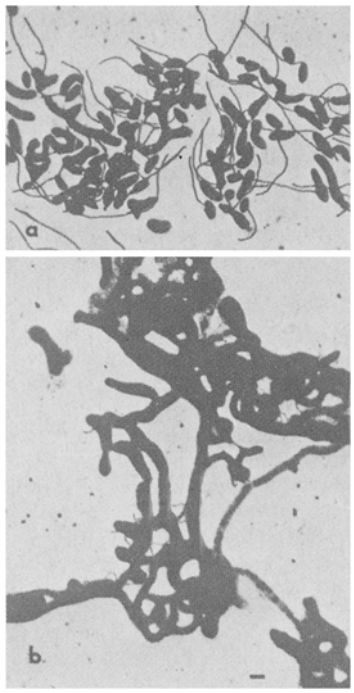

Fig. 4a and b. Electron micrographs of *A. biprosthecum* shadowed with platinum-carbon showing morphology of cells grown on MS-B plus ala, pro, ser, glu, asp (100 µg/ml each) and 0.1% glucose under different conditions of aeration. (a) Morphology of cells which were allowed a 24-hr period of static growth before agitation. (b) Morphology of cells which were agitated immediately after inoculation. Bar 1 µm

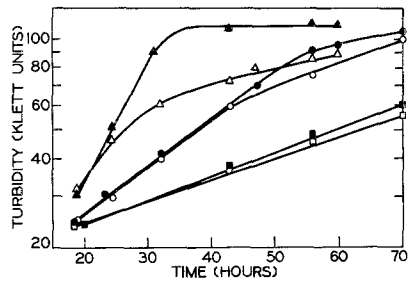

Fig. 5. Growth of *A. biprosthecum* with poor carbon sources under different conditions of aeration. Cells were either grown in static culture (open symbols) or allowed to remain standing for 24 hrs before vigorous agitation (closed symbols). Cultures were grown on MS-B plus pro, ser, glu, asp, ala (100 µg/ml each) and the following carbon sources at a concentration of 0.1%. ○, ● lactose; △, ▲ casamino acids; □, ■ fructose

and swarmer cells were produced, and cells were capable of being subcultured to fresh defined medium as long as the initial period of static growth was maintained. As mentioned earlier, cells exposed to vigorous aeration immediately after inoculation were not viable and appeared very misshapen (Fig. 4b).

Growth and Morphology with Relatively Poor Carbon Sources

Growth of cells of *A. biprosthecum* on MS-B-AA plus 0.1% lactose, fructose or casamino acids (CAA) is shown in Fig. 5. Cells were grown in static culture or

Table 1. Utilization of carbon and ammonium compounds by *Asticcacaulis biprosthecum*

Compound tested[a]	Final O.D. (%)[b]	Growth rate (%)[c]
Nitrogen sources[d]		
$(NH_4)_2HPO_4$	100	100
NH_4Cl	48	47
$(NH_4)_2SO_4$	57	47
$NH_4S_2O_8$	0	0
Ammonium tartrate	43	35
Ammonium acetate	35	29
Methylamine hydrochloride	43	30
Control 1 (minus ammonia)	70	65
Control 2 (minus ammonia) (minus carbon source)	<5	<5
Carbon sources[e]		
Glucose	95	100
Maltose	100	89
Galactose	58	58
Xylose	70	53
Lactose	35	16
Fructose	25	3
Methanol	3	2
Methylamine hydrochloride	0	0
Pyruvate	70	61
Lactate	0	0
Casamino acids	55	63
Citrate	0	0
Control 3 (minus carbon source)	<5	<5

[a] Compounds were tested in MS-B + 0.1% glucose for ammonium utilization. Compounds were tested in MS-B + 0.01% $(NH_4)_2HPO_4$ for carbon utilization.
[b] Percent of final O.D. reached by test culture compared to maximum final O.D. observed.
[c] Percent of growth rate of test culture compared to maximum growth rate observed during logarithmic phase of growth.
[d] Concentration of ammonium sources in each culture normalized to 100 µg/ml ammonia.
[e] Each carbon source tested at two concentrations, 0.05%, 0.1%. Results obtained with 0.1% carbon source are listed.

left standing for 24 hrs before they were vigorously aerated. Cultures which were shaken immediately after inoculation had growth curves similar to cultures which remained standing for 24 hrs before agitation.

The morphology of cells grown on MS-B-AA plus 0.1% lactose is shown in Fig. 1b. Similar results were observed when fructose or CAA were used in place of lactose, or when 0.05% $(NH_4)_2HPO_4$ was used as the nitrogen source in place of amino acids. Cells were allowed a 24-hr period of static growth before they were vigorously agitated. Under these growth conditions, the cells appeared normal (compare with Fig. 1a and c). Cells which were agitated immediately after inoculation also appeared normal when fructose or CAA was the carbon source and appeared only slightly misshapen when lactose was the carbon source (*e.g.*, similar to Fig. 4a).

Table 2. Effect of ammonium concentration on growth of *A. biprosthecum*. 2 ml of a PYE culture were inoculated into 100 ml of MS-B plus 0.01% glucose with no added nitrogen source. 2 ml of this culture in the logarithmic phase of growth were added to fresh media with various concentrations of $(NH_4)_2HPO_4$. Cells were allowed a 24-hr period of static growth before aeration

Concentration of $(NH_4)_2HPO_4$ (µg/ml)	Concentration of nitrogen (µg/ml)	Final O.D. (%)[a]	Growth rate (%)[b]
100	21.2	100	100
50	10.6	100	100
25	5.3	70	62
10	2.1	35	24
5	1.1	20	12

[a] Percent of final O.D. reached by test culture compared to maximum final O.D. observed.
[b] Percent of growth rate of test culture compared to maximum growth rate observed during logarithmic phase of growth.

Carbon and Nitrogen Utilization

Compounds tested as sources of carbon and nitrogen for *A. biprosthecum* are listed in Table 1. Cells were allowed a period of static growth (to a turbidity of 30–35 Klett units) before agitation. Glucose was found to be the best carbon source and $(NH_4)_2HPO_4$, the best inorganic nitrogen source. A mixture of five amino acids included L-ala, which was the only amino acid absolutely required for growth in the absence of ammonia, could be used as a nitrogen source in place of ammonia. The amino acids would not serve as energy sources, however, and glucose or another metabolizable compound had to be included for growth. It was interesting to note the relatively low levels of nitrogen required for growth by this organism. Growth at about 60% of the maximum growth rate and adequate cell yields were obtained on about 5 µg/ml of nitrogen (Table 2).

Effect of Nutrient Concentration on Growth and Morphology

As indicated earlier, cells of *A. biprosthecum* were always misshapen to some extent on MS-B-AA or MS-B-NH_4 when glucose, maltose, galactose or xylose (referred to as glucose groups of sugars), were used as carbon sources. The lowest concentration of these sugars tested was 0.025%. In these experiments, MS-B controls containing no carbon or nitrogen sources were included to determine the amount of growth in

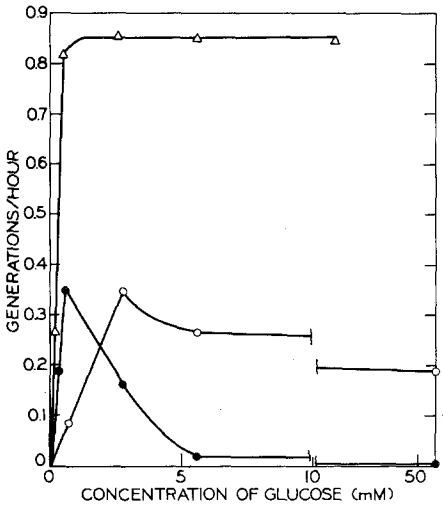

Fig. 6. Growth rate of *A. biprosthecum* with different concentrations of glucose. Cells were grown on MS-B plus 500 µg/ml L-ala (○) or MS-B plus 100 µg/ml each ala, pro, ser, glu, asp (●) and agitated immediately after inoculation. Growth of *Asticcacaulis* strain S-3 on MS-B plus 100 µg/ml each ala, pro, ser, glu, asp (△) is included for comparison

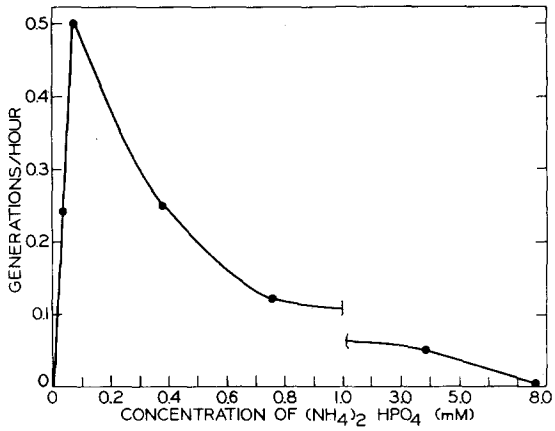

Fig. 7. Growth rate of *A. biprosthecum* with different concentrations of ammonia. Cells were grown on MS-B plus 0.1% glucose and agitated immediately after inoculation

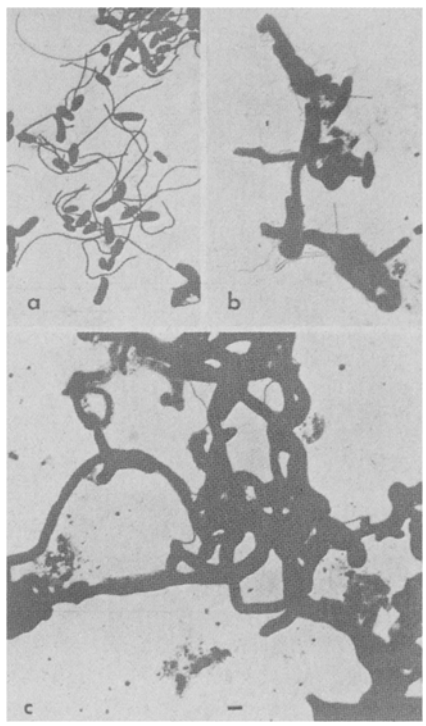

Fig. 8 a–c. Electron micrographs of *A. biprosthecum* shadowed with platinum-carbon indicating morphology of cells grown on MS-B-AA plus the following concentrations of glucose: (a) 0.005%; (b) 0.05%; (c) 0.5%. Cells were agitated immediately after inoculation. Bar 1 µm

defined media due to carryover from the PYE starter culture. It was noticed that cells in these controls did not appear misshapen, though they grew to only low turbidities (5–15 Klett units). Subsequently, when the levels of carbon and nitrogen in defined media were decreased to lower levels than had been used in previous experiments, cells growing on the glucose group of sugars became similar in appearance to cells growing normally on PYE, even with immediate agitation after inoculation. Both prosthecate and swarmer cells were produced, and elongated or branched forms were absent. This type of experiment was repeated on three different media: MS-B plus L-ala (500 µg/ml), MS-B plus ala, pro, ser, glu, asp (100 µg/ml each), and MS-B plus $(NH_4)_2HPO_4$. In MS-B plus amino acid(s), the concentration of glucose was varied with the concentration of nitrogen remaining constant; in MS-B plus ammonia, glucose remained constant at 0.1% and the ammonia was varied for reasons discussed later. The results of these experiments are indicated in Figs. 6 and 7. Notice that the growth rate of cells growing on the different media reaches a maximum and then immediately decreases as the concentration of carbon or nitrogen sources is increased beyond a certain point. Below this point, cells grew normally even with immediate agitation after inoculation and were capable of being subcultured to fresh media immediate agitation. Above this point cells became progressively more elongated and branched with few prosthecae and swarmer cells being produced. At sufficiently high nutrient concentrations, cultures eventually ceased to grow with immediate agitation after inoculation. These morphological changes are shown in Fig. 8 using glucose as the carbon source, though similar results were observed for each of the sugars in the glucose group.

The concentration of either nitrogen or carbon sources could be varied to achieve these growth effects in MS-B plus amino acid(s). With MS-B plus ammonia,

however, the concentration of ammonia had to be varied since mishapen cells were observed at very low concentrations of glucose with a constant concentration of nitrogen [0.05% $(NH_4)_2HPO_4$]; cell yields (*i.e.*, final OD) under these circumstances were too low to permit experimentation (*e.g.*, less than 10 K.U.). Similar results were obtained when maltose, galactose or xylose were used in place of glucose. Cells growing on MS-B plus 0.1% CAA were normal irrespective of the conditions of aeration but they became progressively more misshapen as higher levels of glucose were incorporated in the medium.

In all of these experiments, the minimum generation time (maximum growth rate) achieved during the logarithmic phase of growth was observed to occur at the same concentration of nutrients separating cells with normal morphologies from those with aberrant morphologies. Below the optimal concentration of nutrients, the generation times were longer and the cells did not appear elongated or branched; both prosthecate and swarmer cell types were present. Above the optimum concentration point, cells became progressively more misshapen as the concentration of nutrients was increased and the generation times increased as well. At higher nutrient concentrations where the cells appeared misshapen, turbidity measurements were not an accurate reflection of growth in terms of cell division.

Effect of Dissolved Oxygen Levels on Growth

The relationship between the optical density (OD) reached by cells in static cultures and the ability of these cells to grow when aerated in MS-B plus 0.05% $(NH_4)_2HPO_4$ and 0.1% glucose is shown in Fig. 9. Significant increases in the growth rate began after the cells were allowed to reach a turbidity in excess of 10 Klett units (K.U.) before agitation. The longer the cells were allowed to stand before vigorous aeration (to 35 K.U.) the higher the final OD observed. Similar results were obtained using 0.1% maltose, galactose or xylose in place of glucose or with MS-B plus amino acid(s). With amino acid(s), however, the initial increase in turbidity was not as clearly defined. The levels of dissolved oxygen (O_2) maintained in shaking cultures which were allowed to reach various optical densities before agitation were measured to determine if the ability of cells to grow on MS-B-NH_4 plus glucose was correlated with the maintenance of decreased levels of O_2. It was found that cultures which were agitated immediately after inoculation or allowed to grow to low turbidities (10–15 K.U.) before agitation failed to grow after they were placed on the shaker and could not maintain dissolved O_2 levels before 100% saturation (7.5 mg O_2/l). Cultures which

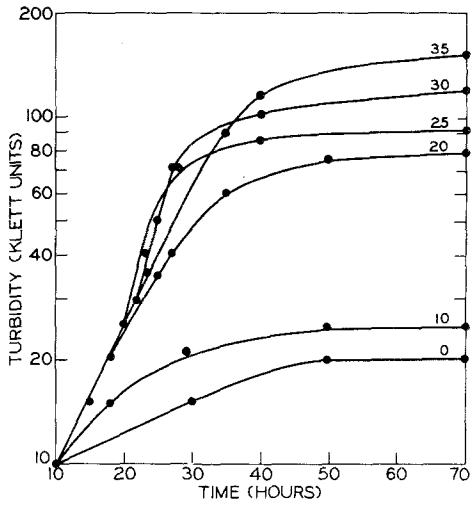

Fig. 9. Effect of static growth of *A. biprosthecum* on subsequent growth with aeration. Cells were grown on MS-B plus 0.1% glucose and 0.05% $(NH_4)_2HPO_4$. Cultures were allowed to remain standing until certain turbidities (Klett units) were reached (numbers in figure) and then aerated by vigorous agitation

remained standing until turbidities of 20–25 K.U. or more had been obtained and then agitated did grow to higher optical densities. These cultures were able to slowly decrease O_2 levels from a maximum of about 90% (± 3%) saturation (6.7 mg O_2/l) to 80% (±3%) saturation (6.0 mg O_2/l) within 5 min.

In a second set of experiments, cultures were grown in a fermenter (concentration of nutrients same as in Fig. 9), and the level of dissolved O_2 was maintained at a constant value throughout growth. Cells exposed to saturating concentrations of dissolved O_2 (7.5 mg O_2/l) failed to grow while those exposed to dissolved O_2 levels of about 90% (± 2%) of saturation (6.7 mg O_2/l) or less did grow. It should be mentioned that the 90% value represents an upper limit above which growth would not occur. This value was obtained with a specified concentration of nutrients and would be expected to decrease as the growth rate increased in response to higher concentrations of nutrients.

Growth with Two Sugars

The growth curves of *A. biprosthecum* on glucose, glucose plus lactose, and lactose are shown in Fig. 10. Although the growth of cells in the glucose culture levels off after glucose is depleted from the medium (data not shown), growth in the glucose plus lactose culture continues on at a reduced rate without a noticeable lag. Two types of experiments were performed to verify this apparent lack of catabolite repression of lactose enzymes during growth with glucose. In the first set of experiments, the con-

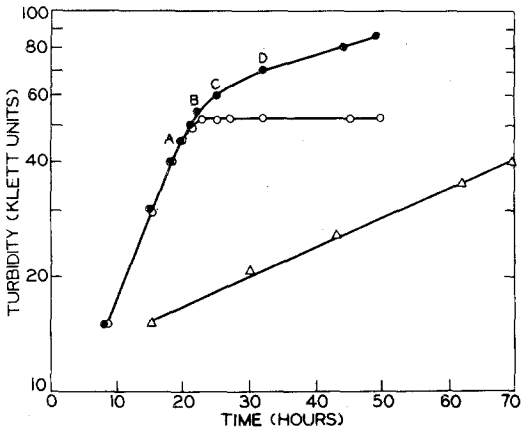

Fig. 10. Growth of *A. biprosthecum* on MS-B plus 0.005% $(NH_4)_2HPO_4$ with the following carbon sources: ○ 0.025% glucose; ● 0.025% glucose plus 0.075% lactose; △ 0.075% lactose. Cells were agitated immediately after inoculation. Letters indicate points in growth curve where samples were taken for enzyme and sugar assays

centrations of glucose and lactose were determined at points throughout the growth curve (indicated by letters in Fig. 10) to determine if glucose and lactose were being used concurrently. Attempts to monitor lactose utilization were not successful since it was difficult to accurately follow the low rate of lactose utilization. Results obtained with glucose were more conclusive. Before glucose exhaustion, about 30–40 µg/ml more glucose was found in the glucose plus lactose culture than the glucose control culture at each test point, even though the optical densities of the two cultures were the same. The glucostat assay used was specific for D-glucose and no reaction was observed with lactose. Apparently, in the glucose plus lactose culture, lactose was being utilized concurrently with glucose and this exerted a sparing effect on glucose consumption.

In the second set of experiments, β-galactosidase was assayed for directly to determine if the enzyme was inducible and subject to catabolite repression. There was no release of enzyme from toluenized cells treated with detergents, mercaptoethanol or urea, so samples were taken at intervals as before (points indicated by letters in Fig. 10), and whole-cell extracts were prepared and analyzed with the results shown in Table 3. Notice that the specific activity of β-galactosidase remained constant in the glucose plus lactose culture and was about the same as the specific activity observed in glucose or lactose control cultures. The activity was quite low, however, which might explain the slow growth by *A. biprosthecum* on lactose, even though the individual sugars, glucose and galactose, were readily utilized.

Table 3. β-Galactosidase activity of cell extracts of *A. biprosthecum*. Cells were grown on MS-B plus 0.005% $(NH_4)_2HPO_4$ and either 0.025% glucose, 0.025% glucose plus 0.075% lactose or 0.075% lactose. Cultures were agitated immediately after inoculation and samples were taken at various points throughout the growth curve (indicated by letters in Fig. 10). Values were averaged from a number of experiments with an approximate range of ± 10%. Optical densities of 0.5 or greater were obtained during enzyme assays

Point in growth curve where samples taken	Carbon source(s) in medium	Specific activity of β-galactosidase[a]
A	glucose	1.7
	glucose + lactose	1.8
	lactose	2.0
B	glucose	1.7
	glucose + lactose	1.7
	lactose	1.8
C	glucose	1.7
	glucose + lactose	1.8
	lactose	1.9
D	glucose	1.8
	glucose + lactose	1.7
	lactose	1.9

[a] nmoles nitrophenol producing/min × mg extract protein.

Table 4. Glucose-6-phosphate dehydrogenase activity of cell extracts of *A. biprosthecum*. Cells were grown on MS-B plus 0.005% $(NH_4)_2HPO_4$ and either 0.05% glucose or 0.05% xylose. Cultures were agitated immediately after inoculation and samples were taken from cultures in the mid-logarithmic phase of growth

Carbon source in medium	Specific activity of glucose-6-phosphate dehydrogenase[a]
Xylose	125
Glucose	193

[a] nmoles NADPH produced/min × mg extract protein.

In contrast to β-galactosidase, another catabolic enzyme, glucose-6-phosphate dehydrogenase, was found to be inducible, though a certain level of constitutive activity was observed which was about 60× greater than that observed for β-galactosidase (Table 4).

Growth of S-3

Cells of *Asticcacaulis* strain S-3 did not exhibit the same lack of growth shown by *A. biprosthecum* when they were agitated immediately after inoculation in the presence of high concentrations of rapidly metabolized compounds (Fig. 6). Under these conditions, a high growth rate (generation time about 70 min)

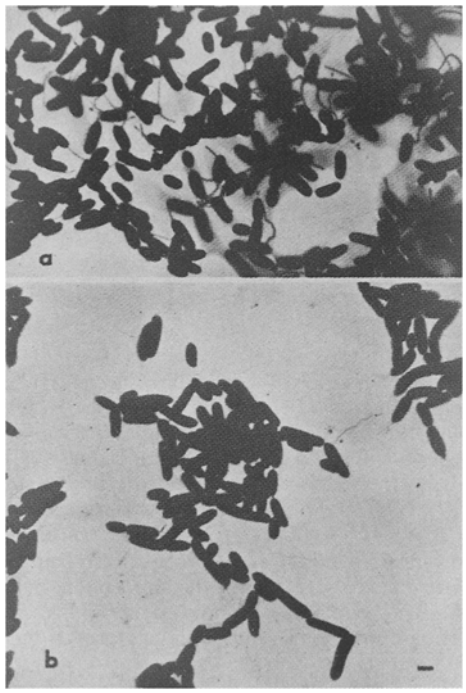

Fig. 11 a and b. Electron micrographs of *Asticcacaulis* strain S-3 shadowed with platinum-carbon indicating the morphology of cells grown on MS-B plus ala, pro, ser, glu, asp (300 µg/ml each) and 0.3% glucose. Cells were agitated immediately after inoculation. (a) Cells growing at reduced rates in the late logarithmic or early stationary phase of growth produce prosthecae. (b) Cells growing at a rapid rate during the logarithmic phase of growth are capable of dividing in the swarmer stage and do not produce prosthecae. Bar 1 µm

and final OD were obtained for S-3, and the cells did not appear elongated or branched (Fig. 11). *Asticcacaulis* strain S-3, which produces one excentral prostheca per cell, is capable of dividing in the swarmer stage without producing prosthecae if the conditions of growth warrant a faster rate of metabolism (Fig. 11 b). Prosthecae are produced in cells in the late logarithmic or early stationary phase of growth where the growth rate is reduced or at reduced O_2 tensions (Fig. 11 a). Preliminary studies also indicate that cells of S-3 growing slowly on MS-B-AA plus fructose produce prosthecae even during the logarithmic phase of growth.

Length of Prosthecae

A comparison of the length of prosthecae of cells of *A. biprosthecum* grown on defined and complex media is shown in Fig. 1 a – c. In all defined media, the length of prosthecae was approximately 20 µm (5 – 10 times the length of the cell) whereas prosthecae of PYE-grown cells were 2 – 4 µm long. The concentration of phosphate in these experiments was 1 mM. No significant increase in the length of prosthecae was observed when cells were grown in defined media lacking phosphate or trace salts, or with low concentration of these compounds.

Discussion

Growth of *Asticcacaulis biprosthecum* on defined media, as measured by final OD and maximum growth rate, was comparable to growth obtained on complex PYE medium as long as concentrations of nutrients and conditions of aeration were carefully controlled. Cells were unusually sensitive to rapid growth, and viability of cultures decreased sharply when the concentration of a limiting nutrient was increased beyond that giving optimum growth. The inability to grow in rich media is a general characteristic of the caulobacter group. In the case of *A. biprosthecum*, a complex medium sufficiently rich to inhibit growth needs only to have a concentration of total organic compounds greater than 0.3%. In a defined medium, growth is inhibited by a concentration greater than about 0.1% of a good carbon or nitrogen source. The ability of these organisms to compete so well in dilute environments is probably releated to the sequential production of specialized organelles: Flagella, holdfast, and prosthecae. But what accounts for their absolute requirement for a dilute environment in order to survive?

The data support the idea that growth of these organisms is inhibited by conditions favoring rapid metabolism rather than by any toxic effects of rich media. High concentrations of good sources of carbon and energy such as glucose or galactose do not inhibit growth under all conditions; growth is observed in cultures which are allowed a period of static growth before aeration or in cultures that are aerated immediately after inoculation but are incubated under reduced levels of oxygen. Oxygen *per se* is not toxic since growth can occur with high concentrations of relatively poor sources of carbon and energy such as fructose or CAA with good aeration. High concentrations of carbon and nitrogen sources which support growth at reduced rates do not lead to the inhibition of growth observed when the same levels of rapidly metabolized compounds are used. Toxicity due to a specific compound is unlikely, since the same effects are produced by a number of compounds. Growth is not inhibited when high concentrations of nitrogen sources are used with lower levels of carbon sources or *vice versa*.

Schmidt and Stanier (1966) have shown elongation of prosthecae of *Caulobacter crescentus* to occur under conditions of phosphate starvation in defined media. In *A. biprosthecum*, long prosthecae were still observed with 1.0 mM phosphate and relatively high concentrations of carbon and nitrogen sources. Significant increases in prosthecal length were not observed when

lower concentrations of inorganic phosphate or trace salts were used. However, prosthecae of cells grown in defined media were generally 5–10 times as long as those produced by cells grown in PYE. The compound or compounds influencing prosthecal length were not identified.

The typical diauxic response during growth with two carbon sources was not observed in cells of *A. biprosthecum* growing on glucose plus lactose. β-Galactosidase was not inducible or subject to discernible catabolite repression but exhibited a low level of constitutive activity, which is in contrast to lac systems in *Escherichia coli* (Magasanik, 1961) and *C. crescentus* (Shapiro *et al.*, 1972). This type of low-level constitutive activity is noteworthy since growth on other sugars, including the component sugars of lactose (galactose and glucose) can proceed at deleterious rates. The activity of another catabolic enzyme, glucose-6-phosphate dehydrogenase, was found to be inducible, although a basal level of constitutive activity corresponding to about 65% of the fully induced level was observed in the absence of inducer. Poindexter (1964) and Shedlarski (1974) have obtained similar results working with the glucose-6-phosphate dehydrogenase of other caulobacters. The level of enzyme activity that they found present in non-induced cultures, however, was much lower than that obtained for *A. biprosthecum*. Shedlarski (1974) reported that the activity of glucose-6-phosphate dehydrogenase of *C. crescentus* was not inhibited by reduced NAD, as it is in enterobacteria. Thus, there is evidence that metabolic pathways in caulobacters are not as carefully coordinated as in bacteria normally in richer environments.

The requirements for these cells to complete the morphogenetic cycle in order to divide, the narrow range of organic compounds metabolized by any given strain, and a decreased ability to modulate enzymes of dissimilatory pathways are probably all consequences of being specialized for life in dilute environments. The same characteristics that give *A. biprosthecum* and other caulobacters an advantage in dilute environments could be disadvantageous or even lethal in environments with increased concentrations of nutrients.

The obligate nature of the morphogenetic cycle of *A. biprosthecum* imposes the requirement that cells produce flagella, holdfast, and prosthecae in a timed sequence before division can occur. The growth rate can be increased up to a certain critical point, and the ability of cells to differentiate, as evidenced by the presence of both prosthecate and swarmer cells, is unaffected. Attempts to increase the growth rate beyond that point results in a reduced rate of growth and misshapen cells. Eventually, at sufficiently high nutrient concentrations, growth is completely inhibited (Figs. 6–8). Apparently beyond a certain critical growth rate, insufficient time is available for expression of the complete morphogenetic cycle, cells become misshapen due to unbalanced growth, and cell division is inhibited. Organisms such as *E. coli* not restricted by an obligate morphogenetic cycle respond to increasing concentrations of nutrients in quite a different manner. Growth rate increases with increasing concentrations of nutrients until a maximum rate is reached. Increasing the concentration of nutrients beyond this point is not inhibitory, and the growth rate remains at its optimal level. *Asticcacaulis* strain S-3 responds to increased concentrations of nutrients like *E. coli* rather than like *A. biprosthecum*. Rapidly dividing cells of strain S-3 bypass the prosthecate stage of the morphogenetic cycle, dividing in the swarmer stage, and growth is not inhibited by increasing the concentration of nutrients beyond that required for optimal growth. No other member of the caulobacter group has previously been reported to bypass the prosthecate stage under specific conditions of growth.

It seems reasonable that mechanisms for cooordinating dissimilatory pathways would be less necessary in dilute environments than in environments where high concentrations of many different organic compounds are frequently available. In a very dilute environment, the concentrations of various nutrient compounds are rarely so high that it would be to the advantage of an organism to metabolize only good sources of carbon and energy while repressing enzyme systems for less-efficiently metabolized compounds. Both types of compounds would be used simultaneously. In such environments, inducible enzyme systems for the metabolism of substrates less commonly found (*e.g.*, lactose) may provide no selective advantage since these substrates may never be present in sufficient concentrations to require increased levels of enzyme activity to compete for them. The presence of very low constitutive levels of certain enzymes in the organisms may be a reflection of the relative abundance of the substrates in the natural environment. However, a bacterium could not afford to maintain a large number of such systems. Even though the total expenditure of resources for one enzyme system would be small when present at such low constitutive levels, the simultaneous presence of thirty or so constitutive systems would completely exhaust the resources of organisms growing in dilute environments. This may explain why any individual strain of the caulobacter group is so limited to the number of organic compounds that can be metabolized.

The evidence suggests that a range of types exists among the caulobacters. At one end is *A. biprosthecum*,

obligately linked to the morphogenetic cycle, extremely sensitive to increased metabolic rates, and the length of prosthecae is less subject to control. Cells of *C. crescentus* also exhibit an obligate morphogenetic cycle, but the length of prosthecae can be controlled by nutritional factors: Rapidly dividing cells produce short prosthecae, and they are not so sensitive to conditions favoring rapid metabolism. Cells of *Asticcacaulis* strain S-3 are able to divide in the swarmer stage, when conditions favor rapid growth, and are not sensitive to high concentrations of nutrients. The small amount of evidence now available suggests there might also be differences among these three types in the control of dissimilatory enzymes, and a complete study of this question would be very interesting.

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