Characteristics of *Arthrobacter* Grown in Continuous Culture

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SUMMARY

When *Arthrobacter globiformis*, *A. simplex*, *A. crystallopoietes* and *A. atrocyaneus* were grown in a chemostat, their morphology was related to growth rate. A transition from rod to coccus occurred at growth rates characteristic for each species; the higher the rate at which this transition occurred, the higher was the maximum specific growth rate. Organism size, concentration and viability were determined for *A. globiformis* at a range of dilution rates between 0.01 h^{-1} and washout. Yield remained constant except at low dilution rates, although the number of organisms varied because of the changes in their size and shape. The percentage of viable organisms in the chemostat remained almost constant at dilution rates above 0.1 h^{-1} (92 %) and was only slightly lowered even at dilution rates as low as 0.01 h^{-1} .

Rods grown in the chemostat and then placed in nutrient-free solutions divided and produced cocci which survived for about 56 days. Cocci survived for about 70 days. The respiration rate of freshly harvested rods was higher than that of cocci but within two days both fell to low levels.

INTRODUCTION

Arthrobacters are pleomorphic bacteria occurring as rods or cocci under different conditions. In batch culture, with appropriate media, they are rod shaped only during the exponential phase of growth, reverting to cocci in the stationary phase. Luscombe & Gray (1971) found that when *A. globiformis* was grown in continuous flow culture the morphology was related to growth rate. A transition occurred within a narrow range of growth rates, above which organisms occurred as rods and below which they grew as cocci. The transition point was temperature dependent. Although changes in size have been reported for other bacteria grown in chemostats at different growth rates (Herbert, 1959), these took place gradually over a wide range of dilution rates, rather than sharply. The aim of the present work was to study this transition in greater detail in *A. globiformis* and to look for it in a range of *Arthrobacter* species. In addition, rods and cocci were grown in the same growth medium and their survival compared in nutrient-free conditions, which may have some relevance to the growth and survival of these bacteria in their natural soil environment.

METHODS

Growth of arthrobacters. Four species of *Arthrobacter* were grown in a chemostat with a working volume of 150 ml and vortex aeration (Luscombe & Gray, 1971). *A. globiformis* (NCIB10683), *A. atrocyaneus* (ICPB2396), *A. simplex* (AC4, J. Antheunisse, Wageningen) and *A. crystallopoietes* (NCIB9499) were grown at 25°C in the simple salts base E used by

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Owens & Keddle (1969) with added biotin (1 µg/l) and a limiting carbon source. Glucose (0.2 g/l) was used for the growth of *A. globiformis*, *A. atrocyaneus* and *A. simplex*, while sodium succinate (0.5 g/l) was used for *A. crystallopoietes*. Yeast extract (2 mg/l) was added to the medium for *A. simplex*. Low glucose concentrations were used to simulate the provision of small amounts of carbon in soil and to give populations where lack of oxygen or the development of acidic pH would not limit growth. The pH of the growth medium following stabilization of the chemostat was 6.75. The bacteria chosen represent strains common in the soil (Lowe & Gray, 1972) and ones used by other laboratories examining the growth of arthrobacters in culture.

A fermentation vessel with a working volume of 2725 ml (Biotec) was used to grow the larger quantities of *A. globiformis* required for studies on endogenous respiration and survival. A glucose concentration of 0.6 g/l was used and dilution rates of 0.05 h⁻¹ and 0.3 h⁻¹ established to produce cocci and rods respectively. Higher glucose concentrations were used in this chemostat since oxygen could be kept at a higher level and pH could be controlled.

Organism size. A drop of 0.033 M-phosphate buffer was placed on the air-dried smear of bacteria and their radii measured at ×1600 magnification using a phase-contrast microscope. Volumes were calculated from these data.

Organism yield. Organisms were counted, using a Helber counting chamber (Hawksley Ltd, London). Dry weights were determined by centrifuging suspensions, washing them and drying to a constant weight at 110 °C.

Harvesting. Bacteria were harvested, washed three times in 0.033 M-phosphate buffer by centrifugation at 3000 g for 15 min, and suspended in further buffer at 5 × 10⁹/ml. This suspension was used for studies on endogenous respiration and survival in nutrient-free conditions.

Viability. This was determined by a modification of the slide culture technique of Postgate, Crumpton & Hunter (1961) using a medium of yeast extract (Difco) 0.7 %, glucose 1.0 % and ionagar (Oxoid) 1.2 %. Blocks (5 × 5 mm) were cut from a layer of agar 0.75 to 1.0 mm deep and inoculated with organisms from the chemostat to give approximately 30 bacteria/field of view. After the inoculum had dried, a coverslip was placed on the agar block which was put in a moist chamber for incubation. Cultures were incubated for up to 16 h, when colonies of 8 to 16 bacteria had formed from viable organisms and dead forms remained visible as single bacteria. Slide cultures were examined using phase-contrast microscopy (×640), and by counting the number of colonies and dead bacteria the percentage viability could be determined.

Endogenous respiration. This was measured as the rate of oxygen uptake of bacterial suspensions in a Gilson differential respirometer (Gilson Medical Electronics, France) and using a polarographic electrode (Rank Bros, Cambridge).

Maximum specific growth rate. This was determined by the washout technique of Jannasch (1969). Bacteria were grown in the chemostat at a dilution rate of 0.35 h⁻¹ until a steady state had been reached and then the dilution rate was increased to 0.43 h⁻¹ for 4 h. Numbers in the culture were determined at this time and after a further 2, 4, 16, 18, 20, 22 and 24 h during washout. Tempest (1970) has shown that

$$\ln x_1 - \ln x_2 = (D - \mu_m)(t_2 - t_1),$$

where x_1 and x_2 are organism concentrations at times t_1 and t_2 respectively, D is the dilution rate and μ_m the maximum specific growth rate. The slope in a plot of $\ln x$ against time is therefore equal to $D - \mu_m$.

RESULTS

Morphogenesis in Arthrobacter species

All four *Arthrobacter* species investigated underwent a transformation from coccus to rod form. *A. simplex* became rod-shaped at dilution rates above 0.05 h^{-1} , *A. crystallopoietes* at dilution rates above 0.15 h^{-1} and *A. atrocyaneus* and *A. globiformis* at dilution rates above 0.25 h^{-1} . The dilution rate at which the transition took place appeared to be a function of the maximum specific growth rate of the organism, since the higher the value for μ_m the higher the dilution rate at which the change occurred. *A. simplex* washed out of the chemostat at dilution rates above 0.1 h^{-1} , *A. crystallopoietes* at rates above 0.25 h^{-1} , *A. globiformis* at rates above 0.35 h^{-1} , and *A. atrocyaneus* at rates above 0.4 h^{-1} . The relationship of the transition point to maximum specific growth rate was also found by Luscombe & Gray (1971) when they studied the effect of temperature on this phenomenon. The highest values for μ_m occurred between 25 and 30°C , and the transition also occurred at the highest dilution rates in this temperature range. The pigmentation of *A. globiformis* also changed markedly with growth rate as the rods produced an indigoidine-type pigment and a carotenoid, while the cocci produced only a carotenoid. Cultures of rods were therefore blue and cocci yellow.

Increases in mean volume and shape (plotted as the ratio of the shorter axis to the longer axis) of *A. globiformis* occurred simultaneously within a limited range of dilution rates between 0.2 and 0.3 h^{-1} (Fig. 1). The increase in size was due to an increase in the length of one axis only. The distributions of individual bacterial volumes at a number of steady states were skewed (Fig. 2); this agrees with the observation by Powell (1956) of a J-shaped distribution of organism ages, most of the organisms in the culture being small and presumably young.

Variation of yield with dilution rate

The yield of *A. globiformis* at different dilution rates is shown both as numbers and dry weights. Numbers of organisms in steady state cultures varied at different dilution rates, reaching the highest values between 0.1 and 0.15 h^{-1} (Fig. 3). At dilution rates between 0.3 and 0.35 h^{-1} the numbers remained constant, but they fell rapidly at higher dilution rates as the culture became unstable. A semi-logarithmic plot of the rate of washout of *A. globiformis* from the chemostat at a dilution rate of 0.43 h^{-1} (Fig. 4) had a slope of 0.06 , and so μ_m must be 0.37 h^{-1} . This value is higher than the highest steady state dilution rate that can be achieved because some of the bacteria in the chemostat are non-viable (see below).

Chemostat theory predicts that organism concentration should remain constant unless the dilution rate is so low that the maintenance energy requirement becomes a relatively important part of the energy used (Veldkamp, 1968). However, this would only be true if organism size were constant over the whole range of dilution rates, which is not the case with *Arthrobacter*. Nevertheless, the yield measured in terms of dry weight should still be constant in these organisms and this proved to be so (Fig. 3). The decrease in the dry weight yield at dilution rates below 0.1 h^{-1} was less than that reported for other organisms, e.g. *Aerobacter aerogenes* (Herbert, 1959), suggesting that the energy required for maintenance of *Arthrobacter* is also smaller. Chapman (personal communication) has now determined the maintenance coefficient for *A. globiformis* NCIB10683 as 0.0088 h^{-1} , confirming this view.

Fig. 1

Fig. 1. Effect of dilution rate on the mean volume and shape of *A. globiformis* NCIB10683. ■, Shape (ratio of the lengths of the shorter axis and longer axis); ●, volume in μm^3 . For details of growth conditions see text.

Fig. 2

Fig. 2. Distribution of individual rod and coccus volumes of *A. globiformis* NCIB10683 grown at different dilution rates in a chemostat. For details see text.

Variation of viability with dilution rate

The percentage of viable organisms in the chemostat cultures was found to be almost constant at dilution rates above 0.1 h^{-1} (92 %) and was only slightly reduced even at dilution rates as low as 0.01 h^{-1} . *A. globiformis* differed markedly from *Aerobacter aerogenes* (Fig. 5). Postgate & Hunter (1962) showed and later Tempest, Herbert & Phipps (1967) confirmed, that although the viability of *A. aerogenes* was between 95 and 100 % at dilution rates above 0.1 h^{-1} , it dropped to about 65 % at dilution rates of 0.01 h^{-1} .

Tempest (1970) pointed out that when non-viable organisms were present in a culture, the viable organisms must grow at a rate greater than the dilution rate since they have to replenish the viable and non-viable organisms lost in the effluent. He showed that

$$\mu_m^* = \frac{D \ln 2}{\ln 2\alpha},$$

where μ_m^* is the maximum specific growth rate for the viable population and α is an index of viability, equal to $V + \frac{1}{2}$ where V is the fraction of viable organisms in the culture.

In the case of *A. globiformis*, the highest steady state dilution rate was 0.35 h^{-1} and the viability index 0.96. This gives a value for μ_m^* of 0.37 h^{-1} , confirming the result obtained earlier by Jannasch's (1969) washout method.

Fig. 3

Fig. 3. Effect of dilution rate on yield of *A. globiformis* NCIB10683 in terms of weight and concentration. ▲, Number of organisms/ml; ●, weight of organisms (mg/l). For details see text.

Fig. 4

Fig. 4. Rate of washout of *A. globiformis* NCIB10683 from a chemostat ($D = 0.43 \text{ h}^{-1}$). O, Rate of washout of organisms; —, predicted rate of washout in the absence of growth.

Rate of endogenous respiration

Suspensions of organisms in 0.033 M-phosphate buffer were incubated at 25 °C on a rotary shaker (150 rev./min) and sampled after 0, 10, 24 and 48 h and 7 days to determine the rate of oxygen uptake. Higher values of Q_{O_2} were obtained using oxygen polarography but, using either technique, the rates of oxygen uptake fell rapidly in both rods and cocci (Fig. 6). Gilson respirometry gave initial Q_{O_2} values of 1.74 for cocci and 7.33 for rods, but after 2 days these had both fallen to a relatively stable level of 0.45. These values are very low compared with the value of 75 obtained by Herbert (1959) for *Aerobacter aerogenes* but are similar to the values 4 to 6 reported by Sobek, Charba & Foust (1966) for *Azotobacter agilis*, another soil organism. A rapid drop in the rate of oxygen uptake during the early stages of starvation has also been reported by Mulder, Deinema, van Veen & Zevenhuizen (1962) and Boylen & Ensign (1970a) for *Arthrobacter*.

Organism numbers and viability during starvation

Measurements of numbers and viability were made after 0, 7, 14, 28, 42, 56 and 70 days in 0.033 M-phosphate buffer at 25 °C. The number of rods, expressed as a percentage of the original suspension, increased during the first 7 days but then declined (Fig. 7). The increase in numbers was accompanied by fragmentation of the rods to form cocci and after 70 days the suspension consisted almost entirely of cocci (Fig. 8). This might account for the convergence of the endogenous respiration rates of cocci and 'rods' observed after 2 to 7 days in the Gilson respirometer. The rate of reduction of total numbers in the suspension was slightly greater for rods than for cocci, despite the fragmentation process. There was a 30 % reduction in the number of cocci and a 40 % reduction in the number of 'rods' (based on the number of organisms present after 7 days) over the 70 day incubation period.

Fig. 5

Fig. 5. Steady state viabilities of *A. globiformis* NCIB10683 compared with *Aerobacter aerogenes* (data of Tempest *et al.* 1967) under carbon-limiting conditions in a chemostat. ■, *A. globiformis* NCIB10683; ●, *A. aerogenes*. For details see text.

Fig. 6

Fig. 6. Oxygen uptake by *A. globiformis* NCIB10683 after pre-incubation for different times in 0.033 M-phosphate buffer (pH 7.0) at 25 °C, measured by respirometry (○, rods; □, cocci) and by polarography (●, rods; ■, cocci). For details see text. Q_{O_2} values were corrected by assuming that only 'viable' organisms respire.

The viability of the 'rods' fell at a faster rate than that of cocci and no viable organisms were observed at the 70-day sampling time (Fig. 9). Presumably, some energy is used in the production of wall material during fragmentation of the rods and so less is available for the maintenance of the organisms. The initial increase in total numbers as rods became cocci was smaller than might be expected if each rod gave rise to at least two cocci. It must be assumed, therefore, that on formation one of each pair of cocci lysed or that rods which did not form cocci lysed.

DISCUSSION

Two theories have been put forward to explain the change of cocci to rods in arthrobacters. Krulwich & Ensign (1969) concluded that certain chemicals, e.g. succinate, were required to induce rod formation in *A. crystallopoietes* and that in the absence of inducers, rods would revert to cocci. Luscombe & Gray (1971) suggested that *A. globiformis* morphology was related to growth rate. The studies reported here have shown that growth rate was a determining factor of morphology in a range of arthrobacters, including *A. crystallo-*

Fig. 7. Changes in total numbers of *A. globiformis* NCIB10683 during incubation at 25 °C in 0.033 M-phosphate buffer (pH 7.0). ■, Rods; ●, cocci.

poietes growing on succinate. In the measurement of organism size, the drying of bacteria in films may have caused some shrinkage but it was considered that this would be relatively uniform. Boylen & Pate (1973) suggested that arthrobacters form two physiologically distinct types of cocci: those formed during exponential growth and those existing in the stationary phase. However, it seems more likely that the cocci found in stationary phase cultures were not distinct types of organism but merely older organisms formed when the rate of growth of the culture was declining.

The loss of viability of bacteria in chemostat cultures at low growth rates led Tempest, Herbert & Phipps (1967) to suggest that bacteria have a 'minimum growth rate'. At growth rates below this, a proportion of the culture becomes non-viable and the remainder continues to grow at the minimum rate. In reviewing evidence for this, Pirt (1972) suggested that below a growth rate equivalent to $0.06 \mu_m$ all or part of a population shows a loss of viability. However, *A. globiformis* showed little loss of viability at growth rates of 0.01 h^{-1} which is $0.027 \mu_m$, suggesting that if a minimum growth rate exists in *Arthrobacter* it is lower than was previously suggested for *Aerobacter* and *Escherichia*.

Bacteria grown under carbon-limiting conditions survived even in the absence of nutrients for remarkably long periods. Boylen & Ensign (1970a) found that *A. crystallopoietes* grown in batch culture survived for similar periods of time. It is not clear whether the long survival times are related to the presence of storage materials and their low rate of utilization. Boylen & Ensign (1970b) found much storage material even after long starvation, but although cocci contained more storage material than rods they survived for similar times. They suggested that rods used up their reserve materials more quickly and, in the present investigation, this may be because of the energy requirement for division of rods in nutrient-free buffer. However, Boylen & Ensign (1970b) found that their rods did not fragment and produce cocci during starvation.

Fig. 8. *A. globiformis* NCIB10683 harvested from the chemostat ($D = 0.3 \text{ h}^{-1}$) and examined either at once (a) or after incubation at 25°C in 0.033 M -phosphate buffer for 7 days (b) or for 70 days (c).

Fig. 9. Loss of viability of *A. globiformis* NCIB10683 during incubation at 25 °C in 0.033 M-phosphate buffer (pH 7.0). ■, Rods; ●, cocci.

These properties are presumably connected with the comparative success of arthrobacters in environments like soil where the ability to retain viability at low growth rates or during resting periods is important. In soil there may be other environmental conditions which are unfavourable, e.g. water availability, pH, etc. The effect of such factors on *A. globiformis* is now being investigated. Boylen & Ensign (1970a) expressed surprise that cocci did not survive longer than rods during starvation. However, the rods and cocci for those experiments were produced in different media and so their survival times are not strictly comparable. We have found that rods of *A. globiformis* do lose their viability a little faster when they are grown in the same medium as cocci. However, this is unlikely to be important to bacteria in natural environments since rods are probably only rarely produced. Gray & Williams (1971) have shown that the generation times of bacteria in soil are not likely to be less than a day, except for very short periods of time, and so most arthrobacters would grow as cocci.

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