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Continuous Culture and Synchronization of *Hyphomicrobium* sp. B-522

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Abstract. A technique was developed for synchronization of *Hyphomicrobium* sp. strain B-522. Bacteria were grown in continuous culture with methanol (0.1 %; v/v) growth limiting. Vitamin B_{12} (2.5 µg/l) was necessary to obtain steady state growth. The critical dilution rate was $D_c = 0.112$; maximum cell output occurred at $D = 0.105$ ($Dx = 30$ mg l^{-1} h^{-1}). Continuous cultures of *Hyphomicrobium* B-522 at $D = 0.110$ were used to obtain cells for synchronization experiments. Synchronization was achieved by trapping young hyphal and budding cells in a glass wool column, while the initial swarmer cells were allowed to pass through. By semi-continuously rinsing the system, newly produced swarmers could be sampled in the effluent. The mean length of these synchronous swarmer cells was 1.25 µm ($s = \pm 0.13$ µm; range 0.6 µm) as compared to 1.40 µm ($s = \pm 0.21$ µm; range 1.2 µm) for swarmer cells of the continuous culture. Division of synchronous swarmer populations was completed after 7 h; the synchronization index was 0.76.

Key words: *Hyphomicrobium* – Continuous culture – Synchronization technique – Synchronous swarmer cells

Visual morphogenetic changes are, in most bacteria, restricted to the events of cell elongation and division. But the group of budding and hyphal bacteria exhibits life cycles with drastic and obligate morphological changes, which renders them especially interesting for morphogenetic studies. Bacteria of the genera *Hyphomicrobium*, *Hyphomonas*, *Pedomicrobium*, and *Rhodomicrobium* have been studied only sporadically (Hirsch 1974a, b). The timing of morphological changes during the life cycle has been published for *Rhodomicrobium vanniellii* (Whittenbury and Dow 1977) and *Hyphomicrobium neptunium* (Wali et al. 1980). The life cycle of *Hyphomicrobium* sp. B-522 was investigated previously by the use of agar slide cultures (Hirsch and Jones 1968), and in partly synchronized swarmer populations (Moore and Hirsch 1973). The motile *Hyphomicrobium* B-522 swarmer cell sheds its flagellum during maturation and becomes immotile. Next it developes a hypha by polar outgrowth, a type of budding. When the hypha reaches a certain length, the formation of a terminal bud is initiated. The bud grows, matures (it becomes flagellated) and at last detaches from the mother cell by cross wall formation and flagellar action. Isolated swarmer populations required 9–21 h (average: 14.25 h) before the first doubling occurred (Moore and Hirsch 1973).

Recently, we were able to significantly improve cell yields and growth rates of *Hyphomicrobium* B-522 by optimizing growth conditions (Matzen and Hirsch 1982). This allowed us to reinvestigate the timing of life cycle events occurring in swarmers. However, first it was necessary to develope optimal procedures for synchronization of swarmer cells. We now report a new protocol for *Hyphomicrobium* B-522 which results in highly synchronous swarmer populations obtained from continuous culture.

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Materials and Methods

Organism. Hyphomicrobium sp. strain B-522, originally isolated from soil (Hirsch and Conti 1964a), was used throughout the tests. In liquid media this organism grows evenly suspended.

Media. A modification of medium 337 (Hirsch and Conti 1964b) was used (Matzen and Hirsch 1982). For batch growth, C-sources were 0.5 % (v/v) methanol, or 3.38 g/l methylamine · hydrochloride, respectively. For continuous culture methanol 0.1 % (v/v) was growth limiting. Chemicals used were of p.a. grade and came from Merck (Darmstadt, FRG), unless otherwise stated.

Growth Measurements. OD_{650} and protein measurements were performed as described previously (Matzen and Hirsch 1982). For dry weight determinations at least 10 ml of a cell suspension were collected on preweighed 0.2 µm membrane filters (Schleicher & Schüll, Dassel, FRG). The washed filters were dried at 105° C.

Colony forming units (cfu) were counted after 1–2 weeks by spreading appropriate sample dilutions on 337 plates (1.8 % Bacto Agar, Difco, Detroit, 3.38 g/l $CH_3NH_2 \cdot HCl$).

Continuous Culture. A "Laborfermentor Biostat" (Braun, Melsungen, FRG) with a working volume of 1.1 l was used to grow *Hyphomicrobium* B-522. The cultures were stirred at 350 rpm at 30° C. Moistened filter-sterilized air (Whatman filter tubes, grade 10, Maidstone, England) was supplied with an external pump at a rate of 1.4 l/h. The pH was kept constant at 7.2. Cultures were initiated by inoculating with 10 ml of a log-phase batch culture and within 2–3 days, fresh medium was fed at the desired constant rate. After introduction of new growth conditions a volume turnover of at least three times was required to reach the steady state again. Growth parameters were determined according to Herbert et al. (1956) and Tempest (1970).

Synchronization Procedure. Synchronization of *Hyphomicrobium* B-522 was achieved in the following way. We

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used a glass column (4.1 cm inner diameter; 39.5 cm length) filled with 100 g of thouroughly washed and dried glass wool. Alternatively, glass wool p.a. (0.05–0.020 mm fiber strength; Merck, Darmstadt, FRG) was used. On top of the glass wool a 2 cm layer of glass beads (2 mm ∅) was placed. For sampling, the column was connected with a 250 ml Erlenmeyer flask by means of silicone tubing and plug. The whole apparatus, which resembled that one used by Whittenbury and Dow (1977), was autoclaved and prewarmed to 30° C before use.

The column was inoculated with 150–200 ml of an asynchronous *Hyphomicrobium* continuous culture. After 10 min the system was rinsed with 400 ml of fresh medium 337 containing 0.1% of methanol. Rinsing of the column with 150 ml medium was then regularly repeated every 25 min. After a total of at least 3 h, two rinses of a 5 min interval freed the column completely from unappendaged cells. With a last rinse (150–200 ml) 20 min later, only newly born swarmer cells were collected, thus representing the synchronous suspension ready for further investigations.

Determination of Synchrony. Hyphomicrobium B-522 swarmer cells differ in length depending on their age (Moore and Hirsch 1973). A relatively homogeneous distribution of average cell lengths in a synchronized culture should, therefore, indicate the degree of synchrony. We compared the average length of swarmer cells of column-collected suspensions with that of swarmers from the original continuous culture. Measurements were made on enlarged micrographs of cells spread on agar-coated slides (1.8% Bacto Agar).

To monitor synchronous growth, colony forming units of swarmer populations were counted. The culture grew aerated and shaken in a 30° C waterbath and was supplied with methanol in the gas phase. Every hour 0.2% (v/v) methanol were added to the gas moistener. A synchronization index was calculated from viable counts according to Wolosker and de Almeida (1979), who modified the original Scherbaum index (Scherbaum 1964).

Results

Continuous Culture

Continuous culture of *Hyphomicrobium* B-522 provided the most active and dependable cells for synchronization experiments. When medium 337 with methanol (0.1% v/v) growth limiting was used, the culture was washed out at a dilution rate of $D = 0.034$ (one volume change every 29.4 h) (Fig. 1a). When, however, 2.5 µg/l of vitamin B_{12} were added to the same medium, steady state conditions were reached fast and easily at $D = 0.034$ (Fig. 1b). The growth parameters of these continuous cultures with varying dilution rates are shown in Fig. 2. The critical dilution rate was $D_c = 0.112$. At a dilution rate of $D = 0.105$ the maximum output of cells was $Dx = 30$ mg l^{-1} h^{-1}. For synchronization procedures, continuous steady state cultures grown at a dilution rate of $D = 0.110$ were used.

Synchronization of Hyphomicrobium B-522

We searched for a synchronization technique with as little physiological stress for the bacteria as possible; the method should provide only newly born swarmer cells. Therefore, filtration of an asynchronous, continuous culture through a column filled with glass wool was tried as was similarly done by Whittenbury and Dow (1977). Cells without hyphae thus could pass through, while cells with hyphae and buds were retained by the glass wool. When such a column then was rinsed with new medium, all unappendaged cells disappeared while mother cells continued to grow and liberated new swarmers. An optimal protocol of such a semi-continuous culture was developed (see Materials and Methods). After several rinses, the last effluent with swarmer cells liberated during the last 20 min interval, was collected. It usually

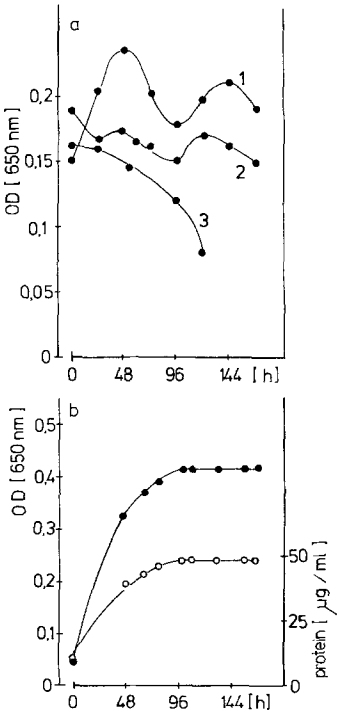

Fig. 1. (a) Growth of *Hyphomicrobium* sp. strain B-522 at different dilution rates using medium "337" (Hirsch and Conti 1964b) with 0.1% methanol. *1*) $D = 0.021$; *2*) $D = 0.030$; *3*) $D = 0.034$. Cells did not grow in steady state. (**b**) *Hyphomicrobium* B-522 steady state growth at a dilution rate of $D = 0.034$. The medium used here was a modification of "337" containing 2.5 µg/l vitamin B_{12}. ●——●: OD_{650}; ○——○: protein

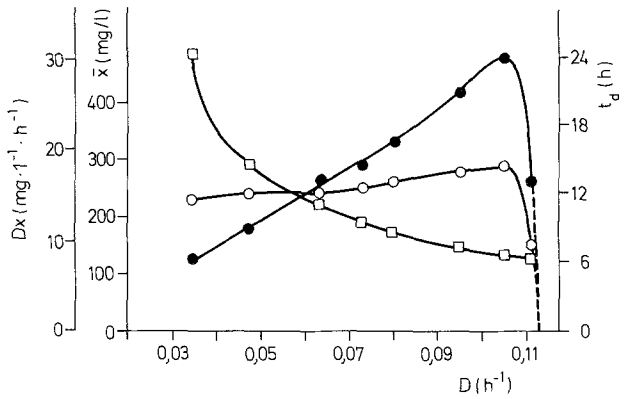

Fig. 2. Steady state parameters of *Hyphomicrobium* B-522 growing in continuous culture at various dilution rates (D). ○——○ cell concentration $\bar{x}$ (mg/l); ●——● out-put of cells Dx (mg l^{-1} h^{-1}); □——□ doubling time t (h)

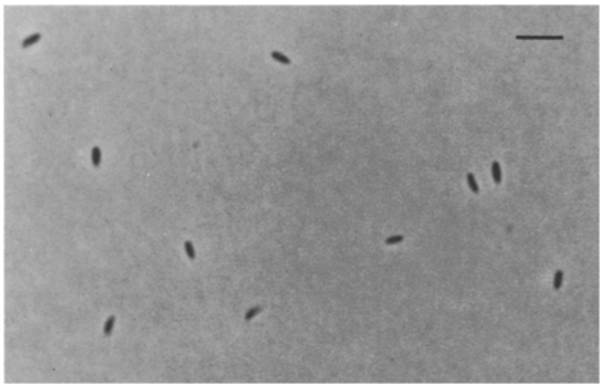

Fig. 3. *Hyphomicrobium* swarmer cells produced by immobilized cells in the glass wool column during a 20 min time interval. Bar represents 3 µm

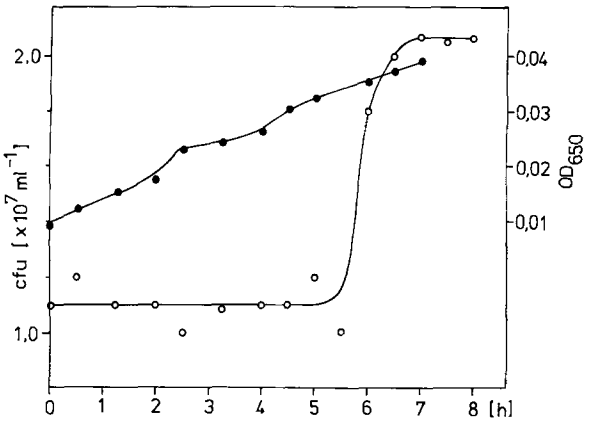

Fig. 5. Changes of OD_{650} (●——●) and stepwise increase of viable counts (cfu) (○——○) in a synchronous culture of *Hyphomicrobium* B-522

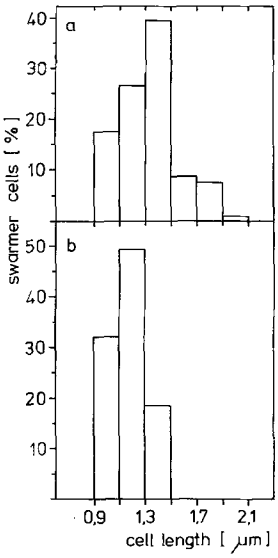

Fig. 4. Comparison of lengths of *Hyphomicrobium* B-522 swarmer cells from continuous culture (**a**) or synchronous population (**b**). Number of cells measured were 218 (a) or 304 (b), respectively

contained $1-2 \times 10^7$ viable cells per ml; contamination with hyphal cells was negligible (0–2%; Fig. 3). This synchronization technique could be varied to a certain degree: we used up to 400 ml of a continuous culture to inoculate the column. The quantity of medium for rinsing had to be raised correspondingly. The times at which the column could be rinsed, could not be changed significantly without a decrease in swarmer production. Especially, the period of 3 h before sampling the swarmer cells could not be shortened.

Characterization of Synchronous Hyphomicrobium Swarmer Cells

a) Swarmer cell length. As the length of B-522 swarmer cells increases with age and stage of maturation (Moore and Hirsch 1973), measurements of the cell length and comparison with swarmers of the original continuous culture were expected to provide information on the degree of synchrony. Therefore, a total of 522 swarmers was measured as shown in Fig. 4. The mean length of 304 synchronous swarmer cells was 1.25 µm with a range of 0.6 µm and a standard deviation of ± 0.13 µm. For comparison, 218 swarmers of the original continuous culture had a mean length of 1.40 µm with a range of 1.2 µm and a standard deviation of ± 0.21 µm. Statistics show that both population size values are highly significant within the 99,9% confidence limits.

b) Growth of swarmer populations. Swarmer populations synchronized by size and time of formation as described above, were grown with aeration and shaking. Samples taken at regular time intervals were checked for turbidity (OD_{650}) and changes of cfu as shown in Fig. 5. Starting with a density of 1.1×10^7 swarmer cells per ml, new swarmer production and division was completed after 7 h with 2.07×10^7 cells per ml. From these data the synchronization index was calculated according to Wolosker and de Almeida (1979). It was S.I. = 0.76. The OD_{650} of synchronous cultures increased in 3 distinct phases. An increase of OD_{650} right from the beginning of the culture suggested that there was no lag in the development of *Hyphomicrobium* B-522 swarmer cells.

One critical point should be mentioned: *Hyphomicrobium* B-522 swarmers were very sensitive to changes of environmental conditions. Significant lag periods were observed before normal development when the temperature was not 30° C. Contact with glass ware not prewarmed was sufficient to cause a lag. Likewise, if the methanol concentration was increased to 0.2% (v/v) or even higher, lag periods of more than 6 h were observed. Wilkinson and Hamer (1972) also noted the influence of the initial methanol concentration on the growth rate of their *Hyphomicrobium* sp.

Discussion

The synchronization degree desired is dependent on the purpose of the investigation. In order to study structural differences between hyphae and nonhyphal cells, one simply needs to separate the two developmental stages by filtration through membranes with a porosity of 1.2 µm, as was done by Moore and Hirsch (1973). But to study timing of morphogenetic events of these bacteria, a higher degree of synchrony is required. Hirsch (1974) pointed out, that the synchrony obtained with *Hyphomicrobium* B-522 was only "fair", hence a more satisfying method had to be developed. Highly synchronous *Hyphomicrobium* swarmer populations should thus

consist of the first offspring of young cells coming from an already quite homogeneous population. That could be achieved by employing cell populations taken from a continuous culture where volume changes remove older cells. Further methods unsuccessfully applied by us or others for the synchronization of *Hyphomicrobium* B-522 were (1) series of filtration steps, (2) differential centrifugation, (3) the use of nalidixic acid (H. Boltes, pers. commun.), (4) sucrose gradients (H. Boltes, pers. commun.), and (5) centrifugation on glycerol shelfs (T. Hempel, pers. commun.).

In the case of *Rhodomicrobium vannielii*, a phototrophic hyphal and budding bacterium, Whittenbury and Dow (1977) achieved synchronization by arresting swarmer cell maturation with a light intensity decrease during the late exponential phase of their culture. After filtering through glass wool (which retained hyphal cells), swarmers passing through were then incubated under suitable light conditions to commence synchronous growth. We did not find such an environmental trigger that could have arrested *Hyphomicrobium* B-522 swarmer development without serious physiological disturbances. Therefore, we resorted to selectively cultivating hyphal cells in a glass wool-packed column and collected newly formed swarmer cells as they were liberated.

By comparing the lengths of synchronized swarmer cells with those of swarmers from the asynchronous continuous culture, we demonstrated successfully a selection of the youngest and smallest cells. Their mean length and narrow size range, and a comparison with the respective data of Moore and Hirsch (1973), confirmed the homogeneity of this swarmer cell population. Viable counts (cfu) of such a swarmer population showed the high degree of synchronization during further development. This synchrony compared favorably to that of swarmer populations used by Moore and Hirsch (1973) or to that obtained in populations of *Hyphomicrobium (Hyphomonas) neptunium* by Wali et al. (1980). The synchronization index of 0.76 also compared favorably to indices obtained for *Escherichia coli* K 12 (0.8), *Escherichia coli* B (0.7), or *Bacillus subtilis* 168 (0.55), as reported by Dwerk et al. (1980).

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