Simplified Vegetative Cell Cycle of *Rhodomicrobium vannielii*

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Homogeneous swarm cell populations of *Rhodomicrobium vannielii*, selected from the late-exponential phase of growth, when cultured photoheterotrophically on solid medium yield two distinct colony types. These reflect two vegetative cell cycle expressions, one composed of the characteristic multicellular complexes, the other of a 'simplified' cell cycle composed of prosthecae, reproductive cells and motile swarm cells. This simplified cell cycle has been characterized and its derivation is discussed. The implication of the CO₂ concentration as a factor in the control of expression of this cell cycle is also considered.

INTRODUCTION

Rhodomicrobium vannielii, a prosthecae 'budding' bacterium of the *Rhodospirillaceae*, was isolated by Duchow & Douglas (1949) and characterized as ovoid cells (2 to $3 \times 1 \mu\text{m}$) linked by cellular filaments or prosthecae (0.3×2 to $30 \mu\text{m}$) (Staley, 1968) which may be branched giving rise to complex multicellular arrays (Murray & Douglas, 1950). Subsequently, motile cells were observed (Douglas & Wolfe, 1959) and, more recently, angular heat-resistant exospores (Gorlenko, 1969). The growth, formation and reproduction of each of the cell types expressed by *R. vannielii* has been documented by Whittenbury & Dow (1977).

Cell growth in *R. vannielii* is by obligate asymmetric polar growth ('budding'). Daughter cell formation follows filament synthesis and, on completion, cell division is by the formation of a plug within the filament. The cells remain physically attached, ultimately generating the characteristic multicellular arrays. The peritrichously flagellated swarm cells produced during the exponential phase of growth are formed sequentially from the filament tips of the multicellular complexes, cell division in this instance being by binary fission. There are therefore two modes of cell separation expressed by *R. vannielii* – binary fission, and the formation of unique and characteristic plugs within the filaments.

We describe here a 'simplified' vegetative cell cycle in which the formation of multicellular complexes is excluded, cell separation being exclusively by binary fission. The derivation and control of expression of this simplified cell cycle is discussed.

METHODS

Organism and growth conditions. *Rhodomicrobium vannielii*, strain Rm5, was from the culture collection at the Department of Biological Sciences, University of Warwick. It was grown in medium containing (g l⁻¹): sodium pyruvate, 1.5; sodium hydrogen malate, 1.5; NH₄Cl, 0.5; MgSO₄·7H₂O, 0.4; NaCl, 0.4; CaCl₂, 0.05. The pH was adjusted to 6.8 with KOH prior to autoclaving, and 50 ml 0.1 M-NaH₂PO₄/Na₂HPO₄ buffer, pH 6.8, was added aseptically after autoclaving. Medium (100 ml) was held in 250 ml Quickfit conical flasks (B19/26) fitted with Suba-seals (W. Freeman & Co., Barnsley, Yorks.). Flasks were gassed with O₂-free N₂ and incubated with shaking at 30 °C with incident light intensities (tungsten lamps) ranging from 1000 to 3000 lx.

Large-scale batch photoheterotrophic cultures of *R. vannielii* were grown in a 15 l batch fermenter (L.H. Engineering Co., Stoke Poges, Bucks.) at 30 °C with an incident light intensity of 6000 lx.

Agar plates [formed by adding agar at 1.5% (w/v) to the above medium] were sealed in autoclavable nylon bags (Portex, Hythe, Kent) using a Calor 'Easyseal' unit and gassed with O₂-free N₂ via syringe needles. Residual O₂ was removed by including in the bag 25 ml saturated pyrogallol to which was added 25 ml alkali [15% (w/v) K₂CO₃ plus 10% (w/v) NaOH] after gassing. Incubation was at 30 °C with an incident light intensity of 1500 lx from tungsten lamps.

Slide cultures. Agar medium was boiled for 15 min under a continuous flow of O₂-free N₂, and the NaH₂PO₄/Na₂HPO₄ buffer was added aseptically when the medium had cooled below 65 °C. Two drops of this medium (about 0.2 ml) were placed on a sterile microscope slide in a sandwich box which was continuously gassed with O₂-free N₂. A sterile coverslip was applied to produce a thin flat film of agar. After 5 to 10 min the cover slip was removed and the agar was inoculated with 5 µl of a suspension of exponentially growing cells. A fresh sterile coverslip was placed on the agar and sealed on to the slide using a petroleum jelly/paraffin wax mixture. Slide cultures were incubated at 30 °C on the microscope stage with an incident light intensity of 1500 lx from a tungsten lamp.

Swarm cell selection. Homogeneous populations of swarm cells were obtained by the glass-wool column technique of Whittenbury & Dow (1977).

Light microscopy. All light microscopy was carried out using Olympus EHT phase contrast microscopes fitted with Olympus PM6 camera units (Olympus Optical Co., Tokyo, Japan). Photomicrographs were taken on Kodak Panatomic X film.

Electron microscopy. Gold/palladium was used for all metal shadowing (angles 15° to 45°). The fixation procedure of Ryter & Kellenberger (1958) was followed for ultrathin sections. These were post-stained for 20 min in 0.5% (w/v) uranyl acetate followed by 5 min in 0.1% (w/v) lead citrate in 0.1 M-NaOH. All preparations were observed using an AEI Corinth 275 electron microscope.

Measurement of N₂, O₂ and CO₂. Dissolved N₂, O₂ and CO₂ were measured by the method described by Phillips & Johnson (1961). Gas analysis was performed after the method of Herbert & Holding (1972) using a gas chromatograph fitted with a katharometer detector head.

Measurement of CO₂ assimilation by intact organisms. Cells were harvested by centrifugation, washed once and resuspended in complete growth medium, gassed with O₂-free N₂ and incubated at 30 °C with an incident light intensity of 2000 lx from tungsten lamps. NaHCO₃, to give 5 mM, was then added followed after 10 min by sterile NaH¹⁴CO₃ [1 µCi ml⁻¹, sp.act. 656 µCi mg⁻¹ (24.3 MBq mg⁻¹)]. At intervals, 1 ml samples were removed, mixed with 5 ml cold 5% (w/v) trichloroacetic acid and filtered through Whatman GF/C glass filters. The filters were washed twice with 10 ml cold 5% (w/v) trichloroacetic acid, dried overnight at 60 °C and then counted by immersion in scintillation fluid [36 g 2,5-diphenyloxazole and 0.45 g 1,4-bis-(5-phenyloxazol-2-yl)benzene in 6 l toluene plus 3 l Triton X-100] using a liquid scintillation spectrometer.

Dry weight estimations. Culture dry weights were measured by filtering suitable volumes through pre-weighed 0.4 µm membrane filters (Oxoid). These were then dried at 60 °C and allowed to cool in a desiccator before weighing. On average, 20 to 30 mg dry weight of cells were filtered.

RESULTS AND DISCUSSION

Swarm cell production by *R. vannielii* occurred throughout the exponential phase of growth (Fig. 1). However, as the culture entered the late-exponential to early-stationary phase of growth, the number of swarm cells present reached a steady value, there being no apparent further increase in cell numbers. When homogeneous swarm cell populations selected from this part of the growth cycle (Whittenbury & Dow, 1977) were cultured photoheterotrophically on solid medium, they gave two colony types (Fig. 2*a*). The first, a rough irregular colony (R), was by far the most common and was composed of multicellular arrays, i.e. the characteristic vegetative cell expression of *R. vannielii* (Fig. 2*b*). The second, a soft mucoid regular colony (S), was composed of single motile cells (swarm or daughter cells), single cells possessing prosthecae (mother cells) and prosthecae cells with daughter cells forming at the prostheca tip (Fig. 2*c*); there were no multicellular arrays. The latter colony type occurred with a frequency of 1 to 2% and constitutes a simplified vegetative cell cycle expression of *R. vannielii*.

Fig. 1. Growth curve of *R. vannielii* (Rm5) grown photoheterotrophically at 30 °C with an incident light intensity of 2500 lx. Culture absorbance at 540 nm (□); swarm cell viable count ml⁻¹ (■); culture pH (●); percentage of CO₂ in solution (○) [CO₂, O₂ and N₂ tensions were measured in solution by the method of Phillips & Johnson (1961) and the CO₂ tension was expressed as a percentage of the total CO₂ plus O₂ plus N₂].

Fig. 2. (a) Colony types expressed by *R. vannielii* (Rm5) grown photoheterotrophically on agar plates; R, rough irregular colonies which are difficult to disrupt; S, smooth mucoid colonies which are easily disrupted. (b) Electron micrograph (gold/palladium shadowed) of a *R. vannielii* multi-cellular array; bar marker represents 2.5 μm. (c) Phase contrast photomicrograph of *R. vannielii* cells expressing the simplified cell cycle; bar marker represents 10 μm.

Simplified vegetative cell cycle

Slide culture (Fig. 3) and electron microscopic studies (Fig. 4) revealed that cell growth and reproduction in this cell cycle resembles that of *Hyphomicrobium* (Leifson, 1964; Hirsch, 1974; Harder & Attwood, 1978), *Rhodopseudomonas palustris* and *Rhodopseudomonas viridis* (Whittenbury & McLee, 1967). The immature swarm cell undergoes a period of obligate differentiation, which includes flagella shedding and synthesis of a prostheca, to become a mature reproductive mother cell. Daughter cell formation is by 'budding' or, more specifically, by asymmetric polar growth from the prostheca tip. On completion of daughter cell synthesis, cell division is by binary fission and yields a prosthecate mother cell and a motile, immature daughter cell. The mother cell can undertake the formation of a second daughter cell immediately after synthesis of a short segment of prostheca (prosthecae are only ever formed at one pole). The daughter cell, however, goes through the differentiation sequence described above. The consequences of asymmetric polar growth and of a dimorphic cell cycle have been discussed by Whittenbury & Dow (1977).

The developmental sequence from swarm cell to mature reproductive mother cell is indistinguishable from the development of swarm cells selected from a culture grown as multicellular arrays (Whittenbury & Dow, 1977), the only exceptions being (i) growth is always from one pole whereas with swarm cells derived from multicellular arrays prosthecae may be formed from both poles and (ii) division is by binary fission and not by plug formation in the prostheca.

Ultrastructure

Cells of the simplified cell cycle possessed the characteristic lamellae membrane system of *R. vannielii* (Conti & Hirsch, 1965; Whittenbury & Dow, 1977) and of *Rhodopseudomonas palustris* (Whittenbury & McLee, 1967; Dow *et al.*, 1976). Their responses to high and low light intensities were similar, i.e. high light intensity, low content of photosynthetic membrane; low light intensity, high content of photosynthetic membrane.

Growth

When grown in liquid medium under an atmosphere of O₂-free N₂ at 30 °C with an incident light intensity of 2000 lx, the doubling time of cells composing the multicellular arrays was 5.3 h and that of cells expressing the simplified cell cycle was 5.0 h. The addition of 5% (v/v) CO₂ to the atmosphere increased the growth rate, giving doubling times of 5.0 and 4.7 h, respectively. There was no significant difference in ¹⁴CO₂ incorporation into cellular material by cells constituting either cell cycle when grown under identical conditions.

The only consistent differences between the two cycles relate to the pH changes apparent during batch growth (Fig. 5) and the amount of carbon assimilated into cell material (Fig. 6), the simplified cell cycle yielding a greater cell mass per gram of substrate carbon than the multicellular array cycle. The significantly higher level of CO₂ apparently produced during the simplified cell cycle (Fig. 5) is a reflection of the lower pH in the later stages of batch growth, i.e. pH markedly affects the CO₂/HCO₃⁻ equilibrium and consequently the CO₂ solubility.

Effect of light intensity on growth

An increase in the incident light intensity increased the growth rate irrespective of the cell cycle expression (Fig. 7). Light saturation, i.e. the light intensity above which any growth rate increase is suppressed, was approached but not reached and in the dark the doubling time approached infinity, i.e. the cells were unable to grow anaerobically in the dark. In Fig. 7, a straight line can be drawn through the points corresponding to 1000 lx and greater (linear regression coefficient of 0.947) giving a theoretical minimum doubling time of 4.9 h for the stated cultural conditions. In addition, however, a straight line (linear

Fig. 3. Sequential phase contrast photomicrographs of a *R. vannielii* slide culture growing photoheterotrophically at 30 °C with an incident light intensity of 2500 lx. Bar marker represents 10 μm .

Fig. 4. Electron micrographs (gold/palladium shadowed) depicting morphogenesis and differentiation in *R. vannielii* expressing the simplified cell cycle: (a) peritrichously flagellated swarm cell; (b to d) swarm cell maturation; (e to h) reproductive cycle. Bar marker represents 1 μm .

Fig. 5

Fig. 6

Fig. 5. pH changes and CO_2 production by *R. vannielii* grown photoheterotrophically in batch culture at 30 °C with an incident light intensity of 2500 lx. pH: simplified cell cycle (■), multicellular arrays (□). Percentage of CO_2 in gas atmosphere: simplified cell cycle (●), multicellular arrays (○) [CO_2 , O_2 and N_2 tensions were measured in the gas atmosphere within 250 ml flasks by the method of Herbert & Holding (1972) and the CO_2 tension was expressed as a percentage of the total CO_2 plus O_2 plus N_2].

Fig. 6. End-point analysis of *R. vannielii* grown on medium containing carbon substrate at different concentrations. Incubation, in 250 ml flasks, was at 30 °C with an incident light intensity of 1000 lx. On reaching the stationary phase of growth, the dry weight (50 ml culture), absorbance (540 nm) and pH were determined for each flask. Over the range of carbon substrate concentrations tested, cultures were carbon-limited, the amount of growth being directly proportional to the substrate carbon concentration. Plots of absorbance against dry weight were linear. Simplified cell cycle (●), multicellular arrays (○).

Fig. 7. Double reciprocal plot of doubling time of *R. vannielii* grown at 30 °C against incident light intensity. Exponential phase cells of *R. vannielii* were resuspended in fresh medium in stoppered glass cuvettes and growth was followed by measuring the absorbance at 540 nm. Different light intensities were obtained by placing the cultures at various distances from a 100 W tungsten lamp; the maximum incident light intensity tested was 2400 lx.

regression coefficient of 0.988) can be drawn through the remaining points indicating that below 1000 lx the doubling time is limited by a factor other than light.

Derivation of the simplified cell cycle

Whittenbury & Dow (1977) have shown that *R. vannielii* swarm cells are formed sequentially from the filament tips of the multicellular arrays, and that the separation process (binary fission) is distinct from plug formation evident in these complexes. It appears, therefore, that the simplified cell cycle is a mechanism for swarm cell production, i.e. constitutive for swarm cell formation, multicellular complexes being repressed (Fig. 8).

Fig. 8. Diagrammatic representation of the cell cycle expressions of *Rhodomicrobium* indicating the derivation of the simplified cell cycle.

Control of vegetative cell cycle expression

On inoculation into fresh medium, an exponential culture of *R. vannielii* (giving expression to multicellular arrays) showed an initial marked decrease in swarm cell production (Fig. 1), the lag phase being characterized almost exclusively by multicellular arrays. From the data available it is most probable that this cessation of swarm cell formation was a consequence of the increased light intensity (decrease in turbidity on inoculation) and the lowered CO_2 concentration. As growth proceeded, however, the CO_2 concentration increased and the light intensity decreased (due to the increase in culture turbidity). These environmental factors led to the initiation of swarm cell formation which then continued throughout exponential growth. Before the onset of the stationary phase the swarm cell number became constant.

When swarm cells selected from points throughout the batch growth curve were plated on solid media and incubated anaerobically in the light, they gave rise to the smooth colony type, indicative of the simplified cell cycle, only towards the late-exponential/early-stationary phase of growth, i.e. at a point coincident with the onset of low light intensity (high culture turbidity) and high CO_2 concentration. This colony type constituted between 1 and 2% of the total population.

When grown in continuous culture under high CO_2 tensions (pH controlled by the addition of gaseous CO_2), *R. vannielii* gave expression exclusively to the simplified cell cycle. However, on reduction of the CO_2 tension (pH controlled by the addition of 0.1 M-HCl) expression of the simplified cell cycle was repressed and the culture reverted to one composed of multicellular arrays (unpublished result). In this series of experiments the factor most influential on vegetative cell cycle expression was the CO_2 tension.

Cells expressing the simplified cell cycle could be maintained in this form indefinitely provided the culture was re-inoculated into fresh medium during the mid-exponential phase of growth. However, subculturing cells expressing the simplified cell cycle into conditions conducive to expression of multicellular arrays (low CO_2 , high light intensity) did not give immediate reversion of the cell cycle expressed; this took a further two to three subcultures and was most rapid when inoculation was from stationary phase cultures.

Fig. 9. Micrographs of the 'tiny' cells produced by *R. vannielii*, expressing the simplified cell cycle, on entering the late-exponential/early-stationary phase of growth: (a, b) gold/palladium shadowed electron micrographs; (c) phase contrast photomicrograph ('tiny' cells are arrowed). Bar markers represent $0.5\ \mu\text{m}$ in (a), $1\ \mu\text{m}$ in (b) and $10\ \mu\text{m}$ in (c).

The failure to obtain reversion of expression immediately on alteration of the environmental conditions pointed to the possibility that the simplified cell cycle was a consequence of mutation. However, the frequency with which the cells giving expression to the simplified cell cycle arose, the reversion rate, and the correlation of the expression of the simplified cell cycle with the CO_2 tension indicate that this was not the case.

The role of CO_2 tension and light intensity in the modification of cell cycle expression in *R. vannielii* remains unclear. However, the involvement of CO_2 as a probable trigger for cellular differentiation in other organisms has been widely implicated (Lones & Peacock, 1960; Bartnicki-Garcia & Nickerson, 1962; Roberts, 1964; Elmer & Nickerson, 1970).

Rhodomicrobium vannielii presents a unique model system in which an obligate differentiation sequence, environmentally induced modification of vegetative cell cycle expression, and formation of a resting cell (exospores) are integrated. The importance of the switch in vegetative cell cycle expression lies in its potential to help elucidate the mechanism(s) of environmentally induced changes in cellular expression.

'Tiny' cells

When grown as the simplified cell cycle, *R. vannielii* did not form exospores. However, on reaching the late-exponential/early-stationary phase of growth such cultures contained cells of unusual but characteristic phenotype in large numbers (Fig. 9). These cells were not resistant to adverse environmental stress and their physiological capabilities have yet to be determined. They did not appear in cultures growing as multicellular arrays; they were unique to the simplified cell cycle.

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