Nuclear Apparatus of *Hyphomicrobium*  

RICHARD L. MOORE and PETER HIRSCH  

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823  

Received for publication 17 September 1973

The nuclear apparatus of *Hyphomicrobium* sp. strain B-522 is examined by various microscopy and radiolabeling techniques to determine its behavior during the reproductive cycle of these bacteria. The young, swarmer cell contains a single nucleoid comprised of a deoxyribonucleic acid (DNA) molecule with a molecular weight of $3.1 \times 10^9$. After development of the swarmer into a mature mother cell with a hypha and bud, the nucleoid replicates and separates into two daughter nucleoids during the initial stages of bud formation. After further development of the bud, one of the daughter nucleoids in the mother cell is rapidly transferred through the hypha to the bud. Half of the old DNA strands pass to each consecutive generation of daughter cells, but only 43% of the stable ribonucleic acid is transferred. The role which the hypha plays in the developmental cycle of these bacteria is discussed, and a mechanism for nuclear transfer is proposed.

---

Reproduction in hyphal and budding bacteria is a complex process. Budding precedes the process of fission, and, contrary to most other bacteria, fission results in formation of two unequal daughter cells. The life cycle begins with a single, motile cell called a swarmer. During development, this cell produces a long filamentous outgrowth termed a hypha and loses its motility. A bud is formed at the tip of this hypha. The bud grows in size, becomes motile, and breaks free as a new swarmer cell. The mature mother cell may then continue to produce more daughter cells after short periods of additional stalk growth (P. Hirsch, and H. E. Jones, Bacteriol. Proc., p. 44, 1968). In some strains, the buds may remain attached to the mother cell hypha, and a number of cells are held together by hyphae. Although this is particularly true for *Rhodomicrobium* spp., it also occurs occasionally in other hyphal, budding bacteria. A reproductive cycle of this type has been reported for *Rhodomicrobium vannielli* (5, 6, 16), *Hyphomicrobium neptunium* (11), *Hyphomicrobium* spp. (8, 21), *Hyphomicrobium* sp. strain B-522 (14) and *Hyphononas polymorpha* (18).

Previous observations on the nucleoids of cells of various ages present in cultures of *R. vannielli* (16) and *Hyphomicrobium* sp. (21) led to the conclusion that nucleoids were able to pass from the mother cell to the bud through the long hypha which connected them. The manner in which this transfer occurred was uncertain. This question is examined in the present study by observing the behavior and appearance of nucleoids during various stages in the reproductive cycle of *Hyphomicrobium* sp. strain B-522.

---

**MATERIALS AND METHODS**

**Bacteria and media.** *Hyphomicrobium* sp. strain B-522 came from the culture collection of one of the authors (P.H.). A modification of medium 337 (8) as described by Moore and Hirsch (14) was used throughout this study. Solid medium contained 1.8% Noble agar (Difco, Detroit, Mich.). Methylamine hydrochloride, at a concentration of 0.34%, was the sole added carbon source. Incubation of cultures was carried out in 250-ml naphelometer flasks in a reciprocating water bath-incubator and at 30 C.

For electron microscopy, cells were prefixed with glutaraldehyde-phosphate buffer and postfixed by the method of Kellenberger et al. (10). The preparations were examined with a Phillips EM-300 electron microscope. Phase-contrast and bright-field photomicrographs were taken on Kodak Plus-X film with a Zeiss Photomicroscope II.

**Autoradiography.** Cells were grown in medium containing generally labeled $10 \mu$Ci of $^3H$-thymidine (1 mCi/ml; 36 $\mu$g/ml) or $^3H$-adenine (1 mCi/ml; 23 $\mu$g/ml) per ml. All radioactive materials were from New England Nuclear Corp., Boston, Mass. Cells were harvested by filtration, washed thoroughly with unlabeled medium, and dried onto glass slides. Where $^3H$-adenine was used, ribonucleic acid (RNA) was removed by alkaline hydrolysis as described below.
The slides were dipped into Kodak NTB 3 photographic emulsion held at 43 to 44 C. They were stored at 4 C in desiccators in light-tight boxes containing desiccant. The slides were developed in Kodak D-76 for 3 min at 24 C after exposure times varying from 5 to 100 days.

DNA content. Cells were grown for four generations in medium containing 20 μg of adenine-8-14C (3.44 μCi/mg) per ml. Swarmer cells were isolated by the method described previously (14), and their titers were determined by direct counts with a Petroff-Hausser cell counting chamber. The counts per minute per cell of adenine-8-14C incorporated into deoxyribonucleic acid (DNA) were determined as described below. DNA was extracted from these cells by the method described previously (13), and the specific activity was determined. The counts per minute per cell, divided by the counts per minute per gram of DNA indicated the quantity of DNA per cell to be 5.57 x 10^-14 g.

The size of the genome was determined from the rate of reassociation of disassociated DNA (1) by the spectrophotometric method described previously (13). DNA was prepared from Escherichia coli and Hyphomicrobium sp. strain B-522 cells which had been grown to late log phase. The DNA was passed through a French pressure cell at 15,000 lb/in.2 and heat denatured to give single-stranded DNA fragments of approximately 300,000 daltons. Reassociation was carried out at 70 C in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7).

The number of DNA molecules (nDNA) per cell was determined from the relationship: nDNA per cell = [(g DNA/cell) (Avogadro's no.)]/ (molecular weight of DNA).

The number of nucleoids per cell was determined by direct counts of Giemsa-stained cells. Cell growth in the sample was first halted by addition of Na2SO4 (0.1 M final concentration), and the samples then were dried on clean, glass slides. These were kept in 70% ethanol until used. The slides were then rinsed with tap water, treated with 1 N HCl at 60 C for 15 min, and again rinsed with tap water before staining for 5 min with dilute Giemsa stain (17).

Macromolecular synthesis. Protein, RNA, and DNA were uniformly radiolabeled with L-[14C]isoleucine (0.01 mCi/ml, 48 μg/ml), 14C-uracil (1 mCi/ml, 11.4 μg/ml), and 14C-adenine (1 mCi/ml, 22.6 μg/ml) (or adenine-8-14C [0.05 mCi/ml, 1.7 mg/ml]), respectively. The radiochemicals were diluted with the appropriate unlabeled compounds before use. For protein or nucleic acid measurements 1-ml samples of culture were incubated with an equal volume of cold 20% trichloroacetic acid. After 30 min at 4 C, the samples were filtered through 0.45-μm pore size filters (Millipore Corp., Bedford, Mass.), washed with cold 5% trichloroacetic acid, dried, and counted in toluene liquid scintillation fluid on a Beckman LS-100 scintillation counter.

Radiolabeled RNA was removed from the adenine-labeled DNA by treating the trichloroacetic acid precipitate with 0.5 ml of 1 N NaOH at 60 C for 1 h. In controls, less than 0.1% of the trichloroacetic acid-precipitable counts per minute in RNA remained after this treatment, whereas the DNA counts per minute remained unaffected. The samples were then neutralized with 1 N HCl and treated as described above. In some cases the quantity of radiolabel in RNA was determined from the difference between total trichloroacetic acid-precipitable material and alkali-stable material.

DNA and RNA transfer from mother to daughter cell. Cells were grown for four generations in medium containing 20 mCi of adenine-8-14C (3.44 mCi/mg) per ml. The swarmer cells were then isolated as described previously (14), washed, and transferred to fresh medium without labeled adenine to give a final concentration of 8 x 107 cells per ml. After 8 h of incubation, the swarmer cells had produced hyphae and buds. By 10 h, 39% of the buds had matured and separated from the mother cell (14). The new daughter cells were isolated by filtration of the culture through a sterile 3-μm membrane filter (Millipore Corp.). Microscopy examination of these samples showed that cells with hyphae were effectively retained by the filter. Cell numbers were determined by direct counts with a Petroff-Hausser chamber, and viable cell numbers were determined by spreading suspensions onto plates. The direct counts were 10% higher than the viable counts and were used for these calculations. The 14C counts per minute in DNA and stable RNA per 107 cells were then determined as described above. The percentage of label in the stable nucleic acids as DNA was 27%. The remaining portion of the isolated first-generation daughter cells was allowed to grow for 10 h; the second generation of daughter cells was then isolated by filtration as before, and the 14C counts per minute in DNA and stable RNA were again determined. This process was repeated for the third and fourth generations.

UV sensitivity. Cells were harvested, washed, and suspended at a concentration of 6.2 x 107 to 2.2 x 109 cells per ml in 6 ml of 0.03 M phosphate buffer, pH 7.2. The ultraviolet light (UV) source was a 30-W General Electric lamp, model G30TB, which supplied an intensity of irradiation of 13 ergs per mm2 per s (G. Yang and R. Brubaker, personal communication). The number of survivors after various periods of irradiation was determined from colony counts of sample dilutions plated on solid medium and incubated in the dark.

RESULTS

Location and appearance of nucleoid. Electron micrographs of thin sections of Hyphomicrobium sp. strain B-522 showed the same diffuse, fibrillar structure typical of other bacterial nucleoids (Fig. 1a). Granules thought to be polyphosphate were associated with the nucleus and could often be seen near one end of the outer periphery of the whorls of nuclear material (Fig. 1b). Like other hyphomicrobia, strain B-522 had a complex system of internal membranes (4). Mesosome-like structures were also observed. Electron micrographs of a large number of cells consistently showed the pres-
ence of nucleoids in mother cells and mature buds; the absence of these structures in most hyphae and very young buds was striking. The notable exception to this was the occasional observation of a nuclear-like structure in the hyphae of old cells (Fig. 1b). The fine structural appearance of these was similar to those seen in the main body of the cell, although the size of such hyphal nucleoids appeared to be considerably less than those of the mother cells. The

Fig. 1. Electron micrographs of sections of Hyphomicrobium strain B-522. (a) Young swarmer cell; (b) part of a hypha of an older cell. The scale marker represents 0.5 μm.
inside diameter of the hypha was approximately 0.15 \( \mu m \). There seemed to be no increase in the density of the nuclear material located here. In some cases, the hypha appeared swollen near a patch of nuclear material.

Figure 2 shows the appearance of cells of various ages from an actively growing culture of *Hyphomicrobium* B-522 after treatment with Giemsa stain. Although the nucleoids were best seen by brightfield microscopy, phase contrast was more suitable for observing the morphology of the cells. Therefore, both phase and brightfield micrographs of the same field are shown. The nucleoids appeared as round, reddish-purple bodies within the cells. None of the bar-shaped nuclear forms occasionally seen in another *Hyphomicrobium* isolate (21) or *R. vanniieli* (16) were observed in this strain. Most swarmer cells, mature buds, and many mother cells contained only a single nucleoid, but some cells possessed two or three nucleoids. These were mother cells with small buds or very old cells with characteristically long hypha and swollen or elongated main cell bodies. Here again, there was no evidence of nuclear material in any of the hyphae or in very young buds.

Earlier investigations with *R. vanniieli* (16) and a *Hyphomicrobium* (21) also failed to detect nuclear material in the hyphae of actively growing cells.

Further attempts to detect nuclear material in hyphae were made. We employed autoradiography of cells containing tritium-labeled DNA. In addition to cells which were radiolabeled over two doublings of the culture turbidity (Fig. 3), others were labeled for only 6% (30 min) of this time to enhance the possibility of detecting newly synthesized DNA which might be present in the hyphae. The slides were examined after 5 days exposure of the photographic emulsion when few grains were present. Slides were also examined after 100 days when the large number of grains completely hid the main body of the cells. The location of the DNA in cells at various stages of growth was the same in these experiments as had been observed in electron micrographs or with light microscopy of Giemsa-stained cells. In both the long-labeled and short-labeled cells, grains were found over swarmer cells, mother cells, and mature buds, but very rarely over hyphae (Fig. 3 arrows) or very young buds.

![Fig. 2. Light micrographs of Giemsa-stained Hyphomicrobium strain B-522 from an actively growing culture. (a) Bright field; (b) phase-contrast. Scale markers: 10 \( \mu m \).](http://jb.asm.org/)
A method for obtaining large populations of swarmer cells from actively growing cultures of *Hyphomicrobium* sp. B-522 has been described (14). These cells were shown to exhibit a fair degree of synchrony during the first cycle of reproduction. The average number of nucleoids per cell determined by direct microscope counts of nucleoids in Giemsa-stained swarmer cell populations was 1.03. When such populations of swarmer cells were transferred to fresh, prewarmed medium, the average number of nucleoids per cell remained unchanged for the first 5 h, corresponding to one-third of the generation time, and then began to increase (Fig. 4). This increase occurred 2.5 h after the first buds were observed in the cell population and 3 h before the separation of the first mature daughter cells from the mother cells was detected (14). After 10 h of incubation, the average number of nucleoids per cell had increased to 1.5, while the proportion of cells with buds had increased to 57%, as shown previously.

These results indicate that, after separation of the daughter nucleoid within the mother cell, one of the nucleoids travels through the hypha to the bud. Failure to find nucleoids in the hyphae of actively growing cells suggests that this process takes place over a relatively short period of time.

**Genome size.** The size of a bacterial genome can be determined from the rate of reassociation of single-stranded DNA compared to the rate of reassociation of the DNA from an organism with a known genome size (1). The $C_{0.6}$ value is calculated from the concentration of DNA in moles of nucleotide per liter, multiplied by the time, in seconds, that the reaction has proceeded at 50% reassociation. A $C_{0.6}$ value of 2.90 was obtained for the *Escherichia coli* control DNA and 2.42 for *Hyphomicrobium* B-522 DNA. The base composition of the DNA of strain B-522 was previously found to be 60% guanine plus cytosine (G + C) (12). To allow for the effect of the 10% difference in base composition of the two DNAs on the rate of reassociation, the *E. coli* value was corrected by a factor of 0.8 as indicated by the data of Wetmur and Davidson (20). The size of the genome of strain B-522 was found to be $4.7 \times 10^8$ nucleotide pairs by using Cairns' (2) value of $4.5 \times 10^6$ nucleotide pairs for *E. coli* DNA. If the average molecular weight of a nucleotide pair is taken as 660, the

![Figure 3](http://jb.asm.org/) Location of DNA in *Hyphomicrobium* strain B-522 by autoradiography. Cells were grown for 18 h in the presence of 10 μg of *H*-adenine per ml. Emulsion-coated slides were prepared as described in Materials and Methods. Exposure time was 39 days. The arrows point to hyphae free from silver grains. Scale marker: 10 μm.
molecular weight of the B-522 genome would be \(3.1 \times 10^9\).

With the use of radiolabeling methods, the quantity of DNA per cell was found to be \(5.57 \times 10^{-10}\). This corresponds to an average of 1.08 molecules per cell and is in agreement with the value of 1.03 nucleoids per cell obtained from direct microscope examination of nucleoids in Giemsa-stained, swarmer cell preparations. This suggests that a limited amount of DNA replication has occurred at the swarmer cell stage of the reproductive cycle.

**Macromolecular synthesis.** A culture of swarmer cells was grown in the presence of various radiolabeled precursors. The rate of synthesis of DNA, RNA, and protein observed during the reproductive cycle is shown in Fig. 5. There is a 60% increase in the rate of synthesis of the three molecular species after 5 to 6 h. The increase in DNA synthesis is most likely due to the increase in the number of replication sites resulting from the larger number of nucleoids per cell present at this time (Fig. 4). The concomitant increase in RNA and protein synthesis would be expected to occur as the gene dosage increases. The timing of this event in the reproductive cycle suggests its importance to the process of bud development.

**DNA and RNA transfer.** Swarmer were isolated from an actively growing culture of cells in which the nucleic acids had been labeled with \(^{14}\)C-adenine (see Materials and Methods for details). The swarmer were allowed to mature and produce daughter cells which were then isolated and incubated until a second generation of progeny was produced. This process was repeated for the third and fourth generation. The proportion of \(^{14}\)C-labeled DNA and stable RNA transferred from one generation of cells to the next is shown in Fig. 6. One-half of the labeled DNA is transferred from the mother cell to the daughter cell at each consecutive generation.

In contrast to the transfer of DNA, only 43% of the stable RNA is transferred from the mother cell to the daughter cell. These results could be accounted for by (i) the production of RNA by the nucleoid which was transferred to...
NUCLEAR APPARATUS OF A HYPHOMICROBIUM

The authors suggested that DNA transfer between the mother cell and daughter cell occurs by (i) the movement of a parental nucleoid into the developing bud, and (ii) by only slow or poor mixing of this newly made material with pre-existing, labeled RNA because of the separation provided by the hypha.

The hypha may not completely prevent the exchange of material between the mother cell and the developing bud. Murray and Douglas (16) observed a basophilic, acid-hydrolyzable granule in the cytoplasm of a mature cell of R. vaniellii. This granule migrated about the cell in a regular fashion. The authors suggested the possibility that protoplasmic streaming might take place in these cells. On rare occasions, we have observed a localized swelling of the hypha of Hyphomicrobium strain B-522, which appeared to move along the length of the hypha. Thus, if protoplasmic streaming took place, it seems reasonable to assume also that this activity might extend into the hypha and very likely into the bud.

The possibility of an exchange of material between the mother cell and bud was also supported by experiments in which the sensitivity to UV by swarmer cell populations was compared after 1 and 11 h of incubation. Sixty-three percent of the population of 11-h cells contained cells which possessed buds. The majority of these could be expected to have one nucleoid in the mother cell and one in the bud. Therefore, the 11-h cell population would have an average of 1.6 nucleoids per cell. This agreed with the value obtained by extrapolation of Fig. 4. The 1-h cells contained an average of only slightly more than one nucleoid per cell and would, therefore, be expected to be approximately 60% more sensitive to irradiation by UV than the 11-h cells. However, the results of such an experiment revealed no differences in the sensitivities of the two cell populations (Fig. 7). Thus, in mature cells containing two nucleoids, the loss of function of either nucleoid rendered the cell nonviable. This suggests there is a means of communication between the two parts of the cell, but one which is limited. Otherwise, one nucleoid should be sufficient to maintain the cell.

DISCUSSION

The swarmer cells of Hyphomicrobium sp. strain B-522 were found to contain a single nucleoid comprised of a DNA molecule with a molecular weight of $3.1 \times 10^6$ and with the characteristic appearance of a bacterial nucleoid when viewed in the electron microscope. The results presented here indicate that DNA
replication occurs during swarmer maturation, hyphal development, and the beginning stages of bud development. The two daughter nucleoids do not separate until some development of the bud has taken place. One of the two daughter nucleoids is then transferred to the bud. At least, with respect to the first generation investigated here, this differs from the findings in another Hyphomicrobium sp. (21) and R. vannielii (16), where separation of the daughter nucleoids is reported to take place before the beginning of bud development, and nuclear material is associated with the buds by the time they are first recognizable as buds.

Transfer of the nucleoid from the mother cell to the bud must be quite rapid, since the various methods employed were normally unable to detect the presence of nucleoids in the hyphae of actively growing cells. However, this could also be accounted for if the nucleoid was transferred through the hypha as a long, thin thread of DNA, even though the time required for transfer might be much longer. This seems unlikely because autoradiographs of cells with tritium-labeled DNA failed to reveal the expected pattern of grain distribution along the hyphae. In addition, the occurrence of two normal nucleoids in mother cells at a stage prior to bud neucleation suggests that the DNA was transferred while in its normal nuclear state. The appearance of the occasional nucleoid observed in the hyphae of old cells also supports this conclusion.

The presence of invaginations in the cytoplasmic membrane observed previously (4) suggest that the membrane may be involved in separating daughter nucleoids as proposed by Jacob et al. (9), but it is difficult to imagine how the membrane might be involved in nuclear transfer to the bud. This holds true particularly for the second and later generations of buds produced by a single mother cell, where membrane growth was unlikely to be sufficient to accomplish nuclear transfer.

One-half of the old prelabeled strands of DNA was passed to each generation of daughter cells (Fig. 6). This is consistent with semiconservative DNA replication and random distribution of old and new strands to daughter cells (19) but differs from the results of others (3, 7), where the old strand seemed to remain fixed to a “segregation structure.” The present study did not examine consecutive generations of bud cells produced by a single, mother cell.

If hyphae are necessary components of the reproductive process in these budding bacteria, as has been suggested (14), their function could involve the control over inter-relationships existing between various cellular processes occurring at the same time in the mother cell and its developing bud. At least some of the processes taking place in the mother cell differ from those in the maturing bud, indicating the presence of different populations of regulatory molecules at the transcriptional, translational or enzyme level. This demands that complete exchange of material between the two poles of the cell be prevented. The observation that mature, binucleate cells have the same UV sensitivity as young mononucleate cells (Fig. 7) demonstrates the inability of a single nucleoid to sustain the cell, whether present in the mother cell or the bud. This failure could be due to interference with the passage of material by the hypha. Likewise, the transfer of stable RNA from the mother cell to the bud was shown to be partially inhibited. One cannot tell from our experiment whether that part of the stable RNA which is ribosomal is transferred as RNA, ribosomes, or polysomes, but it seems reasonable that the presence of the hypha would also affect the transfer of messenger RNA (mRNA). If the mRNA of these cells is heterogeneous in its stability, this would provide a method for selective control over the transfer of different mRNA molecules.

Nevertheless, a considerable amount of material was found to pass through the hypha. Besides the nucleoid and 43% of the stable RNA, substances required for the early stages of bud development may also be transported to this site. It is difficult to imagine from the UV experiments previously discussed how a loss of function of the bud nucleoid could affect the mother cell, since it has the ability to form other hyphae and buds. The loss of function of the mother cell nucleoid could not affect the completion of bud maturation once the bud was nucleated, unless there was a means of communication between these parts of the cell. Diffusion of material through the long hypha cannot account for these observations. Streaming of the cytoplasm would provide a reasonable alternative. The occasional observation of particle movement in these hyphae lends support to this possibility. Malfunctions of this process which might occur in old cells could account for the nucleoid observed in the hyphae of such cells.

Thus, the presence of a partially mobile cytoplasm would explain many of the findings made in this and other investigations on the reproductive cycle of the hyphal-budding bacteria. The rates of flow and the amount of mixing may offer a means to the cell of controlling the degree of communication between the mother cell and its bud.
ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 1 FO2 GM 30699-02 (to R.L. Moore) from the National Institute of General Medical Sciences and by research grant no. GB-7403 (to P. Hirsch) from the National Science Foundation.

We wish to express our appreciation to H. Stuart Pankratz for his assistance with the electron microscopy.

LITERATURE CITED