Studies on the Morphogenesis of Nocardioform Organisms Related to the ‘*rhodochrous*’ Taxon in Synchronous Cultures

By GÉRARD LEFEBVRE, NICOLE MARTIN, GÉRARD KILBERTUS
AND ROBERT GAY

*Laboratoire de Chimie Biologique I, Faculté des Sciences, Université de Nancy I,
Case Officielle 140, 54037 Nancy Cedex, France*

(Received 15 December 1977; revised 29 March 1978)

In nocardioform organisms related to the ‘*rhodochrous*’ taxon (*Nocardia restricta*, *Nocardia* or *Jensenia canicruria*), morphogenesis was affected by nutritional conditions, especially the nature of the carbon source. With glucose, growth was slow and the bacteria divided at each doubling of the cell mass. With succinate or acetate, growth was much more rapid during the exponential phase and cells grew as long rods. Later, when growth slowed down, septa, which were clearly visible in thin sections examined by electron microscopy, gradually appeared and divided the organism into smaller cells. During the stationary phase of growth, cells recovered their original spherical shape. Synchronous growth of these bacteria was achieved in liquid synthetic medium by inoculating fresh medium with spherical cells taken from an advanced stationary phase culture. The synchrony was shown by the periodicity of DNA replication and protein synthesis.

The fragmentation of the rods was studied during incorporation of labelled diaminopimelic acid into cell walls. Septa did not form simultaneously but in distinct phases. The regular periodicity of their formation suggested that this process is coordinated in time and is related to the organism’s growth rate. The data are interpreted using Cooper and Helmstetter’s model for cell division in prokaryotes.

INTRODUCTION

Nocardioform bacteria are pleomorphic organisms existing as filaments or rods without transverse walls or as smaller, round or ovoid cells, depending on cultural conditions. In this, they resemble other species of actinomycetes and coryneform bacteria. Spherical cells incubated in a suitable medium develop into filaments which subsequently fragment with the formation of multiple septa, re-establishing a spherical state similar to the initial one. Some physiological and biochemical aspects of these changes have been studied in *Arthrobacter* (Mulder & Antheunisse, 1963; Ensign & Wolfe, 1964; Krulwich & Ensign, 1968; Luscombe & Gray, 1971, 1974; Massey *et al.*, 1973). In *Nocardia*, morphogenesis has been investigated mainly with the light microscope (Webb & Clark, 1957; Adams & McClung, 1962; Brown & Clark, 1966). There has been one detailed study using electron microscopy (Beaman & Shankel, 1969). Little is known about the influence of nutritional conditions on the morphogenesis of *Nocardia* (Heinzen & Ensign, 1975).

The aims of this investigation were to study the influence of the carbon source on growth rate and morphology of nocardioform organisms in a defined medium, to follow morphogenesis, and particularly fragmentation, by electron microscopy in such cultures and to study the incorporation of labelled diaminopimelic acid by walls and septa of nocardioform organisms in synchronous cultures.

The ‘*Nocardia*’ species used here, *N. restricta* and *Nocardia* or *Jensenia canicruria*, are not in fact *Nocardia sensu stricto* but members of the ‘*rhodochrous*’ taxon or the genus *Rhodococcus* (Goodfellow & Alderson, 1977).

METHODS

Nocardia restricta ATCC 14887, *Nocardia canicruria* ATCC 17896 and *Jensenia canicruria* ATCC 11048 were grown aerobically at 28 °C in a liquid synthetic medium as previously described (Lefebvre *et al.*, 1978). The carbon source was sterilized separately at 120 °C for 30 min and added to the medium to give a concentration of 15 g l⁻¹ for sodium succinate or acetate and 7.5 g l⁻¹ for glucose.

Absorbance at 550 nm was estimated with a Beckman DU spectrophotometer. The methods for synchronization of cultures by inoculation of a fresh medium with spherical cells taken during the advanced stationary phase and the monitoring of synchronous DNA replication by pulsed incorporation of [³H]-thymidine into DNA have been described by Lefebvre *et al.* (1978).

Electron microscopy. Micro-organisms were fixed by adding 1 ml 1 % (w/v) osmium tetroxide to 10 ml culture, centrifuging immediately, dispersing the pellet in 0.1 ml 1 % Bacto-tryptone plus 1 ml 1 % osmium tetroxide, and leaving the suspension overnight. It was centrifuged the next day and the pellet was resuspended in 0.5 ml 2 % (w/v) agar at 50 °C. After the block had hardened, it was stained with 0.5 % (w/v) uranyl acetate and dehydrated in an ethanol/water series. The dehydrated block was embedded in resin (Epon 812), sectioned with an ultramicrotome, and the sections were stained with lead citrate.

Continuous diaminopimelic acid (DAP) incorporation during growth. To 10 ml of a newly synchronized culture were added 122 µg (DL+*meso*)-2,6-diamino[G-³H]pimelic acid (equivalent to 20 µCi; The Radiochemical Centre, Amersham) and 36 µg L-lysine (which reduced DAP degradation). Every 1 h, 1 ml samples were precipitated with 1 ml cold 20 % (w/v) trichloroacetic acid (TCA), passed through Millipore filters (pore diameter 0.45 µm) and washed three times with 4 ml cold 10 % TCA containing 0.5 mg unlabelled DAP ml⁻¹. The filters were placed in a scintillation mixture with the following composition: 70 ml toluene, 25 ml Triton X-100, 0.5 ml water, 400 mg 2,5-diphenyloxazole (PPO) and 20 mg 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP).

Pulsed addition of DAP. Culture samples (1 ml) were taken at intervals and incubated aerobically at 28 °C with 0.5 µg [³H]DAP (corresponding to 1 µCi) and 5 µg L-lysine. After incubation, the samples were precipitated with TCA, filtered, washed and counted as described above.

RESULTS

Influence of the carbon source on growth rate and morphology

In a liquid synthetic medium containing 7.5 g glucose l⁻¹ as the sole carbon source, the doubling time (*T*_d) of *N. restricta* in the exponential growth phase was 10 ± 1 h at 28 °C in aerobic conditions. At the beginning of the exponential phase of growth, the bacteria were spherical or ovoid (Fig. 1) ranging from 0.5 to 1.5 µm in diameter. Thereafter short rod-shaped cells appeared (Fig. 2). When the cell length had doubled, the nucleoid elongated and constricted and the organism divided with the formation of a septum (Figs 3 and 4).

With sodium succinate as the carbon source, growth was much faster: the exponential growth phase lasted for approximately three generations (*T*_d 2 h 15 min at 28 °C) for cells previously adapted to succinate. During this phase the cells elongated without dividing, and reached a length of 5 to 10 µm and a width of 0.5 to 1 µm by the end of the exponential phase (Fig. 5). After 8 h, septa began to appear (Fig. 6) and these were complete after 10 h (Fig. 7). Fragmentation continued until the stationary phase was approached, when small, spherical or ovoid cells were found. Similar growth was obtained on other acids such as carboxylic acids (pyruvate) and short-chain fatty acids (acetate, propionate, butyrate), with *T*_d always between 2 h 15 min and 3 h for cells previously adapted by several transfers in the same carbon source.

With *N. canicruria*, another species of the 'rhodochrous' taxon, results were similar to those with *N. restricta*: rapid growth on succinate (*T*_d 2 h 30 min) and slower growth on glucose (*T*_d 5 h).

Evidence for synchrony

Lefebvre *et al.* (1978) have shown that the synchronization of cultures of *N. restricta* produces steps in the rate of increase of culture absorbance and cell protein content, corresponding to doublings of the cell mass. DNA replication, checked either by measurements

Figs 1 to 4. Thin-section electron micrographs of *N. restricta* grown on glucose, after 2 h (Fig. 1), 9 h (Fig. 2) and 11 h (Fig. 3 and 4) incubation. Bar markers represent 1 μ m.

of DNA content or by pulsed incorporation of [3 H]thymidine, was discontinuous: peaks of incorporation of thymidine into DNA followed one another with a periodicity equal to the doubling time, on succinate or acetate medium. Additional evidence for synchrony was obtained here: during slow growth of *N. restricta* on glucose, peaks of incorporation of [3 H]thymidine into DNA were separated by intervals nearly equal to the doubling time (Fig. 8). Continuous incorporation of [3 H]leucine showed that there was a periodicity of protein synthesis in synchronous cultures of *N. canicruria* (Fig. 9).

Incorporation of labelled diaminopimelic acid

With *N. restricta* growing on succinate, DAP incorporation was enhanced after the end of the exponential phase of growth and was periodic when fragmentation occurred (Fig. 10). The period was the same as the doubling time during the preceding exponential phase.

Pulsed incorporation of labelled DAP into *N. restricta* cells, in the fragmentation phase, confirmed that there were periods of high DAP incorporation followed by periods during which the rate of incorporation was much lower (Fig. 11*a*), with maxima occurring at intervals equal to the doubling time. This suggests that septa did not all form at once, but in several steps, the regular periodicity of which was related to the organism's exponential

Figs 5 to 7. Thin-section electron micrographs of *N. restricta* grown on succinate, after 7 h (Fig. 5), 8 h (Fig. 6) and 10 h (Fig. 7) incubation. Bar markers represent 1 μ m.

growth rate. A similar phenomenon was observed with *N. canicruria* and *J. canicruria*. In glucose medium, in which *N. restricta* grew slowly and its spherical cells divided normally, DAP was incorporated during each doubling of the cell mass throughout the exponential phase (Fig. 11*b*).

Some continuous incorporation curves obtained at the end of the exponential phase of growth of *N. restricta* on succinate medium showed large decreases in radioactivity between DAP incorporations. In order to check whether these were due to a partial turnover of cell-wall constituents, *N. restricta* cultures labelled with [3 H]DAP were transferred to DAP-free medium. The pattern of residual radioactivity in the TCA-insoluble precipitate (Fig. 12) showed that there was little variation during the exponential phase, whereas during the phase of slow growth turnover periods alternated with periods of re-incorporation.

Fig. 8. Pulsed incorporation of [^3H]thymidine into DNA in a synchronized culture of *N. restricta* growing on glucose. At intervals, 1 ml culture samples were taken and incubated with 1 μCi [^3H]thymidine; after 40 min, the radioactivity incorporated into the TCA-insoluble fraction (●) was determined. Bars on symbols represent the duration of the pulse.

Fig. 9

Fig. 10

Fig. 9. Continuous incorporation of [^3H]leucine into cell protein in a synchronized culture of *N. canicruria* growing on succinate. L-4(n)-[^3H]leucine (2 mg; 100 μCi) was added to a 50 ml culture at zero time. Every 30 min, 0.5 ml culture samples were taken, precipitated with TCA, filtered, washed (with unlabelled DL-leucine in the TCA solution) and the radioactivity incorporated (●) was determined as in the method of continuous DAP incorporation. Absorbance of the culture at 550 nm (▲) was also measured. The dotted lines show the theoretical exponential increases.

Fig. 10. Continuous incorporation of [^3H]DAP into cell walls in a synchronized culture of *N. restricta* growing on succinate. [^3H]DAP and L-lysine were added to the culture after 7 h incubation. At intervals, 1 ml samples were taken and the radioactivity incorporated into the TCA-insoluble fraction (▼) was determined immediately.

DISCUSSION

Nocardia restricta responds to changes in nutritional state in a similar manner to *Arthrobacter* (Ensign & Wolfe, 1964). *Arthrobacter crystallopoietes* grows slowly as cocci on glucose, but the addition of compounds such as succinate to the medium allows rapid growth and the formation of rods. Our observations confirm and refine those made by light microscopy for *Nocardia corallina* (Heinzen & Ensign, 1975). This species is not suitable for precise studies of growth and fine microscopic observations because the cells clump in liquid cultures.

Changes in morphology related to carbon source in batch cultures could be interpreted in two ways: (i) by considering that specific substances act as inducers (Ensign & Wolfe, 1964), or (ii) by considering that morphology depends solely on growth rate, whatever the carbon source, which acts only indirectly by influencing the doubling time (Luscombe & Gray, 1971, 1974). In an attempt to resolve this, we are studying continuous cultures of *N. restricta* growing on succinate in a chemostat.

Fig. 11. Pulsed incorporation of $[^3\text{H}]\text{DAP}$ into cell walls in synchronized cultures of *N. restricta* growing on succinate (a) or glucose (b). Every 30 min (a) or 2 h (b), 1 ml culture samples were taken and incubated with $[^3\text{H}]\text{DAP}$ and L-lysine; after 15 min (a) or 30 min (b), the radioactivity incorporated into the TCA-insoluble fractions ($\blacktriangledown$) was determined. Bars on symbols represent the duration of the pulse.

Fig. 12. Turnover of cell-wall constituents, during the fragmentation phase, in a synchronized culture of *N. restricta* growing on succinate. Fresh, DAP-free medium was inoculated at zero time with cells previously labelled with $[^3\text{H}]\text{DAP}$. At intervals, the radioactivity remaining in the TCA-insoluble fraction of 1 ml culture samples ($\blacksquare$) was measured.

Lefebvre *et al.* (1978) have shown that *N. restricta* cultures can easily be synchronized. Additional evidence for synchrony from experiments showing the periodicity of protein synthesis during the cell cycle has now been found.

We took advantage of our ability to obtain synchronous cultures to initiate a study of the time-course of fragmentation, which was previously unknown. Diaminopimelic acid incorporation into cells has shown that septa appear gradually after the end of the exponential phase of growth.

The results of these experiments can be explained by the model of Cooper & Helmstetter (1968) for cell division in *Escherichia coli*. This model divides the bacterial cell cycle into two periods: C, the time necessary for DNA replication, followed by D, the time between the end of a replication cycle and the next cell division. Depending on the values of C and D compared with T_d , a given number of nucleoids are formed per cell.

For some filamentous bacteria, such as some *Bacillus* spp., Paulton (1971) attributes filament formation to D being relatively long and independent of the growth rate. If T_d is less than $C+D$, multinuclear filaments form. The same reasoning could apply to actinomycetes and coryneform bacteria. At low growth rates, $C+D$ would be less than T_d and nucleoid replication and cell division would take place during the same cell cycle, e.g. *N. restricta* grown on glucose. At higher growth rates, $C+D$ would be greater than T_d , the cell mass would increase and nucleoids would replicate faster than septa could form to divide the cell; the resulting cells would be multinuclear. A first septum would be initiated during

the first generation, but it would take part in a division only time D later (more than 6 h in the case of *N. restricta* grown on succinate). Another septum would be initiated 2 h 15 min later but would take part in a division only time D later, and so on. Fragmentation would be delayed and sequential, with septa completion separated by the equivalent of one doubling time.

The Centre National de la Recherche Scientifique is acknowledged for grants to G. Lefebvre (ATP no. 3071) enabling us to carry out this work.

REFERENCES

- ADAMS, J. N. & MCCLUNG, N. M. (1962). Comparison of the developmental cycles of some members of the genus *Nocardia*. *Journal of Bacteriology* **84**, 206–216.
- BEAMAN, B. L. & SHANKEL, D. M. (1969). Ultra-structure of *Nocardia* cell growth and development on defined and complex agar media. *Journal of Bacteriology* **99**, 876–884.
- BROWN, O. & CLARK, J. B. (1966). Fragmentation in *Nocardia corallina*. *Journal of General Microbiology* **45**, 525–530.
- COOPER, S. & HELMSTETTER, C. E. (1968). Chromosome replication and the division cycle of *Escherichia coli* B/r. *Journal of Molecular Biology* **31**, 519–540.
- ENSIGN, J. C. & WOLFE, R. S. (1964). Nutritional control of morphogenesis in *Arthrobacter crystallopoietes*. *Journal of Bacteriology* **87**, 924–932.
- GOODFELLOW, M. & ALDERSON, G. (1977). The actinomycete-genus *Rhodococcus*: a home for the 'rhodochrous' complex. *Journal of General Microbiology* **100**, 99–122.
- HEINZEN, R. J. & ENSIGN, J. C. (1975). Effect of growth substrates on morphology of *Nocardia corallina*. *Archives of Microbiology* **103**, 209–217.
- KRULWICH, T. A. & ENSIGN, J. C. (1968). Activity of an autolytic *N*-acetylmuramidase during sphere-rod morphogenesis in *Arthrobacter crystallopoietes*. *Journal of Bacteriology* **96**, 857–859.
- LEFEBVRE, G., QUENNEDEY, M. C. & MARTIN, N. (1978). Croissance synchrone en milieu liquide d'espèces liées à *Mycobacterium rhodochrous*: *Nocardia restricta* et *Jensenia canicruria*. *Annales de Microbiologie (Institut Pasteur)* **129A**, 285–293.
- LUSCOMBE, B. M. & GRAY, T. R. G. (1971). Effect of varying growth rate on the morphology of *Arthrobacter*. *Journal of General Microbiology* **69**, 433–434.
- LUSCOMBE, B. M. & GRAY, T. R. G. (1974). Characteristics of *Arthrobacter* grown in continuous culture. *Journal of General Microbiology* **82**, 213–222.
- MASSEY, L. K., CLARK, J. B. & JACOBSON, R. A. (1973). Changes in RNA transcription during morphogenesis of *Arthrobacter crystallopoietes*. *Journal of General Microbiology* **77**, 51–60.
- MULDER, E. G. & ANTHEUNISSE, J. (1963). Morphologie, physiologie et écologie des *Arthrobacter*. *Annales de l'Institut Pasteur* **105**, 46–74.
- PAULTON, R. J. L. (1971). Nuclear and cell division in filamentous bacteria. *Nature New Biology* **231**, 271–273.
- WEBB, R. B. & CLARK, J. B. (1957). Cytogenetic study of *Nocardia corallina*. *Journal of Bacteriology* **74**, 31–42.