Autoradiographic Study of the Localization and Evolution of Growth Zones in Bacterial Colonies

By J. REYROLLE* AND F. LETELLIER*

Laboratoire de Physiologie Bactérienne, Université de Caen, 14032 Caen Cedex, France

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Incorporation of [^3H]leucine in the bacteria of 18 to 48 h-old colonies of *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus thuringiensis*, *Staphylococcus aureus* and *Escherichia coli* enabled the localization of bacterial multiplication sites by means of autoradiography of sagittal sections. In colonies where fast diameter expansion occurred, all the bacteria from the peripheral corona contributed to peripheral growth; in colonies where the expansion was slower, the growth rate of the bacteria in this region was heterogeneous. Besides this peripheral growth, a central region of bacterial multiplication was always found, but with variable localization and extension. In aerobic species, such as *P. aeruginosa* and *P. putida*, the central growth site was limited to the zone of oxygen penetration into the bacterial mass. However, in facultatively anaerobic species, bacterial multiplication depended on nutrient supply. For 48 h-old colonies of *S. aureus*, a more complex localization of growth seemed to be affected simultaneously by nutrient penetration and accumulation of toxic substances.

INTRODUCTION

Although in nature bacteria multiply as colonies or related formations (flocs, slimes, clumps), much less study has been devoted to this form of growth than to dispersed growth in liquid medium. In particular, there is little information on the localization of proliferating cells within a colony of bacteria.

It is relatively easy to observe directly the genesis of microcolonies by means of an optical microscope (Bisset, 1938; Hoffman & Franck, 1961; Brock, 1971), but this technique is no longer applicable when a certain stage of development has been reached. Classical histological techniques yield little information on the zones of proliferation since they only show morphological or staining differences. This is especially true for *Mycobacterium tuberculosis* colonies (Arloing & Malartre, 1925; Kahn & Nonidez, 1933) and some mature eubacterial colonies (Legroux & Magrou, 1920; Spanedda, 1958).

Dimensional criteria have also been used to study growth in bacterial colonies. Thus, Pirt (1967), Cooper *et al.* (1968) and Palumbo *et al.* (1971) followed growth by measuring the increase in colony diameter, and occasionally thickness, and demonstrated the presence of a clearly localized peripheral growth zone. The bacteria responsible for the increase in thickness of the colony cannot, however, be located in this zone.

Recently, Wimpenny & Lewis (1977) measured the oxygen consumption of young colonies and deduced that the depth of oxygen penetration varied according to the species and must therefore necessarily lead to different locations for internal colony growth.

We have devised a method for precisely locating the growth zones in a colony, based on

* Present address: Génétique Microbienne Fondamentale et Appliquée, Université de Caen, 14032 Caen Cedex, France.

a technique of section autoradiography (Reyrolle & Letellier, 1974), and have used it to study the distribution of growth zones in 18 to 48 h-old colonies of five bacterial species.

METHODS

Bacteria and growth. The following micro-organisms were used: *Staphylococcus aureus* (Institut Pasteur no. 52156), *Bacillus thuringiensis* var. Berliner (for which we thank P. Boutibonnes, see Boutibonnes, 1975), *Pseudomonas putida*, *Escherichia coli* and *Pseudomonas aeruginosa* (from our laboratory collection). The organisms were cultured on Ryter & Kellenberger's (1958) S synthetic medium, supplemented with Difco yeast extract (3 mg ml⁻¹), except for *P. putida* which was grown on PB semi-synthetic medium containing: Difco peptone, 0.2 g; sodium benzoate, 0.5 g; mineral solution (Kauffmann, 1967), 5 ml; agar, 1.5 g; and water, 100 ml; adjusted to pH 7.2 with NaOH.

Each strain was grown for 16 h at 37 °C (27 °C for *P. putida* and *B. thuringiensis*) in liquid medium and then seeded with a microdrop from a straight metallic thread on fragments of Millipore membrane (HAWP 047) placed on solid medium (S or PB). At different times, a membrane bearing a colony was transferred, under sterile conditions, to a medium of the same composition and depth containing [³H]leucine (5 µCi ml⁻¹, specific activity 250 mCi mmol⁻¹; The Radiochemical Centre, Amersham) for 3 to 6 h. For all media, the depth of agar was 3.2 mm.

Autoradiography. After incubation, the filters bearing the colonies were immersed for 1 h in formaldehyde/70 % (v/v) ethanol/glacial acetic acid (5:15:1, by vol.). Membranes were cut with a razor blade as near to the edge of each colony as possible, and the membrane pieces with attached colonies were washed for 2 h before dehydration and embedding in Spurr's epoxy resin. Semi-fine sections were cut with a glass knife on a Porter Blum Sorvall no. 2 microtome and collected on gelatinized slides (Ilford). The slides were dipped in L4 emulsion (Ilford). After 7 to 8 d exposure at 4 °C slides were developed with D19b developer (Kodak). The sections were exclusively sagittal. Where indicated, they were stained with toluidine blue prior to observation. For each experiment at least five colonies were studied.

RESULTS AND DISCUSSION

The colonies studied were 18 to 48 h-old, during which time the diameter of all colonies increased linearly (*S. aureus*, 0.062 mm h⁻¹; *E. coli*, 0.066 mm h⁻¹; *P. putida*, 0.093 mm h⁻¹; *P. aeruginosa*, 0.097 mm h⁻¹; *B. thuringiensis*, 0.110 mm h⁻¹). According to Pirt (1967) and Palumbo *et al.* (1971), this growth period corresponds to an equilibrium phase that is characteristic of a bacterial colony.

[³H]Leucine was chosen as the labelled precursor since it is almost entirely incorporated into protein (Caro, 1961), thus indicating the sites of bacterial proliferation. In non-proliferating bacteria the incorporation of this amino acid following protein turnover was negligible compared with incorporation in proliferating organisms.

Peripheral growth

Autoradiographs prepared from those colonies which had relatively faster diameter increases, e.g. *P. aeruginosa* (Figs 1a, 1d, 14), *P. putida* (Figs 2a, 2d, 11) and *B. thuringiensis* (Figs 3a, 3d, 18), showed homogeneous labelling of the section extremities. On the other hand, sections of colonies which expanded more slowly, e.g. *S. aureus* (Figs 5a, 5d, 6, 8) and *E. coli* (Figs 4a, 4d, 16), were more intensely labelled at their bases in contact with the growth medium. The regular labelling of the peripheral corona characteristic of colonies with fast diameter growth indicates that all the bacteria of this region are under identical growth conditions. Oxygen therefore diffuses easily through a relatively thin bacterial mass and nutrient supply is satisfactory; this is probably facilitated by the cells' own mobility. Conversely, in colonies with slow diameter growth, a heterogeneity rapidly appears in the growth rate of the bacteria in the peripheral zone.

Figs 1 to 5. Diagrams showing the label distribution in sagittal sections of colonies: (a, d) peripheral zone; (b, c) central zone.

Fig. 1. *Pseudomonas aeruginosa* on S medium. Colonies were grown for 24 h (a, b) or 34 h (c, d) followed by labelling for 4 h.

Fig. 2. *Pseudomonas putida* on PB medium. Colonies were grown for 20 h followed by labelling for 3 h (a, b) or grown for 30 h followed by labelling for 4 h (c, d).

Fig. 3. *Bacillus thuringiensis* on S medium. Colonies were grown for 18 h (a, b) or 32 h (c, d) followed by labelling for 4 h.

Fig. 4. *Escherichia coli* on S medium. Colonies were grown for 24 h (a, b) or 36 h (c, d) followed by labelling for 4 h.

Fig. 5. *Staphylococcus aureus* on S medium. Colonies were grown for 24 h followed by labelling for 3 h (a, b) or grown for 48 h followed by labelling for 6 h (c, d).

Central colony growth

The distribution of label in the central zones of the colonies studied differed according to the species and the age of the colony. In young colonies (24 h) of *P. aeruginosa* (Fig. 1b) and *P. putida* (Figs 2b, 10), the whole depth of the colony was labelled with the same intensity. Thus all the bacteria, whatever their position, must benefit from a nutrient and oxygen (for aerobic strains) supply enabling them to multiply at equivalent rates. In older (34 h) *P. aeruginosa* colonies, no marked variation of label was seen between the base and the summit (Figs 1c, 13). However, in older (30 h) *P. putida* colonies, the label was limited to the top two-thirds of the colony (Figs 2c, 12). In these strictly aerobic species, the diffusion of oxygen probably plays a crucial role, and in a thicker colony only those organisms located in an upper layer, through which oxygen can penetrate, will multiply enabling the colony to increase further in thickness.

For the facultatively anaerobic species studied, the growth zone within the colony was a basal zone whose thickness varied according to the species. For *E. coli* (Figs 4b, 4c, 15) and *S. aureus* (Figs 5b, 7) this zone was quite discrete, whereas for *B. thuringiensis* (Figs 3b, 3c, 17) labelling decreased progressively towards the summit.

Nutrient supply appears to play a key role in the localization of growth zones for facultative anaerobes. The bacteria located at the base of the colonies can multiply faster than those near the surface, despite the opportunity in the latter of an aerobic metabolism with a more favourable yield. Diffusion of substances throughout the colony is probably a function of both the magnitude of the interbacterial volume and the nature of the interbacterial substance. This might explain some differences observed, e.g. in *B. thuringiensis* the basal growth zone embraces a thicker layer than in *S. aureus* or *E. coli* since diffusion of

Sagittal sections, unstained (Figs 6 and 7) or stained with toluidine blue (Figs 8 and 9); bar markers represent 10 μ m.

Figs 6 to 9. *Staphylococcus aureus* on S medium. Colonies were grown for 24 h followed by labelling for 3 h (Figs 6 and 7) or grown for 48 h followed by labelling for 6 h (Figs 8 and 9); Figs 6 and 8 show peripheral zones of colonies, Figs 7 and 9 show central zones.

nutrient substances occurs more easily between the cells. Apart from the basal zones, a diffuse and regular label was also seen, probably due to slow cell proliferation as well as to protein turnover.

In *S. aureus*, as the colony aged, the weak labelling (duration 6 h) was located mainly within a fairly well-defined band below the surface (Figs 5c, 9). A possible explanation for this is that growth occurs in a zone where the opposing effects of two gradients balance, one consisting of toxic substances tending to accumulate at the base of the colony, and the other of nutrient substances migrating from the base (higher concentration) towards the summit. Inhibition of bacterial multiplication at the base of the colony could also result from the spherical shape of the organisms leaving only a relatively small interbacterial space through which oxygen may diffuse (c.f. Wimpenny & Lewis, 1977).

Sagittal sections stained with toluidine blue; bar markers represent 10 μm .

Figs 10 to 12. *Pseudomonas putida* on PB medium. Colonies were grown for 20 h followed by labelling for 3 h (Fig. 10) or grown for 30 h followed by labelling for 4 h (Figs 11 and 12): Figs 10 and 12 show central zones of colonies and Fig. 11 shows a peripheral zone.

Figs 13 and 14. *Pseudomonas aeruginosa* on S medium. Colonies were grown for 34 h (Fig. 13) or 24 h (Fig. 14) followed by labelling for 4 h: Fig. 13 shows a central zone and Fig. 14 shows a peripheral zone.

Sagittal sections, stained with toluidine blue (Figs 15 to 18) or unstained (Fig. 19); bar markers represent 10 μm .

Figs 15 and 16. *Escherichia coli* on S medium. Colonies were grown for 36 h (Fig. 15) or 24 h (Fig. 16) followed by labelling for 4 h; Fig. 15 shows a central zone (basal) of a colony and Fig. 16 shows a peripheral zone.

Figs 17 and 18. *Bacillus thuringiensis* on S medium. Colonies were grown for 32 h followed by labelling for 4 h: Fig. 17 shows a central zone (basal) and Fig. 18 shows a peripheral zone.

Fig. 19. *Staphylococcus aureus* on S medium. Central zone of a colony grown for 24 h followed by labelling for 6 h.

Influence of transfer on localization of the growth zones

The technique used involved transfer to fresh medium containing [^3H]leucine for 3 to 6 h. Under these experimental conditions, and for colonies 18 to 48 h-old, it is unlikely that the transfer caused any modification in the zones of growth localization for the following reasons:

1. Colonies were cultivated on a membrane for 18 or 36 h. For the next 12 h, groups of colonies on filters were transferred every 3 h to medium of identical composition until they were 30 or 48 h-old respectively. During this time, the increase in colony diameter occurred at the same rate as for colonies that had not been transferred. The measurement of diameter increase is a good estimate of colony growth (Pirt, 1975). Therefore, since no significant variation was discernible after several successive transfers had been made, it is probable that a single transfer would have no observable effect on these experiments.

2. Colonies of the same species transferred at different stages of growth did not always give the same result, as might have been expected if the normal conditions of growth were disturbed. Thus, 20 h-old *P. putida* colonies were labelled throughout the whole depth of the central region (Fig. 10), but in 30 h-old colonies the label was limited to the top two-thirds of the colony (Fig. 12).

3. The length of the incubation period (3 to 6 h) in the fresh environment did not modify the distribution of the labelling zones, only their intensity. Thus, when 24 h-old *S. aureus* colonies were transferred to fresh medium with [^3H]leucine and incubated for 3 h (Fig. 7) or 6 h (Fig. 19), the labelling zones were identical and only the labelling intensity was increased.

The study of bacterial multiplication sites within a colony, by means of autoradiography, has provided information concerning their localization and evolution. In the peripheral zone, the main factor seems to be bacterial mobility. Colonies of the most mobile organisms show a faster increase in diameter and are characterized by a homogeneous growth zone. A central growth zone is observed in addition to the peripheral zone. The position of this central zone seems to depend mainly on factors such as oxygen requirement for growth and the composition and extent of the interbacterial space, which affects the diffusion of nutrient and toxic substances and of oxygen. Thus, when studying the complex structure that constitutes a bacterial colony, the crucial part played in its growth and evolution by this 'inner medium' must be taken into account. It should be possible to extend the application of this technique to bacterial clusters that exist in various forms either in nature or in certain technical procedures (e.g. microbial films in fermenters and trickling filters).

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