Oxygen Profiles in, and in the Agar Beneath, Colonies of *Bacillus cereus*, *Staphylococcus albus* and *Escherichia coli*

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(Received 3 September 1986; revised 12 December 1986)

This paper reports the use of microelectrodes to measure O₂ penetration in different aged colonies of *Bacillus cereus*, *Escherichia coli* and *Staphylococcus albus*. In young (18 h) colonies of *B. cereus* and *E. coli* O₂ disappeared at depths of 25–30 µm and 35–40 µm respectively. In young *S. albus* colonies, O₂ reached a minimum but was never completely absent. As colonies aged (24–168 h) the depth to which O₂ penetrated increased.

INTRODUCTION

A bacterial colony obtains nutrients from the solid agar surface on which it develops. Rapid growth leads to steep solute gradients as substrates diffuse upwards into the colony whilst, under aerobic conditions, O₂ diffuses downwards. The depth to which O₂ penetrates has been estimated previously. Pirt (1967) concluded that in colonies of *Escherichia coli*, growing at their maximum specific growth rate on a glucose/salts medium, O₂ would penetrate to 40 µm. Wimpenny & Lewis (1977) investigated the respiration of intact bacterial colonies growing on tryptone-soya agar in respirometer flasks. They calculated that O₂ would penetrate to a mean depth of 31 µm, 37 µm and 9 µm for *E. coli*, *Bacillus cereus* and *Staphylococcus albus* respectively.

A preliminary study by Wimpenny & Coombs (1983) used commercial O₂ microelectrodes to measure the partial pressure of O₂ in and around an 18 h colony of *B. cereus* directly. They found steep O₂ gradients at the colony surface with O₂ penetrating to a depth of 25–30 µm. Bungay *et al.* (1983) have also used O₂ microelectrodes to measure gradients in *Pseudomonas ovalis* colonies. They also demonstrated steep gradients and found that the extent of O₂ penetration varied with substrate concentration. Coombs & Peters (1985) developed a method for producing simple, inexpensive and reliable O₂ microelectrodes with a typical tip diameter of 5 µm. These have allowed further investigations and in this paper we report on the penetration of O₂ in and around colonies of *E. coli*, *B. cereus* and *S. albus* at different times after inoculation.

METHODS

Organisms and culture conditions. Cultures of *Bacillus cereus* C11, *Escherichia coli* K12 and *Staphylococcus albus* NCIB 8558 were obtained from the departmental culture collection. The organisms were maintained and grown on TSBA medium containing (g l⁻¹): tryptic soy broth (Difco), 30; agar (Difco), 15. Plates were poured on a level surface and dried for 1–1.5 h at 37 °C. The plates were inoculated from a confluent lawn plate using a glass needle freshly drawn from a Pasteur pipette. Care was taken to avoid breaking the agar surface. Up to three colonies were inoculated on each plate and the latter were incubated in a water saturated environment at 30 °C. For experiments with *E. coli* and *S. albus* a single *B. cereus* colony was inoculated 24 h before measurements were due to be made. This colony was used to calibrate the electrode zero before it was deployed in a colony in which the characteristic penetration of O₂ was not known. Wimpenny & Coombs (1983) demonstrated that a zone which extended from 25 µm to 100 µm below the surface of an 18 h *B. cereus* colony was anoxic, using electrodes calibrated in a ferrous sulphide slurry. In the investigations described here it was assumed that a 0% partial pressure of O₂ (pO₂) was obtained between 40 and 50 µm below the surface of a 24 h *B. cereus* colony. The 100% pO₂ was assumed to be that partial pressure found on the surface of an air saturated agar plate.

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O₂ measurements. Two different types of electrode were used to collect the data. Work with *B. cereus* was done using a commercially available O₂ sensitive microelectrode (model 723, Transidyne, Ann Arbor, Michigan, USA). All other work was done using electrodes made by the method described by Coombs & Peters (1985). The electrodes were mounted on a micromanipulator (Prior) which allowed vertical movement accurate to 5 µm and horizontal movement in two planes to an accuracy of 100 µm. The microelectrode and a silver/silver chloride reference electrode were connected to a Transidyne chemical microsensor (model 1201) which was used to measure the pO₂. During measurements the reference electrode was inserted in the agar at the edge of the plate. All the apparatus was mounted inside an earthed Faraday cage which reduced unwanted electrical noise. Using the micromanipulator it was possible to position the electrode above the colony and probe the colony and agar below it to a depth of 800 µm. The profile of the colony was also measured by noting the distance between the surface of the colony and a fixed position above it.

RESULTS AND DISCUSSION

Figs 1–3 show the distribution of O₂ in and around different aged colonies of *B. cereus*, *E. coli* and *S. albus*.

In an 18 h *B. cereus* colony (Fig. 1*a*) O₂ penetrated to a depth of 25–30 µm. The anoxic zone extended from 25 µm below the surface to roughly the base of the colony. O₂ was depleted from the underlying agar with a pO₂ 50% of the air saturated value 700–800 µm below the colony base. As the colonies aged (Fig. 1*b, c, d*) the anoxic zone extended into the agar below the colony. The depth to which O₂ penetrated the colony surface increased slightly but never to more than 50 µm. In the 68 h colony (Fig. 1*d*) the anoxic zone extended 250 µm below the colony base. In colonies older than about 120 h (not shown) the anoxic zone disappeared as O₂ diffused back into the colony which by then was probably nutrient limited. In a 168 h colony (Fig. 1*e*) the lowest pO₂ found was 5%. The O₂ gradient from the surface into the colony was much shallower than that found in young colonies.

In an 18 h colony of *E. coli* (Fig. 2*a*) O₂ penetrated to a depth of 35–40 µm below the surface. The anoxic zone extended some 200 µm, up to 50 µm below the base of the colony. The partial pressure of O₂ in the agar was depleted, the 50–70% air saturation isopleth occurring at a depth of 800 µm. In the 24 h colony the position of the anoxic zone had not changed much but the pO₂ had dropped to 40% at 800 µm below the colony base (Fig. 2*b*). As the colonies aged (Fig. 2*c, d*) O₂ penetration increased to 70 µm and O₂ saturation in the agar fell to around 10% at 800 µm in a 68 h colony. In a 168 h colony O₂ penetrated 200 µm at the centre of the colony and the anoxic zone extended below the 800 µm limit of measurement (Fig. 2*e*). O₂ gradients were steeper at the edges of the colony probably because cells in this region were still respiring actively and had not become substrate limited.

In an 18 h *S. albus* colony O₂ penetrated throughout the colony, reaching a minimum of 15% at 50–60 µm below the surface (Fig. 3*a*). O₂ gradients at the periphery of the colony were no steeper than that found at the centre. As the colony aged the minimum pO₂ dropped. At 44 h (Fig. 3*b*) a minimum of 5% was seen 70 µm below the surface. In an 80 h colony (Fig. 3*c*) this value had dropped to 4% and O₂ in the agar was further depleted. The penetration of O₂ into older colonies was not fully investigated; however, preliminary studies in a 168 h colony showed that pO₂ was at about 90–95% throughout the colony and underlying agar. This suggested that respiration rates had fallen to low levels and that O₂ had now diffused back into the system.

When apparent plateaus of measurable O₂ were found in *S. albus* colonies it was feared that the performance of the electrode was in some way affected and that these plateaus were in fact anoxic zones. When recording the pO₂ manually using the chemical microsensor it was only possible to obtain readings to an accuracy of about 1%. Accuracy was improved by logging data from the chemical microsensor on a BBC model B microcomputer. This demonstrated that the limit with *S. albus* represented an O₂ minimum rather than a plateau.

Fig. 4 shows profiles of O₂ penetration through colonies of *S. albus* and *B. cereus*. In the two 48 h *S. albus* colonies a minimum pO₂ was clearly reached although the gradient was very shallow, covering a range of only about 1%. The control profile through a 48 h *B. cereus* colony shows that a true zero is obtained with a large plateau observed throughout the anaerobic zone.

Fig. 1. Distribution of O_2 in and around colonies of *B. cereus* at different ages. (a) 18 h, (b) 24 h, (c) 44 h, (d) 68 h, (e) 168 h. Values shown are a percentage of the air-saturated value; isopleths connect points of similar partial pressure. Individual measurements are represented by points.

Fig. 2. Distribution of O_2 in and around colonies of *E. coli* at different ages. (a) 18 h, (b) 24 h, (c) 44 h, (d) 68 h, (e) 168 h.

Fig. 3. Distribution of O_2 in and around colonies of *S. albus* at different ages. (a) 18 h, (b) 44 h, (c) 80 h.

Fig. 4. Profiles through colonies of 48 h *S. albus*, (▲, ●) and *B. cereus* (■). O_2 concentration is expressed as a % of the air saturated value.

Preliminary work by Wimpenny & Coombs (1983) showed the value of using polarographic oxygen microelectrodes for the direct measurement of O_2 in a bacterial colony. The development of inexpensive, reliable microelectrodes has allowed further investigations to be made.

Values for the penetration of O_2 in all three types of colony can be compared with those

Table 1. *Respiration rates determined from O₂ penetration data at different points across colonies of B. cereus and E. coli*

Colony age (h)	<i>B. cereus</i> *			<i>E. coli</i> *		
	Edge of colony	Midpoint between edge and centre	Centre of colony	Edge of colony	Midpoint between edge and centre	Centre of colony
18	445	445	309	309	309	227
24	445	445	309	445	445	227
48	445	309	227	445	309	174
60	309	227	137	309	227	174
168	—	—	—	445	49	6

* Units expressed as Q_{O_2} , ml O₂ (g organism dry wt)⁻¹ h⁻¹.

—, Not determined as O₂ penetrated throughout the colony.

predicted by Wimpenny & Lewis (1977). They calculated a penetration of 31 µm, 37 µm and 9 µm for *E. coli*, *B. cereus* and *S. albus* respectively. Measured values were 35–40 µm and 25–30 µm for *E. coli* and *B. cereus* respectively, while *S. albus* never appeared to become totally anoxic. One reason for the difference between calculated and measured penetration may be that mathematical calculations were based on the assumption that the shape of a non-spreading colony can be regarded as a segment of a sphere. Whilst this is a good assumption it is not strictly accurate. Another factor which could affect penetration is the rate of diffusion of O₂ into a particular colony. This will almost certainly vary depending on the nature of any extracellular polymers produced by the organisms and on the way the individual cells pack in the colony. The results obtained with *S. albus* are unexpected; they suggest either that cellular respiration rates are fundamentally very slow or, perhaps more likely, that the K_m for cytochrome oxidase enzymes is very high in this organism. Such predictions need to be confirmed.

The time course of O₂ profile changes indicated steep O₂ gradients in the early phases. This was almost certainly due to rapid growth on a plentiful supply of nutrients. As the colonies aged, O₂ moved deeper into the structure, suggesting that cells became more and more nutrient limited. In the oldest colonies of *B. cereus* O₂ was never exhausted, confirming that reductant rather than oxidant now limited growth rate throughout the colony.

The spatial heterogeneity of growing colonies should be emphasized. It is predicted (Pirt, 1967; Wimpenny, 1979) that exponential growth only occurs at the growing colony margin. Here substrates are in excess and oxygen requirements are at their maximum leading to the observed steep O₂ gradients. Pirt (1967) stated that the depth to which a nutrient penetrates a metabolizing tissue is limited by

$$hg = \sqrt{\frac{2Ds_0}{q}}$$

where hg (cm) is the depth of penetration, D (cm² s⁻¹) is the diffusion coefficient for the nutrient, s_0 (g cm⁻³) is the concentration of nutrient at the surface and q [g nutrient consumed (cm³ of tissue)⁻¹ s⁻¹] is the metabolic quotient. By assuming values for D and s_0 for O₂ and having measured the penetration of O₂ it is possible to calculate q at different positions in the colony. These values of q may be converted to Q_{O_2} [ml O₂ (g organism dry wt)⁻¹ h⁻¹] measurements by assuming a cell concentration [g organism dry wt (cm³ colony)⁻¹]. Table 1 shows Q_{O_2} calculations for *B. cereus* and *E. coli* assuming $D = 1.9 \times 10^{-5}$ cm² s⁻¹, $s_0 = 8 \times 10^{-6}$ g cm⁻³ (Pirt, 1967), and that the cell concentration = 0.275 g organism dry wt (cm³ colony)⁻¹ (Wimpenny & Lewis, 1977). The Q_{O_2} decreases from the edge to the centre of colonies and as the colonies age. It is possible to relate Q_{O_2} to the specific growth rate (μ , h⁻¹) as

$$\mu = \frac{Q_{O_2} Y_{O_2}}{x}$$

where Y_{O_2} is the yield coefficient [$\text{g dry wt tissue (cm}^3 \text{ nutrient)}^{-1}$] for O_2 and x is the cell concentration. However, it proved impossible to calculate μ as Y_{O_2} had not been measured and probably varies throughout the colony. The change in Q_{O_2} and hence μ suggest that there is almost certainly a radial substrate gradient from the edge to the colony centre. Spatial heterogeneity implies that the global colony mass increase is only a crude average of a range of values. Indeed Lewis & Wimpenny (1981) showed that mass increase, though close to exponential in the early stages of growth, gradually dropped to lower values as parts of the colony became increasingly nutrient limited.

Fraleigh & Bungay (1986) have simulated O_2 and substrate gradients as a first step in obtaining a detailed colony growth model. Despite major simplifications in the definition of the colony system the model yielded results which were similar to those from microelectrode studies. It is clear that further detailed investigations of solute gradients within colonies are needed as these together with accurate theoretical models will help to explain colony morphogenesis and differences in morphology from species to species.

Adrian Peters was supported by a Natural Environment Research Council research studentship which we gratefully acknowledge.

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