SHORT COMMUNICATION

Biochemical Differentiation in Large Colonies of *Enterobacter cloacae*

By JULIAN W. T. WIMPENNY AND JEFFREY A. PARR

Department of Microbiology, University College, Newport Road, Cardiff CF1 1XP

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Large colonies of *Enterobacter cloacae* which were about 700 μm thick were frozen in liquid nitrogen and sectioned horizontally. The sections were disrupted and several oxidative enzymes were assayed in the crude unfractionated homogenates. In the top 120 μm of the colonies the specific activities of the enzymes were high and characteristic of aerobically adapted cells. Cells nearer the base of colonies had very low enzyme activities.

INTRODUCTION

The bacterial colony is a heterogeneous system in which organisms proliferate in spatially ordered gradients of nutrients. It might be expected that such a structure would exhibit biochemical differentiation in response to changes in the concentrations of solutes. Thus, near the centre of the colony, bacteria at the surface are in direct contact with atmospheric O_2 but are furthest away from nutrients in the agar; conversely, bacteria in contact with the agar might be O_2 -limited. Only at the leading edge of the colony will both nutrients and O_2 be in excess. Previous studies have shown that NADH oxidase and enzymes of the tricarboxylic acid cycle in many bacterial species, including *Enterobacter cloacae*, are most active during growth with excess O_2 (Wimpenny, 1969; Wimpenny & Necklen, 1971). We have therefore determined the activity of some of these enzymes in sections from different positions in large colonies of *E. cloacae* to see whether bacteria have different enzyme activities depending on their location within the colonies.

METHODS

The organism and its cultivation. *Enterobacter cloacae* (strain 6 of the collection in the Microbiology Department, University College, Cardiff) was maintained on Dorset egg slopes (Oxoid) at room temperature. The organism was grown first in stationary flasks of Tryptone Soya Broth (TSB; Oxoid) at 37 °C for 24 h. Plates (6 cm diam.) containing a 7 mm layer of TSB solidified with Bacto-agar (Difco; 12 g l⁻¹) were dried at 37 °C for 90 min. The plates were inoculated by depositing one drop of broth culture on to the centre of the agar using a 5 ml disposable syringe fitted with a 40 mm 20 gauge needle. After all visible fluid had been absorbed, the plates were incubated, inverted, for 48 h at 37 °C. Colonies 15 to 20 mm in diameter were obtained. This technique, in contrast to stab-inocula used by Wimpenny & Lewis (1977) or to the generation of colonies from single cells, produced large colonies which were flat enough to section easily.

Sectioning of colonies. Colonies on agar discs cut from the plate with a cork borer were transferred to circles of blotting paper and frozen on to metal cryostat mounting blocks using liquid nitrogen. The blocks were equilibrated at -25 °C for 30 min and the colony and agar were trimmed to a 5 to 10 mm square before being sectioned on a Pearse Type H cold microtome. Serial sections (10 μm) from at least five colonies were transferred four at a time, with a syringe needle, to wells in an immunological tray kept cold inside the microtome chamber. Sections from the same depth in each colony were collected together in 1 ml of 1.0 M-KH₂PO₄/KOH buffer (pH 7.2) and stored at -40 °C in test tubes covered with Parafilm.

Cell breakage. All the samples, in tubes surrounded with ice, were disrupted by 20 s bursts (2 min total

Fig. 1. Enzyme activities in sections derived from large colonies of *Enterobacter cloacae*: (a) NADH oxidase; (b) succinate dehydrogenase; (c) isocitrate dehydrogenase; (d) fumarase.

time of treatment) using a modified Mullard 500 W ultrasonic generator. Portions of the unfractionated homogenate were used in each enzyme assay.

Enzyme assays. All enzymes were assayed spectrophotometrically. Isocitrate dehydrogenase (NADP^+) [L_s -isocitrate: NADP^+ oxidoreductase (decarboxylating); EC 1.1.1.42] was measured by following the reduction of NADP^+ at 340 nm in the presence of DL-isocitrate (Ochoa, 1955). Fumarase [L -malate hydrolyase; EC 4.2.1.2] was measured by observing the increase in A_{240} in the presence of malate (Racker, 1950). NADH oxidase was measured by following the decrease in A_{340} (Slater, 1950). Succinate dehydrogenase [succinate: phenazine methosulphate oxidoreductase; EC 1.3.99.1] activity was determined by following the reduction of 2,6-dichlorophenolindophenol at 600 nm in the presence of succinate and phenazine methosulphate (King, 1967). Protein was measured by the Lowry method with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The colonies examined were derived from an area of confluent growth rather than from a single point. Whilst the structures generated after 48 h growth were suitable for sectioning since they were broad and almost flat, they were not morphologically the same as colonies which have arisen from a single cell; however, we consider that the term 'colony' is still justified (see, for example, Singleton & Sainsbury, 1978). It is assumed in this paper that any changes in enzyme activities are caused by the presence or absence of diffusible solutes and that this phenomenon will be common to all colonies however they are produced.

There were significant differences in the distribution of activities of oxidative enzymes in large colonies of *E. cloacae* (Fig. 1). Activities of succinate dehydrogenase and NADH oxidase, which are both membrane-bound enzyme systems, as well as those of fumarase and isocitrate dehydrogenase, the two cytoplasmic enzymes, were all highest in the surface sections from 0 to 120 μm into the colony. Experiments with three separate sets of colonies gave substantially similar results.

If it is accepted that high activities of these enzymes indicate conditions of O_2 excess, as suggested by Wimpenny & Necklen (1971), these results show that O_2 can diffuse at least 120 μm into colonies of *E. cloacae*. Pirt (1967) has calculated the range of penetration of O_2 into bacterial colonies using kinetic data derived from liquid culture experiments. For colonies of *Escherichia coli* growing at their maximum specific growth rate on a glucose/salts medium, O_2 should penetrate to a depth of 40 μm , whilst this value rises to 127 μm under maintenance conditions. It is possible that the bacteria in the interior of colonies of *E. cloacae* are respiring at a maintenance rate and thus showing O_2 penetration values close to those calculated by Pirt. It is more likely, however, that the coincidence is fortuitous and explained by the difference in height between colonies of *E. coli* grown on TSB agar or glucose/salts agar (about 200 μm ; J. W. T. Wimpenny, unpublished observation) and colonies of *E. cloacae* grown as described in this paper, which are about 700 μm high. This difference may reflect the reduced metabolic rate of a given volume of colony material, perhaps because of the presence of inert substances such as bacterial polysaccharides which are commonly produced by *Enterobacter* species (Sutherland, 1972). Wimpenny (1979) has suggested that bacteria in the interior of most colonies are growing, but at a slow diffusion-limited rate; it seems, therefore, that rates of respiration in this region are probably higher than the rates required for maintenance.

This paper demonstrates that some of the adaptations to changes in O_2 tension seen in the chemostat also occur within bacterial colonies.

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