pH gradients through colonies of *Bacillus cereus* and the surrounding agar

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pH-sensitive microelectrodes, constructed with a tip diameter of about 4 µm, were deployed through 24 h and 48 h colonies of *Bacillus cereus* incubated on CYS medium (Casamino acids, yeast extract, salts), with and without glucose. Measurements of pH were used to construct pH profiles through the colony and the surrounding agar. pH gradients could be detected for at least 800 µm into the agar beneath a 24 h colony, and to approximately 10 mm horizontally away from the edge of the colony. In older colonies, the lateral gradient extended for over 20 mm. The pH of the underlying agar was increased by up to 1.45 pH units after 48 h growth without glucose. When colonies were grown with glucose, a significant area of acidification was observed within the colony in addition to a zone of alkalization present at its periphery. Acidification was thought to be due to the anaerobic fermentation of glucose producing organic acids whilst alkalization was due to the aerobic oxidation of amino acids releasing ammonia.

Introduction

Bacteria growing on the surface of agar will deplete the nutrients immediately adjacent to the colony (Pirt, 1967). This will lead to the production of steep solute gradients beneath the growing colony. Rieck *et al.* (1973) demonstrated that the growth rate of colonies of *Pseudomonas fluorescens* became limited by the rate of diffusion of glucose when grown on glucose/salts agar containing low concentrations of glucose. In addition, under aerobic conditions, oxygen will diffuse downwards into a colony (Pirt, 1967; Wimpenny & Lewis, 1977). Gradients of oxygen penetration through colonies of *Bacillus cereus* have been measured directly (Wimpenny & Coombs, 1983). The results showed that an anaerobic zone is formed in the centre of an 18 h *B. cereus* colony, incubated at 30 °C. Since *B. cereus* is a facultative anaerobe there is the possibility that cells at the centre of the colony were growing anaerobically and producing organic acids. Kamath & Bungay (1988) also observed that colonies of aerobic species growing on solid media containing glucose receive glucose from the medium below, while oxygen diffuses from the gaseous phase above. The counter-diffusion and uptake of these two species means that there is a possibility that in certain regions, metabolism is limited to oxygen availability while in others, glucose is lacking. This would imply that

there is a significant degree of physicochemical heterogeneity within a colony. This in turn could give rise to the cells in different areas of a colony having different metabolic activities. Cooper *et al.* (1968) postulated that the active growth of a colony is confined to an annulus at the colony margin. Shapiro (1984, 1987) demonstrated that bacterial colonies have a high degree of morphological and biochemical organization, and showed that colonies of *Escherichia coli* expand by the addition of cells only at the periphery. This heterogeneity within a colony might cause characteristic pH gradients to be formed through and around the growing colony.

There are a number of processes which can lead to pH changes. These include the fermentation of carbohydrates to organic acids, so lowering the pH, the complete oxidation of organic acids or the oxidation of amino acids leading to an accumulation of ammonia, both processes leading to a rise in pH. The alteration of pH by the growth of bacteria in liquid batch culture is well documented (Spira & Silverman, 1979; Weimer, 1984). It is suggested that these processes could lead to the formation of pH gradients through a growing colony and into the agar beneath it.

The ability of micro-organisms to alter the pH in their locality, so producing a microenvironment of different pH, is of particular interest to the food industry. Food preserved by a low pH may become compromised by the growth of 'harmless' spoilage organisms raising the local pH. Food-poisoning organisms which would otherwise

Abbreviation: LIX, liquid ion-exchange.

be inhibited may then grow in these microenvironments. The presence of such microenvironments would not be detected by crude pH measurements.

Microelectrodes were originally designed for physiological studies of higher animals (Caldwell, 1954; Hinke, 1959). More recently they have been applied in microbial ecology (Revsbech & Jørgensen, 1986). pH gradients have been measured through complex microbial aggregations such as cyanobacterial mats (Jørgensen *et al.*, 1983; Revsbech & Ward, 1984), marine sediments and algal mats containing colourless sulphur bacteria (Jørgensen & Revsbech, 1983; Nelson *et al.*, 1986) and dental plaque (Brown *et al.*, 1981). By using microelectrodes it has been possible to measure the pH heterogeneity of these systems with a high degree of spatial resolution.

In the experiments described below, microelectrodes were used to determine pH profiles in colonies of *B. cereus* growing in air on an amino-acid-containing medium in the presence and absence of glucose.

Methods

Organisms, maintenance and media. *Bacillus cereus* CRA 666 was obtained from the Campden Food and Drink Research Association. The bacteria were stored as vegetative cells, on glass beads at -70°C (Jones *et al.*, 1984) and maintained at 30°C on nutrient agar containing (g l^{-1} in distilled water): nutrient broth (Difco), 8.0; Bacto-agar (Difco), 15.0. The inoculum was prepared by streaking a plate of the appropriate medium with *B. cereus* and incubating at 30°C for 24 h. The medium used (CYS) contained (g l^{-1} in distilled water): Casamino acids (Difco), 5.0; yeast extract (Difco), 2.0; plus salts solution, 5.0 ml l^{-1} . The salts solution contained (g l^{-1}): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10.0; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2, plus a few drops of concentrated HCl to clarify the solution. This medium, with (CYSG) or without (CYS) 10 g glucose l^{-1} , was solidified with 15 g Bacto-agar (Difco) l^{-1} . The salts solution and the glucose were sterilized separately and added to the sterile medium before pouring. Plates containing 20 ml medium were dried at 37°C for 1–1.5 h. The pH of the agar was then measured using a flat-ended pH combination electrode (no. 91655C, Orion Research UK).

Inoculation procedure. The agar plates were inoculated using a glass needle drawn out from a Pasteur pipette. The tip of the needle was used to transfer cells by placing it onto the surface of a colony and then onto the surface of the sterile agar, without penetrating it. Each plate was inoculated once, at its centre.

Electrode construction and deployment. pH-sensitive liquid ion-exchange (LIX) microelectrodes with a tip diameter of 2.5–6.0 μm were constructed using a method similar to that described by Walker (1971) and Bertl *et al.* (1984). Glass micropipettes with a tip diameter of 2.5–6.0 μm were pulled from 2 mm outside-diameter borosilicate glass (Clark Electromedical Instruments) using a homemade vertical glass puller, as described by Thomas (1978). If necessary the tip of the micropipette was removed to ensure that an unrestricted hole was present. This was achieved by holding the micropipette vertically with a micromanipulator. A second micromanipulator was then employed to position a metal bar directly beneath the downward-pointing micropipette. The micropipette was lowered on to the metal bar and the bench was given a light tap. This provided enough vibration to break the tip of the micropipette. The whole operation was observed through

a horizontally mounted microscope. The tip of the glass was then made hydrophobic by dipping it into a solution of 0.2% (v/v) dimethyldichlorosilane in benzene, so that the solution was drawn up approximately 200 μm into the tip by capillary action. The pipette was then placed in an oven at 90°C for at least 90 min. Prepared micropipettes were stored in a desiccator for up to one week. Liquid ion-exchanger (ionophore no. 95297, Fluka Chemicals) was drawn into the hydrophobic tip by capillary action. The electrode was then backfilled with 1 M-KCl, by using a plastic 1 ml syringe that had been heated and pulled to a fine point. The air between the ion-exchanger and the KCl was removed by inserting a cat's whisker into the electrode until it just touched the surface of the ion-exchanger. The KCl solution ran down the whisker and displaced the air. Any remaining air bubbles were dislodged by gently tapping the side of the electrode. Electrical connection to the KCl was made with a Ag/AgCl wire. The electrode was aged by placing it in potassium phosphate buffer pH 7.0 for 3 h. It could then be calibrated by placing the electrode in CYS broth that had been adjusted to pH values over the range 4.0–8.0. A second Ag/AgCl wire was used as the reference electrode. Electrodes with a drift of more than 5 mV h^{-1} were discarded, as were electrodes that took longer than 3 min to give a stable response in buffer solutions.

The pH was measured through a colony by mounting a pH-sensitive microelectrode on a Prior micromanipulator. The Petri dish was placed on a level board and the electrode was lowered at measured intervals from one side of the colony to the other. The microelectrode and the Ag/AgCl reference electrode were attached to a Dulas Engineering pH meter, which was in turn connected to a chart recorder. The electrodes were deployed in a Faraday cage to reduce electrical noise.

Results and Discussion

The LIX electrode typically gave a linear response of 56 mV per pH unit over the range pH 4–8, compared to the theoretical response at 20°C of about 58.1 mV per pH unit. The response range of the LIX was found to be pH 2.0–8.8.

The pH of the CYS medium was 6.13 ± 0.02 and that of the CYSG medium was 6.15 ± 0.03 . The diameters of the colonies examined were 5.2 mm and 16.5 mm for the 24 h and 48 h CYS colonies and 4.4 mm and 19 mm for the 24 h and 48 h CYSG colonies, respectively (only one colony from each growth condition was measured). Colonies grown without glucose were dome shaped,

Fig. 1. The pH at the surface of the agar, plotted against increasing distance from the colony margin, for 24 h CYS (○), 48 h CYS (●), 24 h CYSG (□) and 48 h CYSG (■) *B. cereus* colonies.

Fig. 2. Vertical pH profiles through the centre (●) and margin (○) of (a) a 24 h *B. cereus* colony grown without glucose, (b) a 48 h *B. cereus* colony grown without glucose, (c) a 24 h *B. cereus* colony grown with glucose, and (d) the centre (●), margin (○) and acid region (□) of a 48 h *B. cereus* colony grown with glucose. The dashed line indicates the surface of the agar.

whilst those grown with glucose had a more complex profile. Of the latter, the young colonies had surfaces showing a ridged appearance, whilst the older colonies had formed a thickened ring just within the periphery. This ring had a smooth and mucoid appearance whilst the colony surface on either side of this region had a rough appearance.

The pH at the surface of the agar was shown in all cases to decrease with increasing distance from the colony margin (Fig. 1), i.e. the colony had an overall alkalinization effect on the surrounding medium. The maximum distance, affected by each colony was approximately 10 mm for the 24 h colonies and 20 mm for the 48 h colonies. The presence of glucose had no significant effect on this. The pH gradient formed was steeper in both cases for the 24 h colonies.

Vertical pH gradients through the centre and margin of the colonies were plotted (Fig. 2a–d). In all cases the pH at the margin of the colony was greater than the pH at the centre. The pH gradients were steepest under the younger colonies and at the surface of the agar, through and immediately adjacent to the colony. The pH gradient through a region near the colony periphery (see Fig. 3d) of the 48 h CYSG colony was also plotted (Fig. 2d). This

showed that there was a region of acid production adjacent to the surface of the agar.

pH values measured through the colonies and the surrounding agar were mapped as pH isopleths. Only half colonies were plotted for the 48 h colonies, to enable more detail to be displayed. Each point on the maps indicates where a pH measurement was taken. The young (24 h) colony grown without glucose increased the pH of the underlying medium by up to 1.8 units, to a maximum of 7.92 (Fig. 3a). The alkalinization occurred throughout the whole colony. After 48 h (Fig. 3b), the maximum pH recorded was 7.6. In this older colony, a region of greater activity at the colony margin could be discerned. It was thought that the reduced activity in the centre of the colony was due to growth in that region being diffusion limited.

The 24 h colony grown with glucose (Fig. 3c) also showed an overall alkalinization, with a maximum pH of 6.78. A pronounced edge effect was detected where the highest pH values were recorded, with lower values (pH 6.26) in the centre of the colony. This effect was thought to be due to anaerobiosis at the centre of the colony (Peters *et al.*, 1987). This would lead to the fermentative production of organic acids from glucose,

Fig. 3. pH profiles of *B. cereus* colonies (a) 24 h, without glucose, (b) 48 h without glucose, (c) 24 h with glucose, (d) 48 h with glucose.

causing a fall in pH. After 48 h (Fig. 3d), the most pronounced alkalization was again at the colony margin (pH 6.90). A region of acidification (minimum pH 5.65) occurred centring on a region 3–4 mm in from

the colony margin, adjacent to the surface of the agar. Between these two regions there was a steep pH gradient. The centre of the colony was more alkaline than the acidic region but less alkaline than the colony margin.

The asymmetry shown by the two 24 h colonies was thought to be due to the time taken to measure the whole profile (approx. 6 h). The colonies were incubated at 30 °C and measured at room temperature (about 22 °C). The reduction in microbial activity at the lower temperature was thought to be responsible for the apparent difference in pH pattern from one side of the colony to the other. The 24 h colonies were measured from left to right.

It seems likely that pH changes on CYSG, which contains glucose as well as amino acids, are the products of two processes. First, in the presence of excess glucose and when the cells are anoxic, anaerobic fermentation reactions lead to the formation of organic acids and a fall in pH. Other measurements in gel-stabilized cultures of *B. cereus* (Coombs & Wimpenny, 1982) indicate that this organism can lower the pH to 5.1 under anaerobic conditions in the presence of glucose.

The second process seen in both colonies grown with and without glucose was assumed to be the oxidative deamination of amino acids, leading to ammonia accumulation. This was also reported by Coombs & Wimpenny (1982), with values in aerobic regions of a gel model up to about pH 7.9. It is suggested that similar processes occur in the growing colony. When the cells are growing in the presence of glucose anaerobically and even aerobically, because of catabolite repression, organic acids are produced by fermentation pathways. Catabolism of glucose as a ready source of energy means that there is little need for the oxidative deamination of amino acids, hence reducing alkali production from this source and reducing the overall increase in pH measured around the colonies grown on glucose.

The method described for constructing the microelectrodes was easily mastered, requiring less skill than the methods used to make microelectrodes with glass as the ion-selective membrane (Thomas, 1978). It took half a day to prepare a batch of twelve pieces of glass and a further fifteen minutes to fill an electrode prior to use. Electrodes with small tips, especially those less than 3 μm in diameter, were very sensitive to any movements made by the operator, despite being used inside a Faraday cage. The high noise-to-signal ratio was the main fault of the system. The wide range of ion-exchangers that are available means that with this technique it is possible to construct microelectrodes to detect a wide range of ions such as Na⁺, K⁺, Ca²⁺ and NH₄⁺.

The microelectrode techniques described are appropriate for measurements in any microbial system displaying spatial heterogeneity. Such systems include

biofilms as found on teeth (dental plaque), slime on water pipes or on submerged rocks, immobilized cells or sediments. Certain food systems also lend themselves to investigation by microelectrodes. Gradients of pH may be expected through foods preserved in an acid solution, e.g. pickled vegetables. Gradients could also occur through mould ripened foods such as soft cheeses (Brie, Camembert, Stilton) or salamis such as Milano (T. P. Robinson & J. W. T. Wimpenny, unpublished data). The growth of the mould on the exterior of these foods may cause deamination of amino acids and an increase in pH. Additionally in fermented foods like salami, tempe or kefir grains, a more complex pH profile might be expected due to the heterogeneous structure of the food.

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