Growth of *Bacillus cereus* in a Gel-stabilized Nutrient Gradient System

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Bacillus cereus consistently formed multiple growth bands in gel-stabilized gradient systems. The development of inoculated and uninoculated systems was followed by gel scanning and by measuring physico-chemical parameters. Factors which influenced band formation included the availability of oxygen and the presence of a glucose gradient. The relative position of bands was dependent on the glucose concentration in the source layer and the strength of the basal medium. Other factors such as agar type and concentration were relatively unimportant. The generation of steep pH gradients in the gel profoundly affected band formation. Thus, in the presence of a high buffering capacity changes in band pattern and pH profile occurred. No bands and no pH gradient were observed in gels incubated anaerobically. Addition of an alkaline overlayer, however, resulted in a pH gradient and band formation. Although pH gradients were responsible for sub-surface bands, it was not clear what caused the 'chopping' of growth into discrete bands. Computer simulations suggested that an asymmetric activation threshold may be responsible, and the mechanism may involve sporulation and germination.

INTRODUCTION

Several laboratory systems have been suggested to investigate microbial growth in solute diffusion gradients (Wimpenny *et al.*, 1981). One approach is to make use of semi-solid culture media achieved by the addition of agar. Such media have been used in the past, e.g. by Spray (1936) to help identify clostridia by their characteristic growth patterns, and by Williams (1938*a, b*; 1939*a, b*) who observed bands of bacterial growth in semi-solid media when they were exposed to different partial pressures of oxygen and carbon dioxide. Tschapek & Giambiagi (1954) also reported banded growth of a species of *Azotobacter* in gel-stabilized cultures.

The phenomenon is not limited to semi-solid media, since bands of *Escherichia coli* have been reported in aqueous systems by Adler (1966) in studies on the chemotactic response of the organism to oxygen and organic nutrients. Furthermore, discrete bands of growth are also seen in natural ecosystems; Perfil'ev & Gabe (1969) have reported that *Gallionella* grew in estuarine muds in well-defined multiple layers which were attributed to step-wise changes in redox potential and to concentration gradients in the mud.

Banded growth has been compared to Liesegang ring formation which is the periodic precipitation of inorganic salts in a gel system containing at least one diffusing reactant. Such phenomena were extensively reviewed by Hedges (1932) and have been discussed sporadically in the literature since. So far, the mechanism for periodic precipitation has not been conclusively established and this can also be said for banded bacterial growth. This paper attempts to explore the phenomenon in greater detail using strains of *Bacillus cereus*, a species which has been found to produce multiple growth bands in a gel-stabilized system.

METHODS

Organisms and maintenance. *Bacillus cereus* C11, D11 and *Bacillus* sp. C19 were obtained from the departmental stock culture collection. Strains C11 and D11 may be the same. Other strains included were *B. cereus* NCTC 2599, NCTC 6474, NCTC 9947 and *B. cereus* var. *mycoides* NCTC 9680. Cultures were maintained on slopes made

from tryptic soy broth (30 g l⁻¹; Difco) solidified with agar (15 g l⁻¹; Difco). Inocula were prepared in 100 or 250 ml conical flasks in 50 or 100 ml amounts of Casamino acids/yeast extract/salts (CYS) broth at full or one-fifth strength, to which was added glucose (final concentration 5 or 10 g l⁻¹). The cultures were incubated overnight, stationary at 30 °C.

Media. The CYS medium contained, in 1 l distilled water: Casamino acids (Difco), 5.0 g; yeast extract (Difco), 2.0 g; and salts solution, 5.0 ml. The salts solution contained, in distilled water (g l⁻¹): MgSO₄·7H₂O, 10.0; MnCl₂·4H₂O, 1.0; FeSO₄·7H₂O, 0.4; CaCl₂·6H₂O, 0.2, and a few drops of concentrated HCl to clarify the solution. The pH of the medium was 6.4 before autoclaving. The medium was sterilized at 121 °C for 15 min. Glucose, where used, was sterilized at 115 °C for 10 min and added separately. In certain experiments, the salts solution was replaced by the basal mineral salts medium (BMSM) of Pope & Skerman (Skerman, 1967), but excluding the minor trace elements.

The gel-stabilized nutrient gradient system. The gel systems were established as described by Wimpenny *et al.* (1981) and consisted of a semi-solid layer containing cells and basal medium, poured over a 'source' layer which contained the same basal medium plus full-strength agar and glucose. In some experiments, systems were established in capped test tubes. In this case each layer contained 7.5 ml of medium and the upper layer was mixed with 0.1 ml of inoculum. In another experiment, comparison of gel systems was made using low electroendosmosis agarose and low gelling temperature agarose (Marine Colloids Inc., Rockland, Me., U.S.A.)

Electrode methods. Needle electrodes for measuring pH, pO₂ and redox potential were mounted on a Prior micromanipulator, and lowered at measured intervals into the semi-solid agar layer. The pH was measured with a needle electrode (MI-405; Microelectrodes Inc., Londonderry, N.H., U.S.A.) using a calomel reference electrode connected via a KCl salt bridge to an Orion ion analyser (Model 801, Cambridge, Mass., U.S.A.). Oxygen tension was measured using a model 760 needle electrode (Transidyne Inc., Ann Arbor, Mich., U.S.A.) and a silver/silver chloride reference electrode connected to a Transidyne chemical microsensor. Redox potential measurements were made using a bare platinum electrode constructed in the laboratory and a calomel reference electrode connected via a KCl salt bridge to the Orion ion analyser.

Sampling the gels. A number 9 cork borer (Gallenkamp) was used to withdraw a sample from the gel system. The core was transferred to a simple gel slicer, constructed in the departmental workshop, which cut 1 mm slices.

Growth estimation. Growth was estimated by measuring the $A_{550}^{1\text{ cm}}$ of the homogenized slice resuspended in 20 mM-phosphate buffer, pH 7.0 or 7.4.

Gel scanning. Cores were removed from the system using a number 4 cork borer (Gallenkamp), immersed in 20 mM-phosphate buffer pH 7.0 or 7.4 and scanned using a Unicam SP500 spectrophotometer with a Gilford gel scanning attachment. Systems established in test tubes were scanned directly.

Chemical determinations. Glucose was measured enzymatically using a Sigma glucose oxidase kit 510 following the method of Raabo & Terkildsen (1960). Absorbance measurements were made on an MSE Spectro-plus spectrophotometer.

RESULTS

Band formation in the standard gel

Incubation of a standard gel system containing *B. cereus* C11 for periods up to 18 d led to the formation of a number of sharply defined growth bands. Each band appeared to consist of densely packed microcolonies and in most of the experiments described, some growth occurred in the remainder of the gel layer irrespective of the number and position of the bands. The time course of band formation was followed (Fig. 1) and demonstrated that bands are generated consecutively rather than together. Earliest growth took place at the surface at the expense of CYS medium and, 48–72 h after this, the first sub-surface bands appeared just above the half-way mark in the semi-solid layer. Later bands appeared at successively higher positions in the layer. Each band grew to a maximum size which remained approximately constant over the period of the experiment.

Physico-chemical changes in the standard gel

Changes in the physico-chemical environment in replicate gels were determined after 0, 1, 3 and 5 d. The systems contained one-fifth strength CYS and 5 g glucose l⁻¹ in the source layer. The results (Fig. 2) showed changes in the physico-chemical composition of the gel over a period of time. pH, pO₂, glucose and redox potential values were determined as a function of depth. The pH profile changed during the first 24 h, showing three main regions: (1) from pH 7.8 at the surface to pH 6.1 at a depth of 11–12 mm; (2) from pH 6.1 to pH 5.3 between 12 and 26 mm;

Fig. 1. Development of bands of *B. cereus* C11 in a test tube gel system. The basal medium was full-strength CYS plus 20 g glucose l^{-1} in the source layer. The gel was incubated at 30 °C and was scanned directly using a Unicam SP500 spectrophotometer with a Gilford gel scanning attachment. The gel was 49.7 mm long at day 0 and 45.7 mm long at day 18 as a result of slight shrinkage.

Fig. 2. Changes in the physico-chemical environment in replicates of an uninoculated gel system and a system inoculated with *B. cereus* C11, and incubated at 30 °C. The basal medium was one-fifth strength CYS plus 5 g glucose l^{-1} in the source layer. (a) Redox potential after 0 (▼), 1 (■), 3 (●) and 5 (▲) d in the inoculated systems; (b) pH after 0 (▼), 1 (■), 3 (●) and 5 (▲) d in the inoculated systems. pH in the uninoculated system (indicated by the broken line) was 6.27 ± 0.12 for 98 determinations; (c) pO_2 in the inoculated system after 1, 3 and 5 d (■), and in the uninoculated systems after 1 (□), 3 (○) and 5 (△) d. (d) Glucose concentration in the inoculated systems after 0 (▼), 1 (■), 3 (●) and 5 (▲) d, and in the uninoculated systems after 0 (▽) and 5 (△) d. The redox potential measurements were made on a different set of replicates from the other measurements.

(3) a plateau region where the pH remained close to 5.3 from 26 mm to the bottom of the semi-solid layer. The first portion of this curve indicated that alkalization of the medium had occurred near the surface. After 3 d, the curve showed only two regions: (1) from pH 7.3–7.6 at the surface to values close to pH 5.3 at a transition point and (2) a plateau region where the pH did not vary much from pH 5.3 between the transition point and the base of the gel. The transition point changed from about 10 mm depth on day 3 to 8 mm on day 5. Thus, the gel system as a whole became progressively more acid with time, although the pH did not fall below 5.3. The pH in the control systems was 6.27 ± 0.12 for 98 determinations. In the inoculated systems, no oxygen was detected below the surface from day 1 onwards. In the uninoculated systems, the concentration varied from 100% at the surface to 39–54% at the bottom of the semi-solid layer. Glucose diffused into the semi-solid layer in both test and control gels, but less was present in the former since it was used by the organisms. Problems were encountered in measuring glucose at the top and the bottom of the gel because of the difficulty of obtaining accurate slices at the extreme ends of the gel, and because surface growth interfered with absorbance measurements of the glucose assay. Redox potential measurements were made on a separate batch of replicate systems. Redox potentials (corrected for pH) were generally quite positive and fell to a minimum in the region of the sub-surface bands and became a little more positive towards the bottom of the semi-solid layer.

The importance of oxygen in band formation

In the experiments described so far, no attempt was made to prevent oxygen from entering the system whilst the gel was being poured. The importance of this dissolved oxygen on band formation was tested. Test and control systems were set up in an anaerobic cabinet and incubated anaerobically or aerobically. Only the inoculated test system incubated aerobically produced bands. For comparison, a gel system was set up aerobically and incubated anaerobically; this produced no bands.

In a separate experiment in which the gels were prepared aerobically, dissolved oxygen tensions were measured at different depths in test and control gels at various times after the gels had set (Fig. 3). In the uninoculated system, oxygen levels at the surface remained close to 100%, while values were nearer to 50% at 10, 20 and 30 mm depth over the measurement period. The sub-surface value reflected the level of oxygen which entered the system when the molten gel was poured. In the inoculated system, oxygen profiles were quite different. At the surface, the level fell from 100% to about 85% after 220 min. At 5 mm depth, the level of oxygen fell to 0% after 145 min and at its lower points in the system (10, 15, 20, 25 and 30 mm) the level of oxygen fell more rapidly and was not detectable after 125 min. At a depth of 35 mm, the oxygen tension fell more slowly and oxygen was still detectable after 220 min. This was probably due to diffusion of oxygen from below, the oxygen having entered the lower source layer when it was poured.

The effect of oxygen partial pressure (up to $3 \times 10^5 \text{ N m}^{-2}$) was tested in gels containing full strength CYS medium plus 20 g glucose l^{-1} . Although band position varied slightly from that seen in gels incubated in air, there was no conclusive difference.

The importance of glucose in band formation

To test the dependence of band formation on glucose diffusion, glucose was incorporated into the upper layer at the same concentration as in the source layer, so that initially there was no bulk diffusion of the solute. No bands were seen over a period of 6 d. In a further experiment, replicate systems were set up in which diffusion of glucose was allowed to proceed whilst growth and metabolism were almost completely inhibited by storing the gels at 4 °C for different times before transferring to a 30 °C incubator. No major bands were seen in those gels which had been refrigerated for 2 d or longer before incubation.

To test the effect of glucose concentration, a number of replicate systems were established, each containing a different concentration of glucose in the bottom layer. The basal medium was one-fifth strength CYS and band position was measured for up to 12 d incubation. The number and position of bands varied with the concentration of glucose. The higher the concentration, the nearer the surface the bands occurred. No bands were seen in the systems containing 0 and 50 g glucose l^{-1} . Most bands were formed in the system which contained 5 g glucose l^{-1} .

Fig. 3. pO_2 measurements in (a) an uninoculated system at depths of 0 (∇), 10 ($\triangle$), 20 ($\circ$) and 30 ($\square$) mm, and (b) a system inoculated with *B. cereus* C11, at depths of 0 ($\blacktriangledown$), 5 ($\blacksquare$), 10, 15, 20, 25, 30 ($\blacktriangle$) and 35 ($\bullet$) mm. The gels were poured and allowed to set under aerobic conditions and measurements were made after the gels had set. The basal medium was one-fifth strength CYS plus 5 g glucose l^{-1} in the source layer.

The importance of basal nutrient concentration and source volume on band formation

The concentration of the CYS medium was varied from full to one-twentieth strength in replicate systems in which the source layer glucose concentration was 5 g l^{-1} . Band position was measured for up to 10 d incubation. The relative depth of the bands varied with the concentration of the CYS medium. At the highest medium concentration, bands were found low down in the gel, but as the medium concentration was reduced, the bands moved closer to the surface. In one-fifteenth and one-twentieth strength CYS, no sub-surface bands were seen, and most bands were formed in one-fifth strength CYS.

The effect of source layer volume on band number and position was tested using half strength CY/BMSM medium. Volumes tested were in the range 10–120 ml. In addition, one system contained a sterile Seitz membrane filter soaked with 10 ml medium plus agar. The total volume of the system was kept constant as was the total glucose concentration. Band position was measured for up to 12 d. Over much of the range, band position was approximately constant, although more bands appeared in systems with a smaller volume source layer.

The effect of agar concentration in the upper layer

The concentration of agar in the upper layer was varied from 0.1% to 1.5% (w/v). Between concentrations of 0.4% and 1.5% (w/v) agar there were only slight differences in the pattern of bands and no marked difference in the position of the lowest band. At the lowest concentrations of agar, 0.3 and 0.1% (w/v), bands appeared at lower positions in the gel, but these bands were not as clear or as sharp as those seen in the other systems.

Effect of agar type and of strain differences in cultures of B. cereus

The band patterns formed by seven different strains of bacteria which were either *B. cereus* or closely related to it were investigated in test tube systems using three different agars. The basal medium was full strength CYS and the glucose concentration in the source layer was 20 g l^{-1} .

Fig. 4. Differences in the band pattern produced by *B. cereus* C11 or closely related strains cultivated in test tube systems. The basal medium was full strength CYS plus 20 g glucose l^{-1} in the source layer. The gels were scanned, after 7 d incubation at 30 °C, using a Unicam SP500 spectrophotometer with a Gilford gel scanning attachment. The length of the gels was 46.2 ± 0.98 mm.

The bands formed were measured over a period of 26 d. In the purified agar and in the low gelling temperature agarose, all strains had formed at least three bands by day 7, except for *B. cereus* NCTC 9947 (Fig. 4). In the low electroendosmosis agarose, fewer bands were produced and this may have reflected the relatively high gelling strength of this agar. By day 26, the greatest number of bands in purified and low gelling temperature agars were formed by *B. cereus* C11, D11 and NCTC 2599. There were few major differences in the band patterns formed by the seven different organisms in purified and low gelling temperature agars, although fewer bands were seen in the low electroendosmosis agar.

Effect of pH

Phosphate buffer at pH 7.0 was added to the gel systems to a final concentration of between 0.01 and 100 mM. The basal medium was full strength CYS and the glucose concentration was 20 g l^{-1} in this and subsequent experiments. Only at the highest concentration (100 mM) were there significant differences in the pH profile and band positions (Fig. 5). In this case, most growth took place near the interface with the glucose source layer and extended for several mm into the upper layer. Two other bands were also seen. pH changed only slowly down the gel in contrast to pH profiles in the other gels.

Altering the initial pH value of the standard CYS medium led to some changes in band distribution. Thus, more growth took place nearer the glucose source layer where gels were initially more alkaline. Results described so far tend to implicate pH as a dominant factor in band formation. It was possible that alterations in nutrient composition might be important and gels were set up using spent CYS and CYS plus glucose media which had already supported overnight batch culture growth of *B. cereus*. These cell-free media were initially pH 8.2 or 5.6, respectively, and gels were established at these pH values and at pH 7.0 after neutralizing each medium. Standard gels were also set up. Results reinforced the view that pH value rather than

Fig. 5. pH profiles (○, ■, ●) and gel scans for three different systems inoculated with *B. cereus* C11. The basal medium was full-strength CYS plus 20 g glucose l⁻¹ in the source layer, initially at pH 7.0: (a) unbuffered; (b) plus 10 mM-phosphate buffer pH 7.0; (c) plus 100 mM-phosphate buffer pH 7.0. Measurements were made after 5 d incubation at 30 °C.

Fig. 6. pH profiles (○, □, ■) and gel scans for three different systems inoculated with *B. cereus* C11. The basal medium was full-strength CYS plus 20 g glucose l⁻¹ in the source layer: (a) incubated aerobically; (b) incubated anaerobically; (c) incubated anaerobically with an overlayer of 20 ml Tris/HCl pH 8.0. Measurements were made after 6 d incubation at 30 °C.

nutrient composition affected band formation since growth was severely restricted in the acid gel, whilst banded growth or zones of growth were seen in each of the other gels.

pH measurements were made on a gel which had been incubated anaerobically. Neither bands nor pH gradients were seen in this system, the pH value remaining close to 4.9 throughout, after incubating the gel for 6 d (Fig. 6*b*). Figure 6*a*) shows a gel scan and pH profile of an aerobically-incubated system.

It is inferred that the existence of an alkaline region near the surface of the systems incubated aerobically is responsible for periodic growth. An attempt was made to inhibit surface growth using a quaternary ammonium compound which binds to insoluble surfaces and strongly inhibits microbial growth. A disc of cotton fabric to which the agent was bound was placed on the gel surface. Surface growth was reduced and a smaller pH gradient developed. Banded growth was seen, but the position of the bands and their appearance was altered.

Further evidence to support the hypothesis that an acid/alkali counter-gradient was important in band formation was provided by the experiment in which gels were incubated anaerobically but with a source of alkali above the inoculated gel layer. At sufficiently high alkali concentrations, band formation was observed despite the anaerobic conditions (Fig. 6*c*).

A table giving information on band formation by *B. cereus* C11 and related strains grown under different conditions in the gel system is available on request.

DISCUSSION

The gel-stabilized nutrient gradient system as devised by Wimpenny & Whittaker (1979) and described in detail by Wimpenny *et al.* (1981) has been used as a laboratory model for investigating the microbial ecology of heterogeneous ecosystems in which growth is dominated by solute diffusion gradients. The observation that under appropriate conditions a variety of micro-organisms generated multiple growth bands in the gel led us to investigate the phenomenon further. *Bacillus cereus* was chosen because preliminary experiments indicated that more growth bands were generated using this organism than with any other. Bands were produced in an initially homogeneous semi-solid layer in a basal medium containing Casamino acids, yeast extract and salts, when opposing gradients of oxygen and glucose were present. Under standard conditions, band formation was quite slow when compared to growth in many commonly used microbial culture systems, taking a week or more for full development. This suggested that the process of periodic growth was diffusion rate-limited as might be expected, since diffusion coefficients for glucose and oxygen in aqueous media (of the order of $2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$) indicate that diffusion is slow. The first growth band was established low down in the gel and successive bands appeared sequentially between this and the surface. The process was relatively insensitive to agar concentration in the upper gel layer (between about 0.4% and 1.5%, w/v), nor was the type of agar employed very important. However, the low gelling temperature agar could be valuable for cultivating organisms with low temperature optima in gel systems. Band formation was probably unrelated to motility, since high agar concentrations did not influence band position and band formation occurred in cultures of non-motile species (Wimpenny & Whittaker, 1979; Wimpenny *et al.*, 1981). Experiments not reported here suggested that temperature did not affect band formation significantly; again, this might be expected if the process was dominated by diffusion rates.

Physico-chemical changes in the gel itself were largely predictable. Thus opposing glucose and oxygen gradients could be measured and lowest E_h values appeared near the subsurface bands. None of these parameters exhibited any sign of periodicity, though this was unlikely anyway since band formation was a sequential process whilst each gel was analysed at a single point in time.

Band formation was controlled by oxygen, by glucose and by the concentration of the basal medium. No bands appeared when oxygen was absent even in gels which initially contained some oxygen. Experiments showed that oxygen originally present was rapidly removed by the culture. By the same token, high or zero glucose concentrations in the semi-solid layer abolished band formation. The presence of a glucose gradient or of extremely low glucose concentrations in the semi-solid layer were necessary for band formation. The greater the glucose concentration in either layer, the nearer the surface were the bands. In addition, reducing the basal nutrient concentration also caused band formation to move nearer the gel surface.

The results of pH gradient determinations were of considerable interest. It was clear that deep in the semi-solid layer *B. cereus* generated acidic fermentation products which led to a pH value close to pH 5.3 or even lower in systems with higher amounts of glucose in the source layer (e.g. Figs 5 and 6). Nearer the surface the pH became more alkaline than in uninoculated controls. Alkalinization was almost certainly due to aerobic oxidation of amino acids. The two processes, fermentation and aerobic oxidation, led to steep pH gradients in the gel whose point of inflexion was close to the position of the sub-surface bands. It seemed likely that acidification inhibited sub-surface growth, so that such growth could only occur when two conditions were fulfilled: (i) sufficient glucose was present to allow fermentation reactions to provide metabolic energy; (ii) the pH value was above 5.3. The pH discontinuity ascended the semi-solid layer (Fig. 2), as did the appearance of the bands (Fig. 1). It seems likely that pH gradients determine growth position. This was confirmed by two further critical experiments. In the first, phosphate buffer at different concentrations was added to the system. The pH profile was altered in the presence of 100 mM-phosphate buffer at pH 7.0 and this led to a change in band position and intensity. In the second, gels incubated anaerobically showed no banded growth and demonstrated a constant acidic pH value. Addition of an upper alkaline gel layer generated both a pH gradient and banded sub-surface growth.

It is clear that pH gradients are responsible for this particular manifestation of sub-surface growth. It is not obvious what causes 'chopping' of the growth into discrete bands. A computer simulation of the system using simple Monod growth kinetics is incapable of generating periodic phenomena. It predicted that growth should ascend the gel when glucose diffuses into alkaline regions as a single smoothly-moving growth front, instead of in separate bands. Spatial periodicities can be generated in the computer simulations if a mechanism involving an asymmetric activation threshold is invoked (S. Jaffe, personal communication). It is not clear what the physiological basis of such a mechanism is except that it might involve sporulation and germination. This explanation is under investigation.

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