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Detection of growth of *Escherichia coli* on two-dimensional diffusion gradient plates

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One drawback of using two-dimensional diffusion gradient plates, the subjective visual assessment of microbial growth, has been overcome. Growth of *Escherichia coli* was detected with pH indicators in the medium or by staining growth with a biochemical stain, L-alanine-*p*-nitroanilide, or a respiratory dye, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride. Stained growth was scanned with a laser densitometer and traces combined in a computer to give a three-dimensional semi-quantitative representation of growth over the gradient plate.

Microbial growth in foods is controlled by factors acting in combination (Roberts *et al.* 1979). Traditional methods of screening potentially inhibitory combinations of antimicrobial substances are cumbersome. The gradient plate system devised by Szybalski (1952) and developed by Wimpenny & Waters (1984) offers the potential of combining two antimicrobials in the plate and incubating replicate plates at different temperatures. Growth detection has been by visual assessment (Wimpenny & Waters 1984; Waters & Lloyd 1985), making it difficult to determine the exact boundary of growth.

Two methods of indicating the extent of growth are presented. The first uses a pH indicator incorporated into the medium, the acid produced by microbial growth inducing a colour change. The second method uses a stain. L-alanine-4-nitroanilide chloride acts as a substrate for an aminoendopeptidase present in Gram-negative bacteria (Cerny 1976), which liberates *p*-nitroaniline producing a yellow colour. 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) is an electron transport system indicator, and is reduced by respiring bacteria to INT-formazan crystals (Kidd-Haack *et al.* 1985) which accumulate in the cells and produce a red stain.

The extent of growth across the plates was measured by a scanning laser densitometer. Traces from the densitometer were digitized and compiled by computer to give three-dimensional images of growth. By combining staining with densitometry, recording the extent of growth on gradient plates becomes accurate, reproducible and objective.

Materials and Methods

ORGANISM AND MEDIA

Escherichia coli I (NCTC 9001) was maintained at 27°C on Nutrient Agar (Difco) (23 g/l glass distilled water). Inocula comprised 0·5 ml of a 24 h/37°C subculture in 20 ml Brain Heart Infusion Broth (BHI) (Difco) (*ca* 10^8 cells/ml) spread over the surface of the plate with a sterile glass spreader.

PLATE PREPARATION

Two-dimensional gradient plates containing sodium chloride and sodium nitrite were prepared by the technique of Wimpenny & Waters (1984). The nitrite gradient was established by

including 0·1 ml (in the pH indicator plates) or 0·15 ml (in the plates to be stained) of a 750 g/l sodium nitrite solution (sterilized by filtration, Millipore, 0·22 μm) in layer 2, prior to pouring.

Plates using pH indicators also included 10 g/l glucose and either 0·8 g/l bromocresol purple (pH 6·3) or 0·05 g/l phenol red (pH 7·9) in layers 3 and 4. The pH of the phenol red layers was adjusted to 8·0 with 0·01 mol/l sodium hydroxide. After autoclaving, media were maintained at 80°C until poured. Plates were left at room temperature for 24 h to equilibrate vertically by diffusion, and were then inoculated.

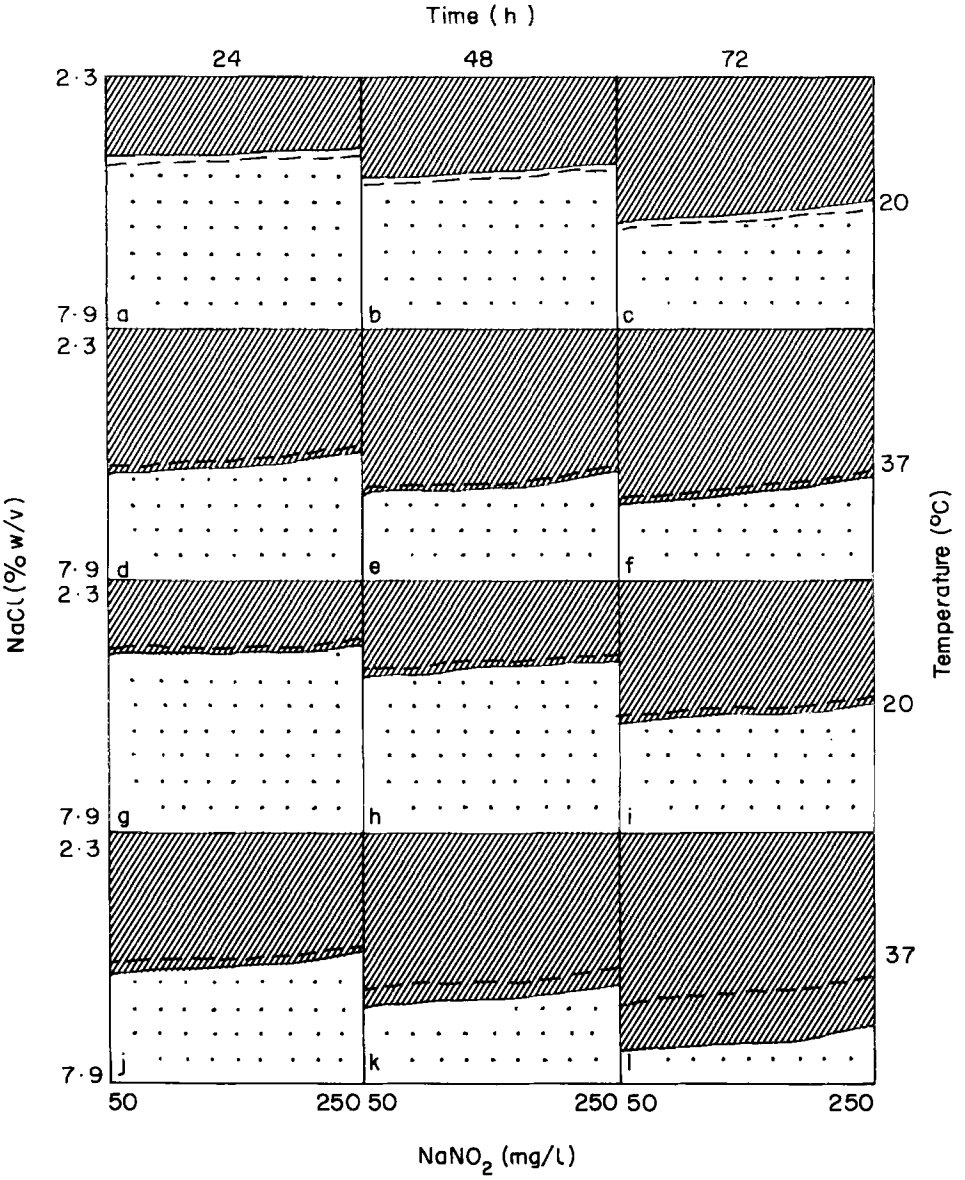

Fig. 1. Growth of *Escherichia coli* on two-dimensional gradient plates of salt and nitrite incubated for up to 72 h at two different temperatures using bromocresol purple (a to f) and phenol red (g to l) as indicators of growth. Areas of colour change are indicated by the hatched areas bordered by solid lines; areas of perceptible surface growth are bordered by dashed lines. The dots indicate 10 mm grid intersections.

NITRITE MEASUREMENT

Nitrite concentrations in cork borer samples were determined according to the method described in the British Standards Institution BS4401 (Part 8, 1976).

APPLICATION OF STAINS

After incubation plates were sprayed with an aerosol of either a 4% (w/v) solution of L-alanine-nitroanilide hydrochloride (Sigma) in distilled water (pH between 6 and 7·5), or a 0·02% (w/v) solution of INT, to cover the entire surface of the plate, and were incubated at 37°C for 60 min.

MAPPING THE RESULTS

The agar medium was carefully removed from each Petri dish and scanned across the salt gradient at 1 cm intervals along the plate using an 'Ultrascan' laser densitometer (model 2202, LKB, Finland). The data were recorded on a LKB 2204 integrator, transferred to a mainframe computer, and the Gino-Surf library at Bristol University Computing Centre used to transform, process, plot and print the data.

Results

GRADIENT REPRODUCIBILITY AND STABILITY

The sodium nitrite gradient was reproducible in replicate plates after storing them for 96 h (24 h at room temperature and 72 h at 37°C), with an S.E. mean of ± 8 μg/ml over the whole plate and with little change up to 96 h. Surface growth of organisms led to significant changes in the concentration of nitrite in the medium; a 72 h culture of *E. coli* reduced the nitrite concentration by 66% where growth had occurred.

MAPPING THE GROWTH ZONE

Indicators of pH change

Growth on each plate was mapped after incubating for 24, 48 and 72 h at 20° and 37°C. Plates containing bromocresol purple were purple in the region of non-growth, red on the boundary of growth and yellow in the region of growth. Colour changes were distinct and coincided with the boundary of visible surface growth. Salt tolerance of *E. coli* was higher at the higher incubation temperature (see Fig. 1 a–f) and appeared to increase the time of incubation. The effect of nitrite was clearly visible at high concentrations, retarding the boundary of growth by up to 1 cm.

Plates including phenol red had two colours visible: red in the region of non-growth and yellow in the region of growth. Colour changes were also distinct but the indicator was more sensitive to pH change in the medium causing the colour boundaries to overtake the boundaries of visible growth, especially at the higher incubation temperature (Fig. 1 g–l).

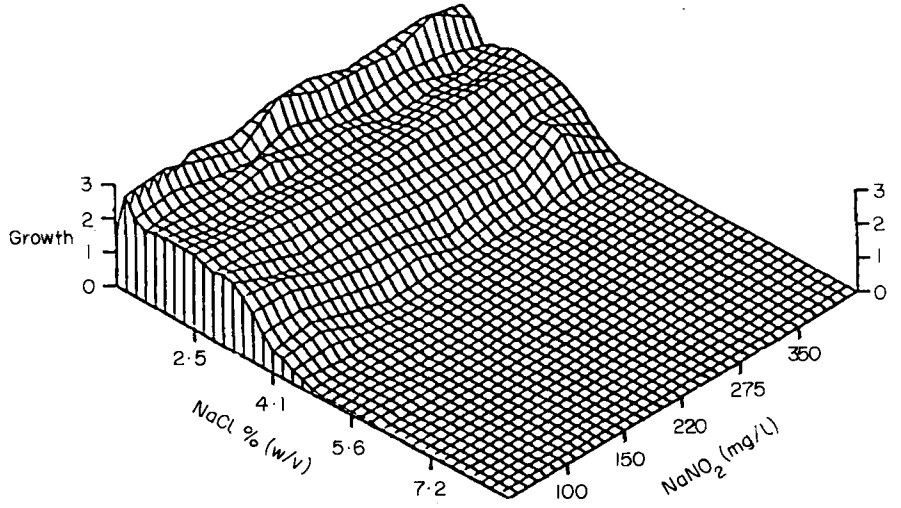

Fig. 2. A three-dimensional image showing the interaction of different values of salt and nitrite concentration on the growth of *Escherichia coli* at 20°C for 72 h. Stained with L-alanine-*p*-nitroanilide.

Aminopeptidase and respiratory stains

After incubation at 20° and 37°C growth was first mapped visually and then by scanning laser densitometer. Boundaries of growth indicated by the two stains were almost identical at both temperatures and after each incubation time with an S.E. mean of 0·2 cm each side of the boundary. Growth of *E. coli* across the plates increased with time of incubation at each temperature. An example of the pattern of growth is illustrated in Fig. 2. Colours produced by both indicators were stable for up to 24 h.

Discussion

The use of pH indicators appeared to be an accurate and reproducible method of visualizing the extent of growth on gradient plates although care is necessary when choosing the indicator. Bromocresol purple seemed preferable to phenol red because of its lower pH value, thereby needing more acid to produce a colour change. Detection of pH change was improved by including a buffer in the medium, retarding the effect of diffusion of acid. However, pH indicators are of only limited use because they give no indication of the number of viable cells present.

Staining cells appears to offer advantages. As well as indicating the boundary of growth across the plate, the intensity of the colours produced is indicative of the number of viable cells present, because the colour changes are only produced by active bacteria. The method of application is simple and the laser densitometer provides a convenient means of measuring growth. The limiting concentrations of salt and nitrite were identical to those seen in the bromocresol purple plates (and phenol red plates incubated at 20°C) under the same conditions of incubation. The respiratory dye, INT, was more easily detected.

Use of the biochemical stain and the respiratory dye improved location of the growth boundary and provided a semi-quantitative measure of growth. Each microbe will respond differently to the stains, depending on growth conditions, therefore comparisons between organisms grown under different conditions may be unwise.

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