The use of gel-stabilized gradient plates to map the responses of microorganisms to three or four environmental factors varied simultaneously

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1. SUMMARY

The Szybalski wedge plate technique has been used to map the responses of bacteria to up to 4 simultaneously varying environmental factors. 2-dimensional NaCl–pH gradient plates were used throughout. Sets of the latter were incubated at a range of temperatures to provide a third dimension. A fourth variable, nitrate concentration, was investigated by incorporating it homogeneously in sets of NaCl–pH gradient plates.

2. INTRODUCTION

Plate diffusion methods were first used quantitatively by Szybalski and his colleagues [1,2]. The method was later used to establish pH gradients [3] and as a single-dimensional double-gradient method to investigate synergism between metal ions and antibiotics [4]. Mapping two dimensions at once was first reported by Baas-Becking and his colleagues [5] who recorded responses of estuarine bacteria to pH and redox potential. An experimental system was devised to grow photosynthetic algae in 2 dimensions of light and temperature [6–8] and Caldwell et al. [9,10] constructed steady-state 2-dimensional gradient plates to investigate microbial growth. We have developed 2-dimensional gradient plates using the Szybalski wedge plate technique [11–13]. More than 2 variables can be changed simultaneously if a number of such plates are incubated at different temperatures or if they each contain a different concentration of a biologically active solute: what is more, both factors can be incorporated into the same experiment. Representative results of 3- and 4-dimensional experiments are reported here.

3. MATERIALS AND METHODS

3.1. Organisms and maintenance

The following organisms were maintained on nutrient agar slopes at 4°C as already described [13]: Micrococcus luteus NCIB8845 and NCIB8553; Serratia marcescens strains NCIB2302, NCIB4612, NCIB9155 and NCIB9523; NCTC1377, NCTC2847, NCTC10211 and NCTC10912.
Fig. 1. Three-dimensional experiments where cells are growing on 2-dimensional salt pH gradient plates at a range of different temperatures. (a) M. luteus strains NCIB8845 and NCIB8553. (b) S. marcescens strains NCIB2302, NCIB4612, NCIB9155, NCIB9523, NCTC1377, NCTC2847, NCTC10211 and NCTC10912. All organisms were maintained and grown as described earlier [13]. The heavily shaded area indicates red pigmentation: the lightly shaded area indicates pink colouration.
3.2. Preparation of two-dimensional gradient plates

2-dimensional pH vs. NaCl gradient plates were constructed in 10-cm square disposable plastic petri dishes as described in [13]. After pouring wedges for the pH gradient, plates were rotated through 90° and the second (salt) gradient was formed. The systems were dried and allowed to equilibrate for 24 h. An inoculum was spread over the surface of the plate, and the latter were incubated at the selected temperature. Values for pH and NaCl gradients, temporal changes, reproducibility of the technique and its application to a group of aerobic heterotrophic bacteria were reported by us [13].

The third environmental factor selected for investigation was temperature. Either 6 or 7 different temperatures were chosen, and plates or sets of plates incubated appropriately.

The fourth environmental factor was nitrate concentration. 6 sets of 6 plates each were made up at a range of KNO₃ concentrations from 0–0.75 M. Nitrate was incorporated in each of the 4 layers of the gradient plate so that concentrations in each plate were uniform.

3.3. Determination of growth zones

All plates were photographed at 24, 48 and 72 h. Boundaries of the growth zones were either
traced directly from prints or measured as X–Y coordinates and the data stored and replotted using a computer graph plotting routine.

4. RESULTS

4.1. Three-dimensional experiments

3-dimensional experiments were carried out for a number of strains of 2 species of bacteria, *S. marcescens* and *M. luteus*. In these experiments the third variable was incubation temperature, which ranged from 5–35°C.

8 strains of *S. marcescens* and 2 of *M. luteus* were obtained from British stock culture collections. Growth zones were determined (Fig. 1). From these results the two micrococci are easy to distinguish, in particular by their sensitivity to NaCl. Whilst the 2 micrococci show similar temperature responses, these are quite different from those of the *Serratia* strains. The latter also show the extent of pigment production. It is well known that this phenomenon is temperature-sensitive. In each of the strains examined here, pigment production ceases at temperatures between 30–35°C. Some of the strains are weak pigment producers, and here pigment production is also affected by pH and salinity. Even in well-pigmented strains pigment production is sensitive to high salt concentration, especially at more elevated temperatures. What is clear from the growth zones is the very significant difference between most of the strains. The exceptions to this generalization are strains NCIB2302 and NCTC1377, which appear to be identical, and NCIB9523 and NCTC10211, which are poor pigment producers and also resemble one another closely.

4.2. Four-dimensional experiments

A representative strain of *S. marcescens* was grown on 2-dimensional salt–pH gradient plates containing a range of 6 different nitrate concentrations. 6 identical sets of these plates were incubated at different temperatures. The 36 plates are shown in Fig. 2. Nitrate concentration inhibits both growth of this organism and to a lesser extent its ability to produce prodigiosin. There appear to be differences in colour produced. Thus, at higher temperatures and higher nitrate concentrations, the pigment is more purple than elsewhere on the plates.

5. DISCUSSION

The experiments outlined in this report suggest a powerful tool in discriminating between closely related species or between strains of the same organism and imply a value in identification and taxonomy. There are a number of other useful applications of these techniques, especially in the area of biodeterioration of materials and food spoilage. Using appropriate variables it is easy to see by inspection what combination of environmental factors is needed to prevent microbial growth, since outlining the growth zone for an organism clearly also delineates the regions where growth is prevented. The technique has applications in microbial ecology. For instance the microflora of one habitat can be easily compared with that of another on such plates. Competition studies are possible using mixed populations. Preliminary results indicate that certain pairs of bacteria can be differentiated on gradient plates, however, further more detailed experimental work is needed here, especially in discriminating 2 organisms from confluent mixtures on the plates. There are applications on the theoretical side of microbial ecology, too. Hutchinson [14,15] has suggested that the niche of an organism consists of an n-dimensional hyperspace of environmental factors and cellular responses to such factors. It has not always been easy to visualize an n-dimensional hyperspace, however, the techniques outlined here suggest ways of realizing at least 4 dimensions in a simple practical experiment. 4-dimensional experiments are by no means an upper limit. For example, time may be regarded as a fifth dimension in these experiments if photographs of sets of plates are taken at known intervals. Yet another dimension could be incorporated if each of a number of sets of the 36 plates shown in Fig. 2 were to contain a different concentration of another solute.
REFERENCES