Investigation of the Effect of Combined Variations in Temperature, pH, and NaCl Concentration on Nisin Inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*

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Gradient plates were used to investigate the effects of varying temperature, pH, and sodium chloride (NaCl) concentration on nisin inhibition of *Staphylococcus aureus* and *Listeria monocytogenes*. Nisin was incorporated into the plates at 0, 50, 100, 250, and 500 IU ml⁻¹. Gradients of pH (3.7 to 7.92) at right angles to NaCl concentration (2.1 to 7% [wt/vol]) were used for the plates, which were incubated at 20, 25, 30, and 35°C. Growth on the plates was recorded by eye and by image analysis. The presence of viable but nongrowing cells was revealed by transfer to nongradient plates. Lower temperatures and greater NaCl concentrations increased the nisin inhibition of *S. aureus* synergistically. Increasing the NaCl concentration potentiated the nisin action against *L. monocytogenes*; the effect of temperature difference was not so apparent. Between pH 7.92 and ca. pH 5, a fall in pH appeared to increase nisin's effectiveness against both organisms. At more acid pH values (ca. pH 4.5 to 5), the organisms showed resistance to both nisin and NaCl at 20 and 25°C. Similar results were obtained with one-dimensional liquid cultures.

Nisin is a small polypeptide bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*, which has been used for some years as a preservative by the food and dairy industries. Nisin inhibits the vegetative growth of gram-positive organisms, such as the food-borne pathogens *Listeria monocytogenes* and *Staphylococcus aureus*, and prevents the outgrowth of *Bacillus* and *Clostridium* spores. Nisin acts on the cytoplasmic membranes of vegetative cells, forming pores which allow the efflux of potassium ions, ATP, and amino acids; the action of nisin also results in the dissipation of the proton motive force (15, 19, 20).

Two-dimensional gradient plates (3, 21, 31) have been used to investigate the combined effects of environmental variables on bacteria as single species (17, 25, 29–31) or as a group of species from the same genus (26) and on competition between different bacterial species (22, 23). The gradients of these plates have included pH, sodium chloride (NaCl) concentration, sodium nitrite concentration, and temperature (13, 14, 17, 25, 29, 32). The combined effect of three variables has been investigated by incubating plates with gradients of pH and NaCl concentration at different temperatures (32). A fourth variable, such as an inhibitory substance, can be incorporated homogeneously at different concentrations in separate sets of plates (24–26).

In the present study gradient plates were used to investigate the effect of simultaneous variations in temperature, pH, and NaCl concentration on the inhibitory effect of nisin against the gram-positive food-borne pathogens *L. monocytogenes* and *S. aureus*.

MATERIALS AND METHODS

Organisms and maintenance. *S. aureus* CRA410 and *L. monocytogenes* F44642 were obtained from the Campden Food and Drink Research Association, Chip-ping Campden, Gloucestershire, United Kingdom. These were grown at 35°C on BHIYEG medium containing, per liter of distilled water, 37 g of brain heart infusion (Difco Laboratories, Detroit, Mich.), 3 g of yeast extract (Difco), 3 g of

glucose (BDH Chemicals Ltd., Poole, United Kingdom), and 15 g of agar (Difco). Broth cultures were grown in the same medium without agar. Organisms were maintained on glass beads at –70°C (12).

Plate production. The gradient plates consisted of four 15-ml agar layers in 10-cm² wettable-surface petri dishes (Bibby Sterilin Ltd., Stone, Staffordshire, United Kingdom). BHIYEG medium was made in 200-ml portions; acid and alkali were added for the first and second layers, respectively (25). The plates were raised by 3 mm at one end so that the first layer, which was acidic, set as a wedge. Once the first layer set, the plates were levelled and the second, alkaline layer was poured. The plates were turned through 90° and raised as described above, and the third layer, containing 15% (wt/vol) NaCl, was dispensed. Finally, the plates were levelled for the fourth layer, which was BHIYEG medium. Nisin (obtained from Aplin & Barrett Ltd., Beaminster, Dorset, United Kingdom) was prepared as a stock solution of 2.5×10^5 IU ml⁻¹ in 0.02 N HCl, filter sterilized, and added to each layer of the gradient plates to yield nisin concentrations of 0, 50, 100, 250, and 500 IU ml⁻¹.

Measurement of the gradients. Gradients were measured after 24 h, before inoculation. The pH gradients were determined at intervals of 1 cm across the plate surface with a flat-ended pH electrode (Orion Research Incorporated, Boston, Mass.). The NaCl gradient was measured by taking 5-mm core samples of agar across the plate at 1-cm intervals and melting these in 10 ml of distilled water. The conductivity was recorded with a Corning conductivity meter, model 220. Calibration curves were constructed by using BHIYEG medium containing known amounts of NaCl. The gradients were reproducible and similar to those in previous experiments (32); they remained stable over the incubation period.

Plate inoculation. Gradient plates were inoculated by flooding with 2 ml of mid-exponential-phase broth cultures followed by removal of any excess fluid. The plates were allowed to stand for a few minutes until the surface was dry, and then each was placed in the appropriate incubator. After 48 h of incubation at 20, 25, 30, and 35°C, an image of each plate was recorded by direct observation and by the image analysis system described below. Growth on the gradient plates was transferred to nongradient BHIYEG plates not containing nisin, by using sterile velvet cloths attached to a wooden block (10 by 10 cm) (22). The transferred cells were incubated for 48 h at 20, 25, 30, or 35°C, and images of these plates were recorded as before. This procedure enabled the detection of cells which were viable but nongrowing on the gradient plates.

Mapping growth. (i) **Direct observation.** Each plate was placed on a light box, and the outline of visible growth was traced onto graph paper. This procedure was used to generate Fig. 1b and 3.

(ii) **Image analysis.** Each plate was photographed by transmitted light with a monochrome TV camera (WV1800; National Panasonic, Industrial Video Systems, Cwmbran, United Kingdom). A digitized image was stored on a framestore (Synapse; Synoptics Ltd., Cambridge, United Kingdom) and processed with image processing software (SEMPER 6 Plus, version 6.3; Synoptics Ltd.) installed on an IBM-compatible microcomputer with an 80486 processor and an 80387 math coprocessor (System 300; Dell Computers Ltd., Bracknell, United Kingdom). Subtraction of the backgrounds of uninoculated plates was done by the program. By the application of Beer-Lambert laws (16), the image data were converted to absorbance values so that the growth was presented in optical

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FIG. 1. (a) Representation of a gradient plate showing the pH values and NaCl concentrations across each plate in panel b and Fig. 4. (b) pH-NaCl gradient plates with 0, 50, 100, 250, and 500 IU of nisin ml⁻¹. The plates were inoculated with *S. aureus* and incubated at 20, 25, 30, and 35°C. Visible growth after 48 h incubation is shown by shading.

density units. A region of no growth was selected on each plate to set the background to approximately zero. The data were exported to a commercial spreadsheet application (Lotus 123 [Lotus Development UK Ltd., Windsor, United Kingdom] and 3DGraphics [4-5-6-World, Colchester, United Kingdom]) and expressed as wire frame graphs (18).

Preliminary liquid culture experiments. BHIYEG cultures (20 ml each) were

prepared with appropriate pHs and concentrations of nisin and NaCl. The conditions were chosen to reflect the results and trends shown by the gradient plates. Triplicate cultures were inoculated with 20- μ l samples of mid-exponential-phase liquid cultures which had previously been incubated at the test temperature (20, 25, or 35°C). The cultures were incubated at the same test temperature for 18 h. Viable counts were taken in triplicate to determine the

FIG. 2. Wire frame representation of 48-h growth of *S. aureus* at 25°C on gradient plates with nisin at concentrations of 100 IU ml⁻¹ (a) and 500 IU ml⁻¹ (b).

inoculation count and the count achieved after incubation. The counts were expressed as log₁₀ CFU per milliliter ($\pm$ standard deviation).

RESULTS

***S. aureus*. (i) Gradient plate experiment.** Figure 1a indicates the pH and NaCl concentration gradients for all the gradient plates. Areas of visible growth of *S. aureus* on each plate after incubation are shown in Fig. 1b. Differences in the density of

growth within these areas are not indicated; these were recorded by the image analysis system. The growth area found after transfer is not shown since growth covered the whole area of the majority of the plates. The inhibitory effect of nisin was assessed by comparison with the control nisin-negative plates. The gradient plates (Fig. 1b) showed that decreased temperatures and increased NaCl concentrations worked synergistically with nisin to inhibit growth. The visible growth on certain plates, particularly those with higher concentrations of nisin (>100 IU ml⁻¹) incubated at 20 and 25°C, was confined to an arc-shaped region which was located in an area of the plate at ca. pH 5. The growth in this region of the plates was usually denser. Examples of wire frame graphs derived from the image analysis of two plates are shown (Fig. 2). Figure 2a shows growth on plates containing 100 IU of nisin ml⁻¹ incubated at 25°C. The growth in the acidic region was denser and extended over more of the NaCl concentration gradient than the growth at more neutral pH values on the same plate. At 25°C with 500 IU ml⁻¹ (Fig. 2b), visible growth was seen only under more extreme conditions (i.e., in an area centered around pH 4.8 and >2% [wt/vol] NaCl).

(ii) Liquid culture experiments. The effect of NaCl concentration on the nisin inhibition of *S. aureus* was further investigated by measuring the growth achieved after 18 h of incubation at 25°C in liquid BHIYEG cultures containing different concentrations of NaCl, at pH 7.05 with 100 IU of nisin ml⁻¹. Counts in nisin-containing cultures that contained 0.5 to 6.5% (wt/vol) NaCl were at least 2 log cycles below the counts in the

TABLE 1. Effect of initial NaCl concentration on nisin activity

Initial NaCl concn (wt/vol)	Viable count at indicated nisin concn (IU ml ⁻¹) ^a			
	<i>S. aureus</i> ^b		<i>L. monocytogenes</i> ^c	
	100	0	500	0
0.5	6.6 $\pm$ 0.17	8.9 $\pm$ 0.066	5.7 $\pm$ 0.26	8.6 $\pm$ 0.22
1.5	6.9 $\pm$ 0.24	8.7 $\pm$ 0.20	5.5 $\pm$ 0.30	8.9 $\pm$ 0.14
2.5	6.1 $\pm$ 0.29	8.6 $\pm$ 0.22	4.2 $\pm$ 0.31	9.0 $\pm$ 0.036
3.5	5.8 $\pm$ 0.66	8.7 $\pm$ 0.081	4.0 $\pm$ 0.58	9.1 $\pm$ 0.10
4.5	5.0 $\pm$ 0.33	8.6 $\pm$ 0.080	2.0 $\pm$ 0.38	9.0 $\pm$ 0.12
5.5	3.7 $\pm$ 0.72	8.5 $\pm$ 0.17	2.0 $\pm$ 0.10	8.5 $\pm$ 0.25
6.5	3.4 $\pm$ 0.56	8.3 $\pm$ 0.055	ND ^d	7.6 $\pm$ 0.18

^a Viable count data (expressed as mean log₁₀ CFU ml⁻¹ $\pm$ standard deviation) from 18-h liquid BHIYEG cultures at pH 7.05.

^b Inoculated at 4.9 $\pm$ 0.041 CFU ml⁻¹ and incubated at 25°C.

^c Inoculated at 4.7 $\pm$ 0.12 CFU ml⁻¹ and incubated at 35°C.

^d ND, no counts detected.

TABLE 2. Effect of initial pH on nisin activity

Initial pH	Viable count at indicated nisin concn (IU ml ⁻¹) ^a			
	<i>S. aureus</i> ^b		<i>L. monocytogenes</i> ^c	
	500	0	100	0
6.5	5.9 ± 0.10	9.0 ± 0.050	5.5 ± 0.22	9.0 ± 0.072
6.2	2.7 ± 0.58	8.4 ± 0.058	2.7 ± 0.76	8.8 ± 0.10
5.9	3.9 ± 0.28	8.2 ± 0.022	ND ^d	8.5 ± 0.05
5.6	3.4 ± 0.27	7.8 ± 0.15	1.7 ± 0.36	8.3 ± 0.17
5.3	3.6 ± 0.39	7.6 ± 0.088	1.2 ± 0.40	7.9 ± 0.069
5.0	3.9 ± 0.13	6.5 ± 0.042	1.6 ± 0.26	7.3 ± 0.10
4.7	4.4 ± 0.078	5.8 ± 0.20	1.8 ± 0.44	4.9 ± 0.14
4.4	3.8 ± 0.25	5.2 ± 0.33	1.2 ± 0.17	4.3 ± 0.074
4.1	2.5 ± 0.22	4.4 ± 0.11	1.1 ± 0.18	3.1 ± 0.39
3.8	1.1 ± 0.17	3.5 ± 0.15	ND	2.7 ± 0.71

^a Viable count data (expressed as log₁₀ CFU ml⁻¹ ± standard deviation) from 18-h liquid BHIYEG cultures containing 2% (wt/vol) NaCl.

^b Inoculated at 5.5 ± 0.047 CFU ml⁻¹ and incubated at 25°C.

^c Inoculated at 4.4 ± 0.093 CFU ml⁻¹ and incubated at 25°C.

^d ND, no counts detected.

nisin-negative controls (Table 1). At an initial NaCl concentration of 6.5% (wt/vol) the total counts in the nisin cultures were ca. 5 log cycles less than the total counts in the controls. As expected for *S. aureus*, growth in the absence of nisin was not inhibited by 6.5% (wt/vol) NaCl, which was the maximum initial NaCl concentration tested. The effect of pH on nisin inhibition was investigated in BHIYEG cultures containing 2% (wt/vol) NaCl and 500 IU of nisin ml⁻¹ at different pH values. After 24 h of incubation at 25°C the increase in acidity from pH 6.5 to 6.2 resulted in an increase in the efficacy of the nisin (Table 2). Between pH 5.9 and 4.7, the counts in the cultures containing nisin were greater than those at pH 6.2; within this range, counts were highest at pH 4.7. Between pH 4.4 and 3.8, a fall in pH correlated with increased effectiveness of the nisin. Counts of the control cultures showed a steady drop which correlated with increasing initial acidity.

***L. monocytogenes*. (i) Gradient plate experiment.** The area of growth of *L. monocytogenes* on gradient plates was not as extensive as that of *S. aureus* (Fig. 3). No arc-shaped growth region was seen in acidic areas of the plates as had been observed for the staphylococcus (Fig. 1b). The listeria grew in a confluent area from the corner of the plate with neutral pH

FIG. 3. pH-NaCl gradient plates with 0, 50, 100, 250, and 500 IU of nisin ml⁻¹. The plates were inoculated with *L. monocytogenes* and incubated at 20, 25, 30, and 35°C. Dark shading, visible growth after 48 h incubation on the gradient plates; medium and light shading, areas of dense growth and individual colonies, respectively, after transfer to nongradient plates that did not contain nisin and that were incubated at the same temperatures for 48 h.

FIG. 4. Wire frame representation of 48-h growth of *L. monocytogenes* at 35°C on gradient plates with nisin at a concentration of 500 IU ml⁻¹.

and low NaCl values. In the presence of nisin, an increase in NaCl concentration and acidity together reduced the area of visible growth on the plates (Fig. 3 and 4). The effect of temperature was more difficult to assess. Transfer to nongradient plates revealed areas on the gradient plates where cells had remained viable but had not grown at particular combinations of pH, NaCl, temperature, and nisin. The bactericidal effect of nisin at extremes of pH and NaCl was greater at 35 than at 20°C; at the latter temperature, cells remained viable over much of the NaCl gradient. At 20 and 25°C, a separate arch-shaped area of nongrowing but viable cells was revealed at a more acidic region of the nisin plates centered around ca. pH 4.5. The edge of this area corresponded to the pH limit of viability on the nisin-negative control plates (Fig. 3). The shape and location of this region were similar to those seen on gradient plates inoculated with *S. aureus*.

(ii) Liquid culture experiments. The effect of NaCl on nisin activity was investigated. BHIYEG cultures at pH 7.05 containing 500 IU of nisin ml⁻¹ plus differing NaCl concentrations were inoculated with $4.7 (\pm 0.12) \log_{10}$ CFU ml⁻¹. After incubation for 18 h at 35°C in the presence of nisin, a drop in cell numbers correlated with an increase in NaCl (Table 1). At 6.5% (wt/vol) NaCl no counts were detected in the nisin culture, compared with a count of 7.6 in the control culture. The increase in initial NaCl concentration (0.5 up to 5.5% [wt/vol]) had little effect on the growth of *L. monocytogenes* in the absence of nisin. The effect of pH on nisin inhibition was examined next. Cultures containing 100 IU of nisin ml⁻¹ and 2% (wt/vol) NaCl were inoculated with $4.4 (\pm 0.093) \log_{10}$ CFU ml⁻¹. After incubation in the presence of nisin for 18 h at 25°C, a fall in the number of surviving bacteria correlated with a change of initial pH values from pH 6.5 to 5.9 (Table 2). At pH

FIG. 5. Comparison of the effect of variation in NaCl concentration (a) with the effect of variation of pH (b) on the efficacy of 100 IU of nisin ml⁻¹ against *S. aureus* grown at 25°C. The activity ratio is the ratio of growth in the absence of nisin and growth in the presence of nisin.

5.9 no viable cells were detected, yet cultures with an initial pH between 5.6 and 4.1 contained at least $1.2 \log_{10}$ CFU of organisms ml^{-1} after incubation. Counts in the nisin-negative controls showed a gradual fall which correlated with an increase in the initial acidity of the cultures.

DISCUSSION

Gradient plates do not provide exact data but instead demonstrate overall trends and the influence of combined variables on growth. In the present study several general observations were noted and were confirmed in liquid culture experiments. For *S. aureus* decreasing the temperature apparently increased the sensitivity of the cells to nisin. Staphylococci are generally resistant to high concentrations of NaCl, but the gradient plates showed that lower temperatures and higher NaCl concentrations together increased the efficacy of the bacteriocin. Decreasing the temperature led to a reduced bactericidal effect of nisin on *L. monocytogenes*, which is similar to previous findings (1). Transfer from the listeria plates showed that at higher temperatures, greater NaCl concentrations worked synergistically with nisin to produce a bactericidal effect. At lower temperatures, higher NaCl concentrations increased the bacteriostatic effect only.

The unexpected finding with the gradient plates was that cells were resistant to the action of nisin when grown within a narrow pH range below the pH at which the cells were sensitive to the bacteriocin and approaching the pH at which they are unable to grow in the presence or absence of nisin. This value for *L. monocytogenes* was reported by Conner et al. (5) to be pH 4.0 when HCl was used as the acidulant. Gradient plates have been used to examine the effect of temperature, pH, NaCl concentration, and a preservative (sodium benzoate, sodium nitrite, or potassium sorbate) on the growth of food-borne pathogens (*Bacillus cereus*, Vero-cytotoxigenic *Escherichia coli*, and *S. aureus*) (24). Growth zones on these plates were always confluent and occupied a single area extending from the corner of the plate at neutral pH and low NaCl values. The separate growth area on the plates observed in the present study was new to our experience. Liquid culture experiments confirmed that the organisms showed unexpected resistance to the bactericidal effects of nisin within a narrow pH and salt range. Figure 5 (derived from data in Tables 1 and 2) plots the ratio of the growth of *S. aureus* in the absence of nisin to that in the presence of nisin as a function of pH and NaCl concentration. This contrasts the synergistic effect of NaCl concentration and nisin (Fig. 5a) with the more complex result for pH and nisin (Fig. 5b). Our observation that these food-borne pathogens may be less sensitive to nisin at certain acidic pH values has important practical implications.

So far we have no explanation for this observed drop in nisin's effectiveness under these particular conditions of acidity and NaCl concentration. Other workers have shown that the efficacy of nisin and other bacteriocins increases at acidic pH (2, 4, 28). Many factors are known to affect nisin activity, in particular the size and age of the inoculum, the composition of the medium, and the pH of the solution in which the nisin has been dissolved. Nisin is an acidic molecule which is more stable and easier to dissolve at lower pH (7, 11). For the purposes of our study the nisin was dissolved in 0.02 N HCl, filter sterilized, and used immediately. The action of nisin was compared in plates prepared at the same time and in the same way, with incubation temperature and nisin concentration as the only variables. It is believed that in order to be effective, nisin must first adsorb to the cytoplasmic membrane (10, 19, 27). It is interesting that this adsorption has previously been shown to

be reduced at acid pH (19) and that the amount of nisin adsorbed correlated with the sensitivity of *L. monocytogenes* to nisin (6).

Few studies have examined the combined effect of these variables on nisin inhibition. We suggest that gradient plates should be used as the basis for investigations into the effects of combined environmental variables on growth. A further variable that could be incorporated is a modified atmosphere. The effect of carbon dioxide atmospheres on nisin inhibition of *L. monocytogenes* and *Pseudomonas fragi* has been investigated (8), but the study did not take into account the additional effects of pH and NaCl concentration. Gradient plates provide a shortcut, enabling an informed approach to the design of future experiments. Gradient plates could also be used to compare the effectiveness of combinations of bacteriocins (9) and to isolate spontaneous nisin-resistant variants.

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REFERENCES

1. Abee, T., F. M. Rombouts, J. Hugenholtz, G. Guillard, and L. Letellier. 1994. Mode of action of nisin Z against *Listeria monocytogenes* Scott A grown at high and low temperatures. *Appl. Environ. Microbiol.* **60**:1962-1968.
2. Becker, B., W. H. Holzapfel, and A. Vonholty. 1994. Effect of pH and the bacteriocin carnocin 54 on growth and cell morphology of 2 *Leuconostoc* strains. *Lett. Appl. Microbiol.* **19**:126-128.
3. Caldwell, D. E., and P. Hirsch. 1973. Growth of microorganisms in two-dimensional steady-state diffusion gradients. *Can. J. Microbiol.* **19**:53-58.
4. Campbell, L. L., Jr., and E. E. Sniff. 1959. Nisin sensitivity of *Bacillus coagulans*. *Appl. Microbiol.* **7**:289-291.
5. Conner, D. E., V. N. Scott, and D. T. Bernard. 1990. Growth, inhibition, and survival of *Listeria monocytogenes* as affected by acidic conditions. *J. Food Prot.* **53**:652-655.
6. Davies, E. A., and M. R. Adams. 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. *Int. J. Food Microbiol.* **21**:341-347.
7. Delves-Broughton, J. 1990. Nisin and its uses as a food preservative. *Food Technol.* **44**:100-118.
8. Fang, T. J., and L. W. Lin. 1994. Growth of *Listeria monocytogenes* and *Pseudomonas fragi* on cooked pork in a modified atmosphere packaging/nisin combination system. *J. Food Prot.* **57**:479-485.
9. Hanlin, M. B., N. Kalchayanand, P. Ray, and B. Ray. 1993. Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. *J. Food Prot.* **56**:252-255.
10. Hirsch, A. 1954. Some polypeptide antibiotics. *J. Appl. Bacteriol.* **17**:108-115.
11. Hurst, A. 1981. Nisin. *Adv. Appl. Microbiol.* **27**:85-123.
12. Jones, D., P. A. Pell, and P. H. A. Sneath. 1984. Maintenance of bacteria on glass beads at -60°C to -76°C , p. 35-40. In B. E. Kirsop and J. J. S. Snell (ed.), *Maintenance of microorganisms*. Academic Press, London.
13. McClure, P. J., and T. A. Roberts. 1986. Detection of the growth of *Escherichia coli* on two-dimensional diffusion gradient plates. *Lett. Appl. Microbiol.* **3**:53-56.
14. McClure, P. J., T. A. Roberts, and P. Otto Oguru. 1989. Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium. *Lett. Appl. Microbiol.* **9**:95-99.
15. Montville, T. J., and M. E. C. Bruno. 1994. Evidence that dissipation of proton motive force is a common mechanism of action for bacteriocins and antimicrobial proteins. *Int. J. Food Microbiol.* **24**:53-74.
16. Peters, A. C. 1990. Using image analysis to map bacterial growth on solid media. *Binary Comput. Microbiol.* **2**:73-75.
17. Peters, A. C., L. V. Thomas, and J. W. T. Wimpenny. 1991. Effects of salt concentration on bacterial growth on plates with gradients of pH and temperature. *FEMS Microbiol. Lett.* **77**:309-314.
18. Peters, A. C., J. W. T. Wimpenny, L. V. Thomas, and J. Griffiths. 1991. Mapping bacterial growth on gradient plates using image analysis. *Binary Comput. Microbiol.* **3**:147-153.
19. Ramseier, H. R. 1960. The mode of action of nisin on *Clostridium butyricum*. *Arch. Mikrobiol.* **37**:57-94.
20. Ruhr, E., and H.-G. Sahl. 1985. Efflux of low Mw substances from the

- cytoplasm of sensitive cells caused by the staphylococcal-like agent Pep 5. FEMS Microbiol. Lett. **16**:75–79.
21. Szybalski, W. 1952. Gradient plates for the study of microbial resistance to antibiotics. Bacteriol. Proc. **1952**:36.
 22. Thomas, L. V., and J. W. T. Wimpenny. 1993. Method for investigation of competition between bacteria as a function of three environmental factors varied simultaneously. Appl. Environ. Microbiol. **59**:1991–1997.
 23. Thomas, L. V., and J. W. T. Wimpenny. Int. J. Food Microbiol., in press.
 24. Thomas, L. V., J. W. T. Wimpenny, and J. G. Davis. 1993. Effect of three preservatives on the growth of *Bacillus cereus*, Vero cytotoxigenic *Escherichia coli* and *Staphylococcus aureus*, on plates with gradients of pH and sodium chloride concentration. Int. J. Food Microbiol. **17**:289–301.
 25. Thomas, L. V., J. W. T. Wimpenny, and A. C. Peters. 1991. An investigation of the effects of four variables (pH, temperature, sodium chloride and sodium nitrite) on the growth of *Salmonella typhimurium* CRA663 using two types of gradient gel plates. Int. J. Food Microbiol. **14**:261–275.
 26. Thomas, L. V., J. W. T. Wimpenny, and A. C. Peters. 1992. Testing multiple variables on the growth of a mixed inoculum of *Salmonella* strains using gradient plates. Int. J. Food Microbiol. **15**:165–175.
 27. Thorpe, R. H. 1960. Action of nisin on spoilage bacteria. I. Effects of nisin on heat resistance of *Bacillus stearothermophilus* spores. J. Appl. Bacteriol. **23**:136–143.
 28. Tramer, J. 1964. The inhibitory action of nisin on *Bacillus stearothermophilus*, p. 25–33. In N. Molin (ed.), Microbial inhibitions in foods. Almqvist and Wiksell, Stockholm.
 29. Waters, P., and D. Lloyd. 1985. Salt, pH and temperature dependencies of growth and bioluminescence of three species of luminous bacteria analysed on gradient plates. J. Gen. Microbiol. **131**:2865–2869.
 30. Wimpenny, J. W. T., H. Gest, and J. L. Favinger. 1986. The use of two-dimensional gradient plates in determining the responses of non-sulphur purple bacteria to pH and NaCl concentration. FEMS Microbiol. Lett. **37**:367–371.
 31. Wimpenny, J. W. T., and P. Waters. 1984. Growth of microorganisms in gel-stabilised two-dimensional gradient systems. J. Gen. Microbiol. **130**:2921–2926.
 32. Wimpenny, J. W. T., and P. Waters. 1987. The use of gel-stabilized gradient plates to map the response of microorganisms to three or four environmental factors simultaneously. FEMS Microbiol. Lett. **40**:263–267.