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ORIGINAL ARTICLE

A model experimental gel surface for the growth of bacteria on foods

T. F. Brocklehurst*, G. A. Mitchell and A. C. Smith

A method is presented for the growth of bacteria on the surfaces of gels as a model of the changes that occur on food surfaces, and is demonstrated for Salmonella typhimurium*. The method is based on a gel cassette, which consists of a frame holding a layer of gel, in this case gelatin, formed between two PVC windows. After formation of a sterile gel, one of the PVC windows is removed to expose the gel surface for inoculation. Using this technique, the effect of sucrose and sodium chloride on the growth rate of* S. typhimurium *growing as surface colonies was compared with that of immersed colonies and planktonic cells in broth and was found to follow the order: broth>immersed colonies>surface colonies. The maximum numbers of cells decreased with increasing sucrose concentration and this decrease was more marked for surface colonies than for immersed colonies or planktonic cells. These results indicate that predictions of the growth rate of bacteria on food surfaces may be erroneous where these predictions are based on data collected in broth.*

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Introduction

Microbiological contamination of foods can result in the growth of micro-organisms in the bulk of the food or on its surface. In liquid medium, growth occurs planktonically, whereas growth can occur as discrete colonies in gels (Robins et al. 1994), gelled regions of food (Dodd 1990, Dodd and Waites 1991, Wimpenny et al. 1995), model emulsions (Brocklehurst et al. 1995d, Parker et al. 1995), on meat muscle surfaces (Mattila and Frost 1988a,b), and on other surfaces, such as cut vegetable tissues (Brocklehurst 1994).

Growth of bacteria on surfaces has been measured following direct inoculation of food (e.g. Canadian wieners, McKellar et al. 1994, pate, Farber et al. 1995), agar gels in petri dishes (Cooper et al. 1968, Wimpenny and Lewis 1977, Wimpenny 1979, Thomas et al. 1991, McKay and Peters 1995), an agar film coating a microscope slide (McKay and Peters 1995) or it can be modelled (e.g. Nicolai et al. 1993, who assumed growth was in a surface-film of liquid).

Typically, the increase in viable cells in surface-growing colonies shows exponential growth, although deviations occur as the colony expands and growth becomes limited. The extent of growth of colonies on a surface is influenced by the proximity of other colonies (Cooper et al. 1968). Model experimental systems based on agar gels in petri dishes have also been used to measure local chemical changes around and within colonies (Wimpenny and Coombs 1983, Peters et al. 1987, Robinson et al. 1991).

Food surfaces are rarely invariant with time and position, and a major influence on their potential to support bacterial growth is the availability of water (Clayson and Blood

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Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich NR4 7UA, UK

*Corresponding author

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1957). Drying of a food surface may be deliberate in order to inhibit growth, and methods for the study of desiccation of micro-organisms have been reviewed by Potts (1994), but gradual drying may also occur during storage. The same can be true of agar within a petri dish, where water loss occurs from the gel surface. Such water loss results in a water gradient extending from within the bulk of the food or gel to its surface. Micro-organisms growing on this surface are thus subjected to conditions which vary with position and time, and the local conditions are difficult to characterize.

We have developed a method to allow the measurement of growth of bacteria on the surfaces of gels poised to a constant water content. Ultimately, these data will be used to construct a model for the growth of bacteria on surfaces subject to continuous water loss as occurs on a drying food surface.

This paper reports preliminary experiments that compare the growth of colonies of *Salmonella typhimurium* on a steady-state surface with that of immersed colonies and planktonic cells. The bacteria were grown on gelatin, as a representative gel, that contained different concentrations of sodium chloride (NaCl) or sucrose, and that were housed in a cassette which also makes them amenable to microscopical examination.

Materials and Methods

Bacteria

S. typhimurium, strain LT2 (NCIMB 10248) was obtained from the National Collection of Industrial and Marine Bacteria, UK. The study reported here involved growth in a medium where the water activity was adjusted using sucrose, which could not be assimilated by this strain.

Culture media

Stock cultures were maintained on heart infusion agar (Difco) slopes, incubated at 25°C for 1 day, subsequently stored at 1°C and sub–cultured monthly.

Liquid growth medium consisted of trypticase soy broth (TSB, Baltimore Biological Laboratory), or TSB plus yeast extract (Difco), 0·3% (w/v) and glucose, 1% (w/v) (TSBYG) adjusted to pH 7·0 with HCl, 1 mol l^{-1}. Sucrose or NaCl was added to the TSBYG as required, and the medium sterilized by filtration through a 0·22 μm membrane filter (Millipore, UK).

The medium for gel cassettes that were used for growth of bacteria either within gel or on a gel surface was prepared using TSBYG solidified with gelatin. The TSBYG adjusted to pH 7·0 and with or without added sucrose or NaCl was prepared at twice the final concentration. The gelatin (approx. 225 bloom from bovine skin, Sigma) was prepared as a 20% (w/v) or a 40% (w/v) solution and adjusted to pH 7·0 by the addition of NaOH, 5 mol l^{-1}. It was sterilized by autoclaving at 121°C for 15 min, cooled to 32°C and combined with an equal volume of concentrated TSBYG. Culture media were thus prepared that contained either 10% (w/v) or 20% (w/v) gelatin.

Preparation of inocula

Bacteria were grown successively in TSB (10 ml) at 25°C for 24 h, and then at 20°C for 24 h. The A_{650} of the second culture was measured in a spectrophotometer (SP600, Pye Unicam) and the concentration of viable bacteria determined from a calibration curve. Inocula were prepared by dilution of a sample of the culture in peptone salt dilution fluid (PSDF, ICMSF 1978) to give a suspension that contained the required number of viable bacteria.

Preparation of gel cassette

The cassette comprised an acetal frame which was 2 mm thick and had outer measurements of 130 mm × 145 mm and a window within the frame that measured 100 mm × 100 mm, sealed within a sleeve of polyvinyl chloride (PVC) packaging film, 15 μm in thickness. Cassettes were formed by enveloping the frame within a sleeve of PVC that was heat sealed on three sides as described in Brocklehurst et al. (1995a,c). The cassettes were sterilized by autoclaving

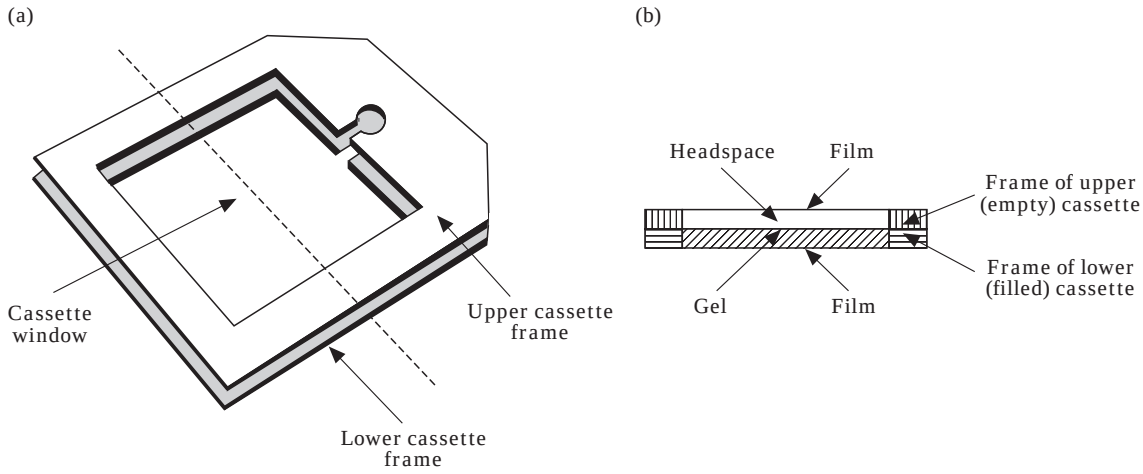

Figure 1. Line drawing of the modified gel cassette used for growth of surface colonies. (a) general view, where the dotted line represents the cross section shown in (b).

at 121°C for 15 min and the PVC on either side of the sterile cassette made taut in a stream of hot air.

Determination of the growth rate of bacteria

Growth in liquid medium was measured by inoculation of TSBYG (50 ml) in a 250 ml Erlenmeyer flask to give an initial inoculum concentration of approximately 10^2 viable bacteria ml^{-1}. Incubation and sampling were as described by Brocklehurst et al. (1995b).

Growth of immersed colonies of bacteria was in TSBYG solidified with 10% (w/v) gelatin housed in gel cassettes, as described by Brocklehurst et al. (1995a,c). The inoculum was added to TSBYG/gelatin medium to give a concentration of approximately 10^3 viable bacteria ml^{-1}. The inoculated medium was loaded into the gel cassettes, which were incubated at 20°C, and sampled periodically as described by Brocklehurst et al. (1995a,c).

For growth of surface colonies a gel surface was prepared using uninoculated TSBYG solidified with either 10 or 20% (w/v) gelatin. The latter concentration of gelatin was necessary to provide a gel that would support its own weight when placed vertically with the PVC film removed in order to allow exposure to flowing air of various relative humidities that will be required in further phases of this work. These gel cassettes were modified for growth of bacteria on the surface by removal of one of the two films covering the gel faces of a sterile cassette. Bacteria were inoculated onto the gel surface using a Model D spiral plate maker (Spiral Systems Inc.) which was fitted with a uniform cam, and thus allowed delivery of a constant volume of suspension with time. The total volume of inoculum deposited was 50 µl. This contained either 20 or 2000 viable cells and was deposited in a spiral fashion over 648 mm^2 of the surface of the gelatin gel in the gel cassette in such a way that did not damage the surface. An empty sterilized gel cassette was secured over the inoculated surface and the outer window of this cassette sealed with PVC film to provide a sterile head space for growth of colonies (Fig. 1). Inoculated cassettes were incubated as above. At intervals during incubation the upper PVC film was removed from a cassette and the gel removed and placed in a sterile Stomacher bag. The gel was melted by immersion in a water-bath at 36°C, or at 42°C in the case of high sucrose or 20% (w/v) gelatin gels. The molten gel was agitated vigorously to ensure uniform distribution of bacteria, diluted as appropriate in PSDF (or PSDF warmed to 32°C in the case of the high sucrose or 20% (w/v) gelatin gels) and viable counts made as described above.

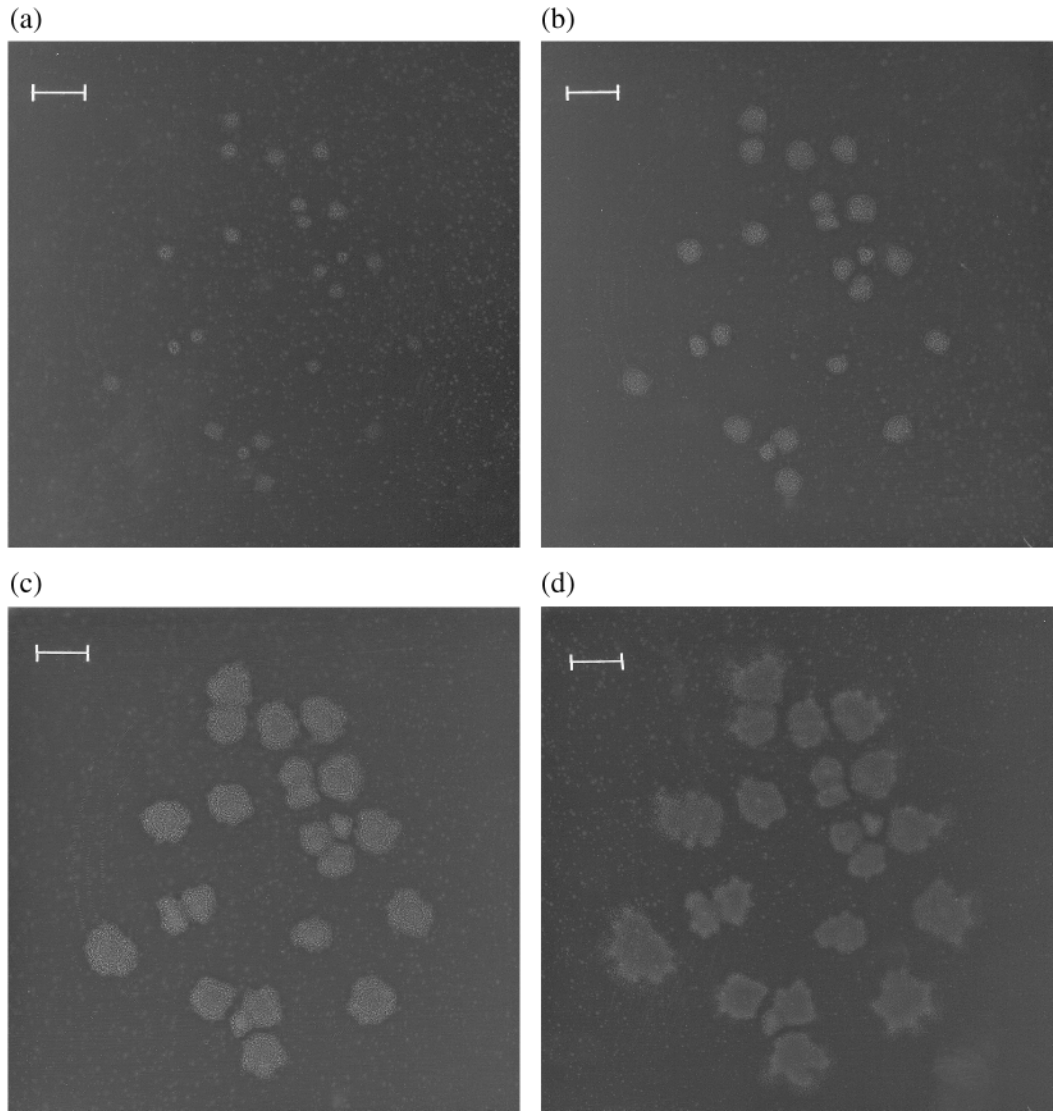

Figure 2. Photographs of colonies of *Salmonella typhimurium* LT2 on the surface of a gel cassette incubated at 20°C for (a) 40·5, (b) 46, (c) 63·5 and (d) 161 h. In all cases the scale bar=10 mm.

Enumeration of bacteria

Bacteria in liquid culture in TSBYG, or on or within gels of TSBYG solidified using gelatin were enumerated on duplicate plates of plate count agar (Oxoid CM325) (PCA) and on duplicate plates of PCA containing 3·5% (w/v) NaCl. The latter medium was calculated to be of the same water activity as media that contained either 3·5% NaCl or 30% (w/v) sucrose, the highest concentrations of humectants used (Lueck 1980), and had been shown to increase the viable count of bacteria recovered from media containing either NaCl or sucrose (Brocklehurst et al. 1995b,c, Mitchell et al. 1995). The use of high concentrations of sucrose alone in PCA was not possible due to the adverse effect on the gel strength of the agar.

Derivation of growth rate

Growth curves were fitted using a non-autonomous differential equation (Baranyi et al. 1993) in order to determine the doubling time.

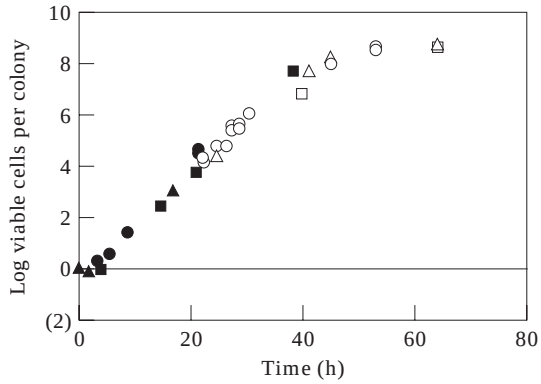

Figure 3. Numbers of viable cells colony^{-1} on the surface of modified gel cassettes during incubation of *Salmonella typhimurium* LT2 at 20°C. Counts are from three replicate experiments and are expressed as viable cells colony^{-1} as described in the text. Those sets of data represented by closed symbols (●, ▲, ■) are derived from cassettes inoculated with approximately 2000 cells and open symbols represent data derived from cassettes inoculated with approximately 20 cells.

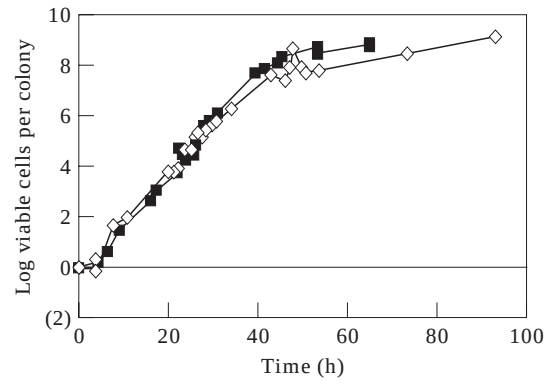

Figure 4. Effects of 10% w/v (■) and 20% w/v (◇) gelatin concentration on the increase in the numbers of viable cells of *Salmonella typhimurium* LT2 on the surface of gelatin gel during incubation at 20°C.

Measurement of pH

The pH of the growth media was measured at 20°C with a Radiometer digital pH meter (PHM92) and combined glass and reference electrode (Radiometer, type GK2421C).

Results

Determination of the growth rate of surface colonies of bacteria

The use of a spiral plate maker fitted with a uniform cam enabled the deposition of cells of bacteria over the surface of gelatin gels (Figs 2(a)–(d)). It was assumed that each cell inoculated onto the gel surface grew to produce an individual colony. The viable counting procedure resulted in a viable count that was a combination of all viable cells in all colonies on the gel surface. These data were divided by the initial inoculum concentration of the surface from which they were derived and hence represent the mean number of viable cells colony^{-1}. In the early stages of growth it was not possible to obtain reliable viable counts from gels inoculated with approximately 20 cells. Hence, those surfaces inoculated with 2000 viable cells were sampled. Using these methods, reproducible viable counts of the numbers of cells in colonies of *S. typhimurium* growing on the surface of gelatin gels were determined (Fig. 3), and also showed that equivalent growth occurred on gels that contained a final concentration of either 10% (w/v) or 20% (w/v) gelatin (Fig. 4).

Viable counts of bacteria recovered from surfaces of gelatin gel that contained 3·5% (w/v) NaCl or 20% or 30% (w/v) sucrose were higher in number when plated onto PCA that contained 3·5% (w/v) NaCl compared with standard PCA (Fig. 5). This phenomenon was also observed for growth of *S. typhimurium* in liquid culture (Brocklehurst et al. 1995b, Mitchell et al. 1995) and in gel (Brocklehurst et al. 1995c).

Comparison of growth rate of bacteria on surfaces, immersed in gel, and in liquid culture

The effect of NaCl and sucrose on the growth rate of *S. typhimurium* on the surface of gels, immersed in gels and in liquid culture is shown in Table 1. The maximum numbers of viable cells achieved in each system are shown in Table 2 as normalized ratios. These data show that an increase in the concentration of either NaCl or sucrose results in a decrease in the growth rate of surface

colonies, immersed colonies and of planktonic cells. As the concentration of NaCl or sucrose increases, the maximum number of cells achieved during growth decreases more in the case of surface colonies than in immersed colonies or liquid culture. Some of the growth rate data are re-expressed in Fig. 6 where the effect of sucrose concentration on the reciprocal of the doubling time is shown. This demonstrates clearly that at any concentration of sucrose, the rate of growth of *S. typhimurium* follows the order: broth>immersed colonies> surface colonies.

Discussion

The techniques described in this work enable growth of surface colonies to be characterized under carefully controlled, steady-state conditions. This is the first stage in prediction of growth of bacteria on surfaces subjected to a non-steady-state water content (i.e. during surface drying or rehydration).

The growth rate of *S. typhimurium* immersed in gel was slower than in broth, and slower on a gel surface than immersed in gel, as was also observed by Wimpenny and Lewis (1977). The increase in the numbers of viable cells on gel surfaces was exponential, consistent with observations of Wimpenny (1979), McKay and Peters (1995) and Maxcy (1976).

The lag times and growth rates on surfaces at 20°C were affected by the concentration of NaCl or sucrose in the gel, but not by gel strength in the range of 10–20% (w/v) gelatin. The effects of the NaCl and sucrose were consistent with the effects observed in liquid culture by Li and Torres (1993) and Brocklehurst et al. (1995b). This contrasts, however, with the work of McKay and Peters (1995), where increasing the concentration of NaCl over the range of 0·5–3·5% (w/v) had no effect

Table 1. Effect of concentration of NaCl and sucrose on the doubling time of *S. typhimurium* growing on gel, within gel and in liquid medium

Solute and concentration (%w/v)		Doubling time (h)		
NaCl	Sucrose	On gel surface	In gel	In liquid medium
0·5	0	1·5	1·3[a]	1·2[a]
0·5	10	1·9	NT	1·2[a]
0·5	20	2·9	2·2	1·9[b]
0·5	30	NG	3·6	3·2[b]
3·5	0	3·1	2·6[a]	2·0[a]

NT; not tested.
NG; no growth in 120 h.
a; data from Brocklehurst et al. 1995c.
b; data from Brocklehurst et al. 1995b.

Table 2. Effect of concentration of NaCl and sucrose on the normalized maximum numbers of viable cells achieved during growth of *S. typhimurium* on gel, within gel and in liquid medium

Solute and concentration (%w/v)		Maximum numbers of viable cells normalized by those achieved in the presence of 0·5% (w/v) NaCl and 0% (w/v) sucrose		
NaCl	Sucrose	On gel surface	In gel	In liquid medium
0·5	0	1	1	1
0·5	10	0·47	NT	0·6
0·5	20	0·0059	0·48	0·34
0·5	30	NG	0·15	0·25
3·5	0	0·0033	0·35	0·29

NT; not tested.
NG; no growth in 120 h.

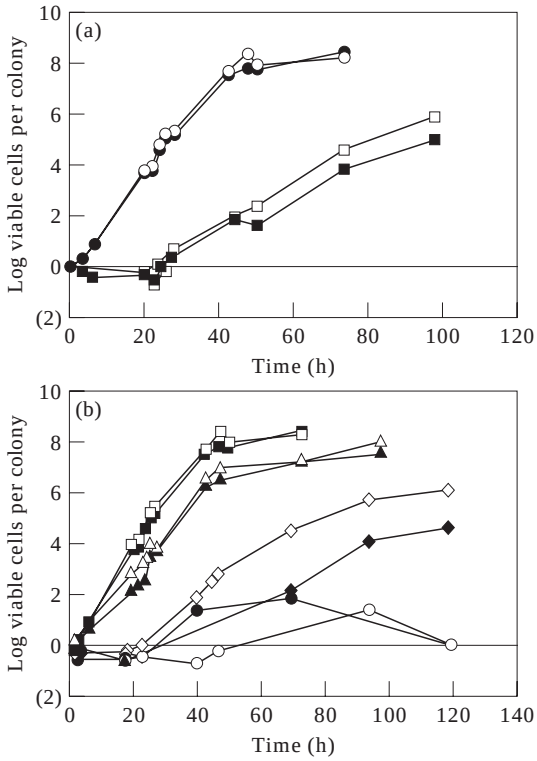

Figure 5. Effect of (a) 0·5% w/v (●, ○) and 3·5% w/v (■, □) NaCl and (b) 0% w/v (■, □), 10% w/v (▲, △), 20% w/v (◆, ◇), and 30% w/v (●, ○) sucrose on the growth of *Salmonella typhimurium* LT2 on the surface of gelatin gel incubated at 20°C. Enumeration was on standard PCA (closed symbols) or PCA+3·5% w/v NaCl (open symbols).

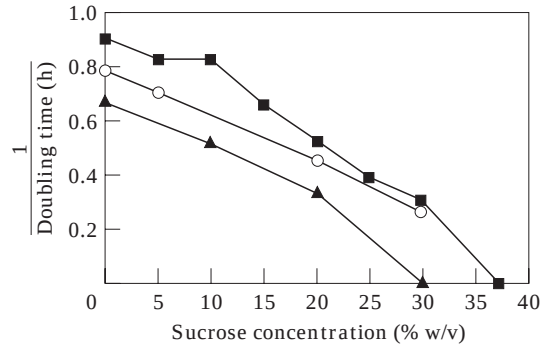

Figure 6. Effects of sucrose concentration on the growth rate of *Salmonella typhimurium* LT2 during growth in liquid medium (■), in bulk gelatin gel (○) and on the surface of gelatin gel (▲) during incubation at 20°C.

on growth rate of surface colonies of *S. typhimurium* at 30°C, although the lag time was increased. Thomas et al. (1991) showed that NaCl affected the growth of colonies of *S. typhimurium* on agar plates containing gradients of NaCl, although the measuring techniques used did not differentiate lag times and growth rates. They do, however, point out the importance of temperature of incubation on the limiting concentrations of NaCl for growth.

Growth of bacteria in gel has been shown to increase the minimum pH for initiation of growth when compared with liquid media (Brocklehurst, unpublished). The absence of growth on the surface of a gel containing 30% (w/v) sucrose indicates that the boundary conditions for growth on surfaces also differ not just from liquid culture, but from growth within the bulk of gel too. Inhibition of growth of colonies on a gel surface may result from limitation of diffusion of nutrient from the gel to all parts of the growing colony, although gradients of pH and O_2 occur within the gel beneath the colony and within the colony (Robinson et al. 1991, Peters et al. 1987). Such modification of the local environment will affect the local growth rate of the cells and hence contribute to inhibition, as well as result in less metabolic activity in some regions of the colony (Wimpenny and Parr 1979).

The effect of water activity on the inhibition of growth of micro-organisms is dependent on the type of solute (e.g. Li and Torres 1993, Chirife and Buera 1994). This is noticeable in the data presented here concerning growth in the presence of 30% (w/v) sucrose and 3·5% (w/v) NaCl. Growth on the surface of these gels was markedly different, although differences were also apparent in the bulk of the gel and in liquid culture. However, the calculated water activity of solutions that contained these concentrations of solutes were equal at 0·98 (Lueck 1980).

The maximum numbers of viable bacteria achieved within surface colonies were affected by the concentration of solutes in the gel, but would also be influenced by the proximity of other colonies (Cooper et al. 1968). Solutes had a much greater inhibitory effect on the maximum numbers of viable cells achieved during growth on the surface than during growth immersed within the gel or of plank-

tonic cells, indicating the greater vulnerability of surface colonies to inhibition.

All of the colonies studied in this work using either 10 or 20% (w/v) gelatin gels were discrete colonies. Some colonies, however, adopt a swarming growth regime, which allows them to overcome inhibition due to restricted diffusion (Wimpenny and Lewis 1977). This is an important consideration for the growth of bacteria on the surfaces of foods, and can be a response to an increase in the water content of a surface (Harshey 1994).

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