

# Solute-specific effects of osmotic stress on *Staphylococcus aureus*

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## ABSTRACT

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**Aim:** To determine if cell death from osmotic stress is because of lack of sufficient energy to maintain cell metabolism. Additionally, the solute-specific effect of five humectants on bacterial osmoregulation and cell survival was examined.

**Methods and Results:** *Staphylococcus aureus* was placed into 84% relative humidity (RH) broth (five humectants used individually). ATP, ADP and cell viability measurements were determined over time. The results indicate that ATP is not the limiting factor for cell survival under excessive osmotic stress. Although the same RH was achieved with various humectants, the rates of cell death varied greatly as did the sensitivities of the cell populations to osmotic stress.

**Conclusions:** The results from this study provide strong evidence that mechanisms of osmotic inactivation depend on the solute. The molecular mobility of the system may be an important means to explain these differences.

**Significance and Impact of the Study:** By bringing together an understanding of solute-specific effects, microbial physiology and genetics, the mechanisms of inactivation of micro-organisms by solute-specific osmotic stress may be elucidated, and this knowledge may then be exploited to ensure the production of high quality, safe foods.

**Keywords:** ATP, glass transition temperature, osmotic stress, *Staphylococcus aureus*.

## INTRODUCTION

Reducing the available moisture content of foods is a food preservation method that has been used since ancient times. *Staphylococcus aureus* has the ability to grow over a much wider range of relative humidities (RH; typically referred to as 'water activity' or  $a_w$ ) than any other food-associated bacterial pathogen (Bergdoll 1989). This micro-organism is the aetiological agent in food-borne staphylococcal poisoning and is one of the most prevalent causes of gastroenteritis worldwide (Olsen *et al.* 2000). It has been well documented

that *Staphylococcus aureus* is highly resistant to NaCl, surviving even in conditions of 25% NaCl (w%) (ICMSF 1996). This characteristic sets it apart from most other prokaryotic micro-organisms as well as the halophilic micro-organisms that require NaCl to grow. Although NaCl is the humectant typically used in staphylococcal studies, it has been documented that the humectant used to adjust the RH of the growth medium has a significant effect on the growth of *S. aureus* (Scott 1957; Marshall *et al.* 1971; Christian 1981; ICMSF 1996). Stewart *et al.* (2002) reported the limiting RH for growth of *S. aureus* to be ca 88% (pH 7.0) when sucrose–fructose (50 : 50, w/w) was used, 86% when glycerol was used and 85% when NaCl was used to adjust the RH of the system. The literature on microbial osmoregulation via compatible solutes has also shown that the type of humectant rather than the absolute value of RH is

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critical (Gould 1985; Townsend and Wilkinson 1992; Vijaranakul *et al.* 1995, 1997).

For the past several decades in the food science literature, the term 'water activity' introduced by Scott (1953) has been widely used to predict microbial growth and many common food deterioration reactions. Although  $a_w$  or RH is a better indicator for food stability and safety than the water content of a system, it is not always a reliable predictor. In recent years, increasing evidence has shown that molecular mobility may deserve further attention as it is related to many important diffusion-limiting properties of foods (van den Berg 1986; Slade and Levine 1991, 1995; Champion *et al.* 2000). Molecular mobility is governed by glass transition temperature ( $T_g$ ), a solute-specific property that is inversely linearly related to the measured RH of a system. The use of molecular mobility in conjunction with RH to understand microbial response to osmotic stress is discussed in further detail by Stewart *et al.* (2002).

Some of the mechanisms *S. aureus* utilizes to survive under osmotic stress are similar to those of gram negative enterics such as *Escherichia coli* and *Salmonella typhimurium*, both of which accumulate compatible solutes including proline and glycine betaine via transport. There are, however, several major differences, as enterics generally can only grow in NaCl concentrations of up to  $0.8 \text{ mol l}^{-1}$ , where *S. aureus* has been reported to grow at NaCl concentrations as high as  $4 \text{ mol l}^{-1}$  (Miller *et al.* 1991). Enterics have a two-phase process in which they adapt to osmotic stress. The process first allows the co-transport and rapid accumulation of potassium and glutamate ions, followed by the activation of transport systems that lead to the accumulation of compatible solutes (Booth *et al.* 1988). In contrast, when *S. aureus* is under osmotic stress, it increases turgor by initially increasing the compatible solute pools in the cytoplasm, precluding the transport of potassium and glutamate for regulating turgor (Pourkomialian and Booth 1992).

Glycine betaine and proline are the principal compatible solutes of *S. aureus*, with glycine betaine being the more potent osmoprotectant (Miller *et al.* 1991). Transport of these osmolytes is mediated by three constitutive systems: an osmotically sensitive, low affinity glycine betaine/proline transport system (BPII) and two osmotically insensitive, high affinity transport systems; one specific for proline (PPI) and the other specific for glycine betaine (BPI) (Bae and Miller 1992; Pourkomialian and Booth 1992, 1994; Townsend and Wilkinson 1992; Pourkomialian 1998). The active transport of glycine betaine and proline in *S. aureus* is achieved by sodium-dependent transport, and thus is energized by sodium motive force (Pourkomialian and Booth 1994). Additionally, the retention of the compatible solutes within the cell is an active process (Pourkomialian and Booth 1994).

The purpose of this study was to determine if cell death from osmotic stress is because of lack of sufficient ATP energy because most of the cell's energy is being spent on osmoregulation. Information on the mechanism of cell death would be critical in modifying the food safety dogma that states that all foods are at risk of supporting *S. aureus* growth if their RH is above 85. Two strains of *S. aureus* were utilized; RN4220 and an osmoregulatory mutant of RN4220, BP108, that lacks the low-affinity transport system for glycine betaine and proline. By using five humectants a comparison of their effects on microbial osmoregulation could be made including: (i) the effects of different glass transition temperatures on microbial survival, (ii) the effects of membrane permeable vs membrane impermeable glass-formers on microbial survival, (iii) the effects of ionic solutes vs glass-forming solutes on microbial survival and (iv) discriminating the effects of ions from the effects of molecular mobility as determined by  $T_g$ .

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

Two *Staphylococcus aureus* strains, RN4220 (from Dr C. Rees, University of Nottingham, UK) and BP108 (from Dr I. Booth, Marischal College, Aberdeen, UK), a transposition mutant of RN4220, lacking the low affinity transport system as described by Pourkomialian and Booth (1994) were used in this study. Their identification as *S. aureus* was confirmed by use of a Riboprinter<sup>®</sup> (Qualicon, Wilmington, DE, USA). RN4220 stock culture was grown overnight at  $37^\circ\text{C}$  in brain-heart infusion broth (BHI; Difco, Detroit, MI, USA), and BP108 stock culture was grown overnight at  $37^\circ\text{C}$  in BHI amended with  $10 \mu\text{g ml}^{-1}$  erythromycin (BHIE; erythromycin resistance used as the mutation marker). Both were suspended in glycerol freezing medium (66% glycerol, 1.6% each  $1 \text{ mol l}^{-1}$  KCl and NaCl, and 0.2%  $0.2 \text{ mol l}^{-1}$   $\text{MgSO}_4$ ) and stored at  $-80^\circ\text{C}$  until needed.

Quarter strength BHI broth was reconstituted with the appropriate ratio of water to sucrose-fructose (50 : 50, w/w), glucose-fructose (50 : 50, w/w), glycerol, NaCl or LiCl to achieve 84% relative humidity (RH). Quarter strength BHI was used because the amount of LiCl required to achieve a broth of 84% RH caused precipitation of full strength BHI. The appropriate amount of BHI powder was added to 1/2 of the required water and the appropriate amount of LiCl to the other 1/2 of the required water and then the LiCl solution was slowly added to the BHI solution. Growth curves with RN4220 indicated that similar growth was achieved utilizing either quarter strength or full strength BHI (data not shown). For experiments with strain BP108, broths were amended with erythromycin as described previously. Final RH was determined using an

Aqualab CX-2 water activity meter (Decagon Devices Inc., Pullman, WA, USA). The broths were filter sterilized and stored at 4°C until needed.

## Chemicals

All chemicals were obtained from Sigma (St Louis, MO, USA) unless otherwise stated.

## ATP determination

RN4220 and BP108 were removed from -80°C storage and one loopful was transferred separately into BHI or BHIE broth (9 ml) respectively. The inoculated broths were incubated for 18 h at 37°C, so that cells were in stationary growth phase. The BP108 culture was centrifuged for 10 min, the supernatant was removed and cells were resuspended in the minimal amount of BHIE to achieve an OD<sub>530</sub> = 1.0 (Perkin-Elmer 35 spectrophotometer). The overnight cultures (1.5 ml) were separately added to 150 ml of each of the 84% RH broths in glass screw-capped flasks and were incubated at 37°C. Samples for ATP assays and plate counts were taken at appropriate time intervals (over a period of up to 172 h). All experiments were duplicated on separate days.

Measurement of ATP and ADP levels were performed using a method adapted from Bracey *et al.* (1998) and Chapman *et al.* (1971). ATP was measured using the Celsis Personal Care Product (PCP) Kit (Celsis Inc., Edison, NJ, USA). For determination of ATP and ADP levels, two 50 µl samples of cell suspensions were each added to 200 µl of ATP Releasing Agent (from the PCP Kit) in sterile cryovials, mixed for 10 s, frozen in liquid nitrogen and stored at -80°C until assays could be performed. A total of 500 samples were analysed.

Samples were thawed at room temperature for 15 min, transferred to appropriate cuvettes, and placed in the luminometer (Celsis Advance Coupe; Celsis Inc.). The standard PCP Kit assay was modified. The luminometer was programmed to inject 65 µl of Chapman buffer (75 mmol l<sup>-1</sup> potassium phosphate buffer, pH 7.3, 15 mmol l<sup>-1</sup> MgCl<sub>2</sub>, and 0.5 mmol l<sup>-1</sup> phosphoenolpyruvate). The samples were then incubated for 15 min in the luminometer, 100 µl Bioluminescence Reagent was automatically injected and the light emission was measured after a 1-s delay over an integration period of 10 s. To measure ADP levels, the same procedure was carried out except the Chapman buffer contained 25 µg pyruvate kinase (PYK)/65 µl Chapman buffer to convert ADP to ATP. Total ATP (measured ATP plus converted ADP) minus measured ATP gives ADP levels.

To measure extracellular ATP and ADP, 300 µl samples were taken and cells were removed with a 0.22-µm syringe

filter (Fisher Scientific, Pittsburgh, PA, USA). The ATP and ADP levels in the cell-free broth were determined as described above. A total of 500 samples were analysed.

To determine the ATP and ADP concentration in the uninoculated broth, 50 µl of each broth was used and levels were determined as described above. Five replicate samples were analysed. The average relative light units (RLU) luminometer reading from the uninoculated broth was used as the blank.

To determine the effect of the broth on the bioluminescence response to ATP, the ATP and ADP assays were followed as described above with five replicate samples of 50 µl of each broth containing 10 µl ATP Positive Control (PCP Kit; Celsis Inc.). This was repeated as above, substituting the broth with 50 µl sterile (filter sterilized) water. The broth response to ATP was determined with the following calculation:

Response to ATP

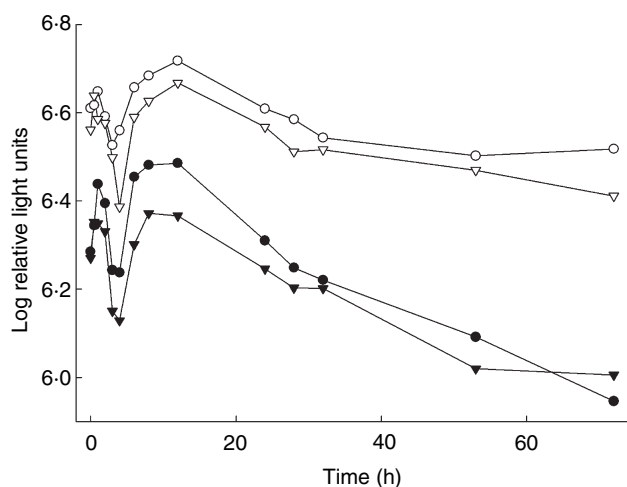
$$= \frac{(\text{Mean RLU of broth} + 10\mu\text{l ATP}) - \text{Mean RLU of broth}}{(\text{Mean RLU of water} + 10\mu\text{l ATP}) - \text{Mean RLU of water}} \times 100\%$$

The response to ATP for the broth is expressed as a percentage of that with water, where the water response is 100%. This was carried out to remove the effect of certain species, such as Na<sup>+</sup> and Cl<sup>-</sup>, which can quench the light produced by bioluminescence.

## Determination of intracellular levels of ATP and ADP

Calculations to determine the intracellular level of ADP were made as follows: A is the ATP level of the cells plus broth, B is the ATP + ADP level of the cells plus broth, C is the level of ATP in the cell free broth and D is the level of ATP + ADP in the cell free broth. Intracellular ATP was determined by A - C and intracellular ATP + ADP was determined by B - D. Intracellular ADP levels were determined by [(B - D) - (A - C)]. Intracellular energy balance was calculated by (A - C)/[(B - D) - (A - C)]. In many instances the resulting RLU value corresponding to intracellular ADP level was a negative number, making its use impractical to calculate the intracellular energy balance. The measured RLU values in the raw data sets were all valid as the ADP + ATP RLU values were always higher than the ATP RLU values. Additionally, the cells plus broth RLU values for both ATP and ATP + ADP determination were higher than the cell free broth RLU values in the vast majority of the data, as shown, for example, in Fig. 1. The trends in this figure are typical for all 10 data sets.

There are several factors that we believe contribute to the difficulties in our calculations. First, the Celsis PCP



**Fig. 1** Raw data of log relative light units over time for *Staphylococcus aureus* RN4220 in 84% relative humidity sucrose-fructose broth. ATP, cells and broth (●); ATP + ADP, cells and broth (○); ATP, broth after cells removed (▼); ATP + ADP, broth after cells removed (▽)

kit has been designed for determination of microbial contamination levels in personal care products or equipment and microbial ATP levels are determined at a single time point after growth under optimal conditions. The measured RLU values are directly proportional to the cell numbers in the linear range of the luminometer response when the intracellular ATP per cell or total net adenosine nucleotide pool per cell is constant. In our experiments, the cells were under extreme osmotic and ionic stress, far from optimal conditions, and the cell numbers were changing over time. Therefore, both the net pool per cell and the cell population were changing over time, so the measured RLU values represent the convoluted combination of the total pool per cell and the cell number. Secondly, because of the nature of the experiments, there were high levels of background ATP and ADP in the samples leading to high nontest variation or 'background noise'. The broths were filter sterilized rather than autoclaved to avoid caramelization of sugars in the three broths with high sugar concentrations and to avoid water loss from the media, which leads to difficulties in achieving the targeted RH. As the media were not autoclaved, high levels of background nucleotides present from both the water and the dehydrated BHI broth can be expected.

It was noted that the RLU values for any of the samples taken from the LiCl and NaCl broths or the uninoculated broths were several orders of magnitude smaller than those of samples taken from the other three media. Various chemicals and ions can cause quenching, and even after the samples were diluted as per the assay protocol, the solutes used to achieve the desired media

**Table 1** Concentration of solutes in 84% RH 1/4 strength BHI broth used in experiments and in solutions used for ATP assays

Solute	Concentration in broth (mol l <sup>-1</sup> )	Concentration during ATP assay (mol l <sup>-1</sup> )
Sucrose-fructose	6.70	0.92
Glucose-fructose	7.90	1.08
Glycerol	9.28	1.27
NaCl	4.33	0.59
LiCl	3.55	0.49

RH were still at high levels (Table 1). The extent of quenching caused by the high concentration of sugars ranged from 21.1 to 33.9% and the extent of quenching caused by the high concentrations of LiCl or NaCl ranged from 91.6 to 97.1%.

Taking into account all of these issues, it follows that the calculation of the intracellular level of ADP [(B - D) - (A - C)] introduces error by compounding the individual nontest variation already incurred in A - C and B - D. Additional error is introduced by the calculation of the energy balance for ATP/ADP (A - C)/[(B - D) - (A - C)]. As we are ultimately interested in determining how intracellular ATP changes with the total intracellular pool when the cells are under osmotic stress, and the total intracellular pool is ATP + ADP, we assume that the same information for energy balance is contained in total ATP/total ADP as in intracellular ATP/intracellular ADP. Therefore, total values (intracellular + extracellular) for ATP and ADP were determined by averaging the raw RLU values from duplicate experiments, subtracting the blank value, taking the log values, and calculating total ADP (B - A) and ATP/ADP [A/(B - A)]. These values are utilized in the remaining results and discussion sections of this paper.

### Measurement of cell numbers

Cell numbers were measured whenever samples were collected for ATP determination. Samples (1 ml) were taken, diluted appropriately in sterile 0.1% peptone water, and plated on BHI agar using an Autoplate<sup>®</sup> 4000 spiral plater system (Spiral Biotech, Bethesda, MD, USA). The plates were incubated at 37°C for 24 h before counts were determined using a QCount<sup>™</sup> plate reader (Spiral Biotech). A total of 250 samples were enumerated.

### Weibull curve fitting

Data were transferred to a Microsoft Excel spreadsheet. Curves were fit to experimental data using the Weibull equation in the Solver function in Excel.

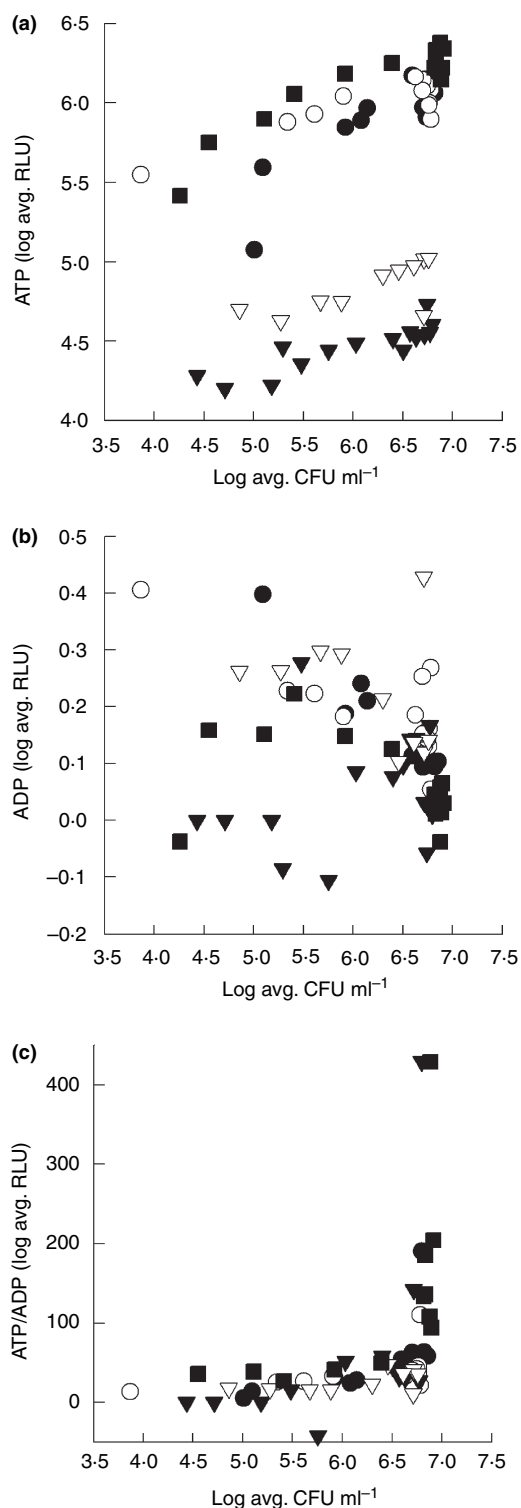
## RESULTS

### ATP, ADP and ATP/ADP levels

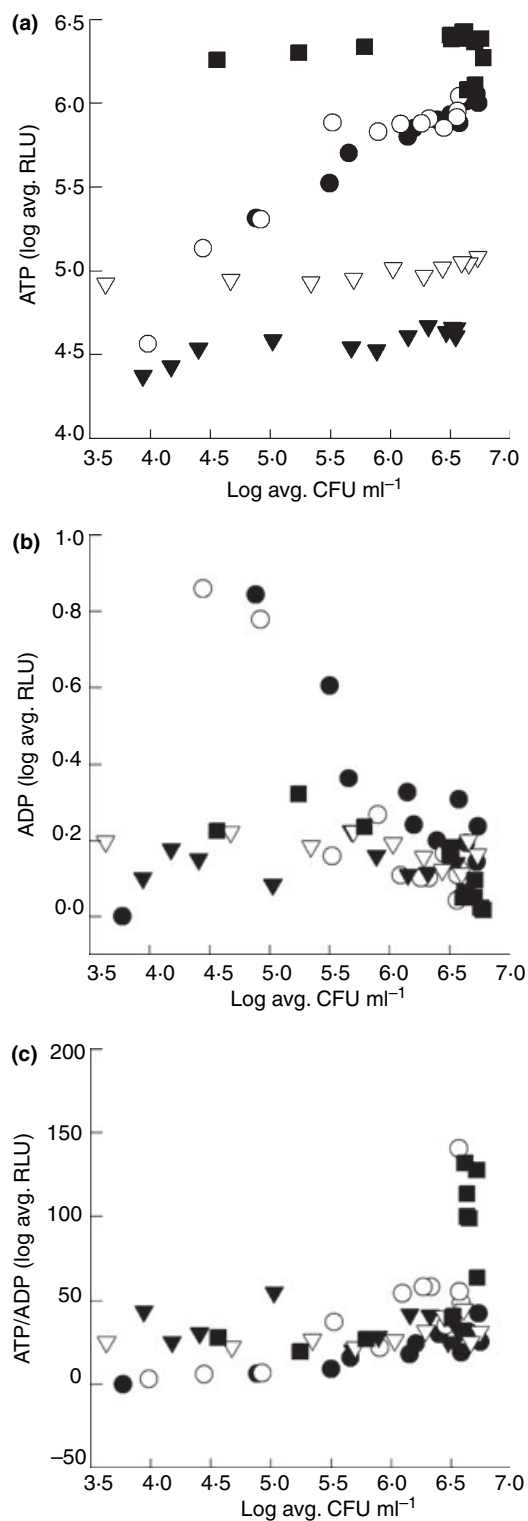
If a cell is using most of its energy for osmoregulation when under high osmotic stress, then it may not have the energy (i.e. ATP) required to continue metabolism and consequently dies. Assuming this is the mechanism of cell inactivation, then experiments should show a depletion of ATP with a concomitant increase in ADP over time prior to a decrease in cell numbers. No such patterns were found when ATP, ADP and ATP/ADP levels over time were examined for each experiment performed with either strain RN4220 (Fig. 2) or BP108 (Fig. 3). Regardless of the humectant or strain, the ATP levels remained steady until the number of viable cells decreased and then ATP levels decreased with decreasing cell numbers. When sucrose–fructose or glucose–fructose was the humectant, ADP levels tended to increase with a decrease in viable cells; when glycerol was the humectant, ADP levels first increased and then decreased and cell numbers decreased; and when NaCl or LiCl was the humectant ADP levels were variable but fairly constant. After an initial drop, ATP/ADP remained at a steady level even when cell numbers decreased (Figs 2 and 3). The exceptions with the BP108 strain were observed in the LiCl, NaCl and glycerol experiments, which showed relatively steady levels of ATP even when cell numbers were decreasing. From these patterns, it is apparent that ATP is not the limiting factor for the ability of cells to survive and subsequently grow when under extreme osmotic stress.

### Cell viability over time

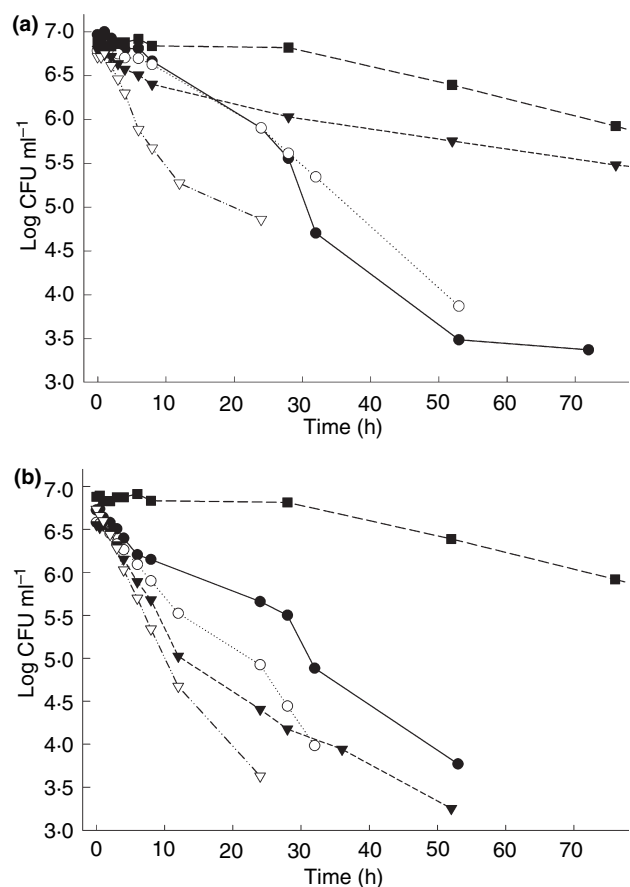
In this study, five humectants were used to achieve a broth RH of 84%. Despite the fact that the same RH was achieved, the rates of cell death varied greatly, depending on which of the humectants was used, with similar patterns seen for both strains (Fig. 4a,b). For the parent strain, RN4220, glycerol (a glass-forming solute with a reference  $T_g = -65^\circ\text{C}$ , membrane permeable) was most protective initially, as seen by extended ‘shoulder’ until viable cell count began a gradual decrease. Cells in the NaCl broth (a nonglass-forming ionic solute which may affect various ion channels in the cell) showed a sharp initial decrease in viable cell numbers, followed by the most gradual decrease in numbers over time. Both glucose–fructose (glass-forming solutes with a reference  $T_g = -42^\circ\text{C}$ , not membrane permeable) and sucrose–fructose (glass-forming solutes with a reference  $T_g = -37^\circ\text{C}$ , not membrane permeable) were initially protective, as seen by the ‘shoulder’ time until viable cell counts began to decrease, followed by a decrease in viable cell numbers that was more rapid than that observed for glycerol or NaCl, with sucrose–fructose eventually



**Fig. 2** Effects of osmotic stress (84% relative humidity broths) on *Staphylococcus aureus* RN4220 cell ATP and ADP levels as cell numbers decrease. (a) ATP; (b) ADP; (c) ATP/ADP. Sucrose–fructose (●); glucose–fructose (○); NaCl (▼); LiCl (▽); glycerol (■)



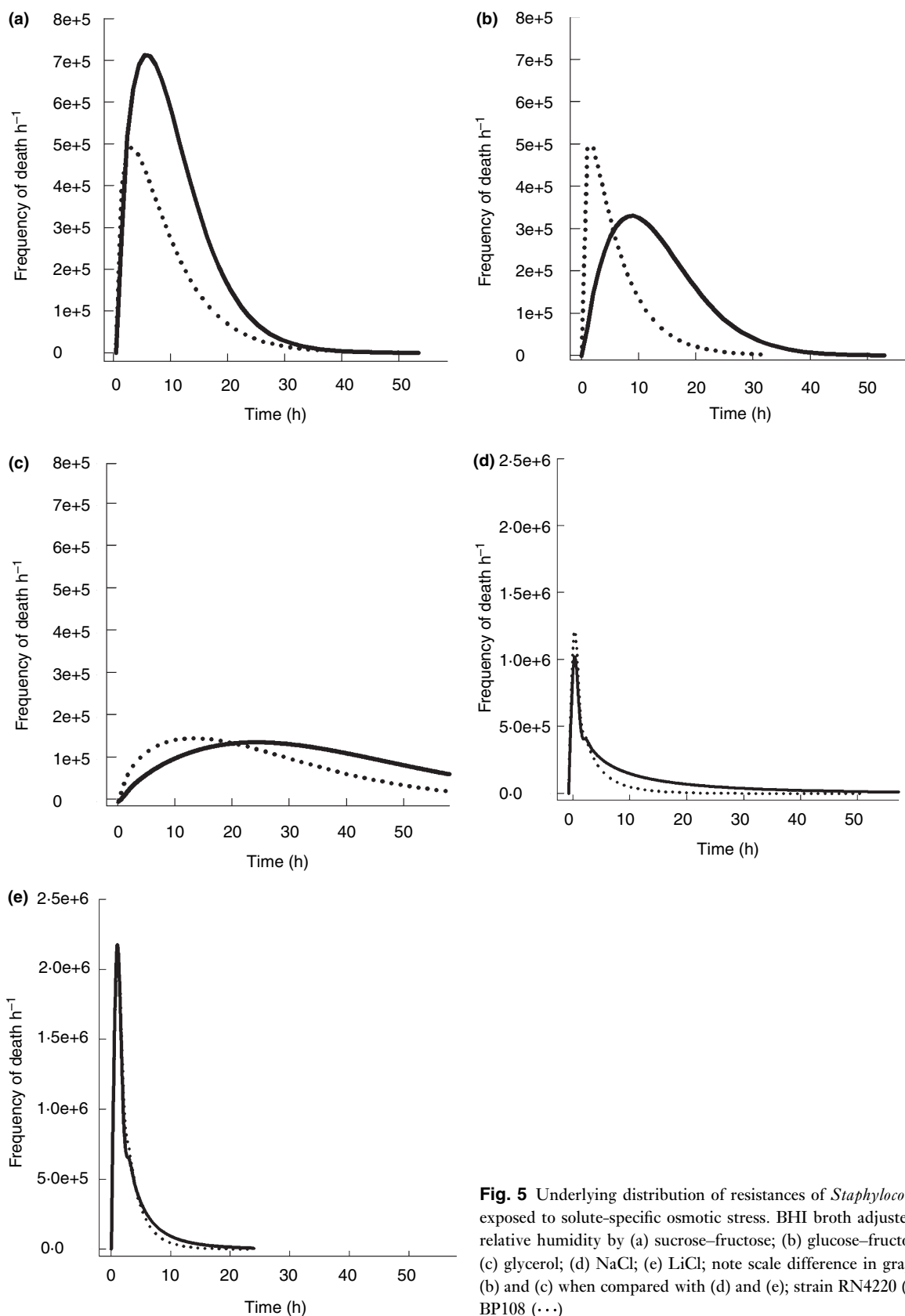
**Fig. 3** Effects of osmotic stress (84% relative humidity broths) on *Staphylococcus aureus* BP108 cell ATP and ADP levels as cell numbers decrease. (a) ATP; (b) ADP; (c) ATP/ADP. Sucrose-fructose (●); glucose-fructose (○); NaCl (▼); LiCl (▽); glycerol (■)



**Fig. 4** Survival curves of *Staphylococcus aureus* exposed to solute-specific osmotic stress (84% relative humidity broths) (a) strain RN4220; (b) strain BP108. Sucrose-fructose (●); glucose-fructose (○); NaCl (▼); LiCl (▽); glycerol (■)

causing a more rapid decrease in viable cells than glucose-fructose. LiCl (a glass-forming ionic solute which may affect various ion channels in the cell, reference  $T_g$  greater than that for glucose-fructose) appeared to be quite toxic to the cells, as a rapid decrease in viable cell numbers was observed over the entire experimental time frame. Similar patterns were seen for BP108, except that it was more sensitive to NaCl, as shown by the relatively rapid decrease in viable cells over time, when compared with RN4220 as well as with BP108 exposed to all other humectants except LiCl. In all instances, BP108 showed more sensitivity to osmotic stress caused by the same humectant when compared with RN4220.

One could consider the various shapes of the survival curves shown in Fig. 4 as different cumulative forms of distributions of osmotic stress resistances or sensitivities. The Weibull distribution has been used to describe a cumulative distribution of osmotic sensitivities for both



**Fig. 5** Underlying distribution of resistances of *Staphylococcus aureus* exposed to solute-specific osmotic stress. BHI broth adjusted to 84% relative humidity by (a) sucrose-fructose; (b) glucose-fructose; (c) glycerol; (d) NaCl; (e) LiCl; note scale difference in graphs (a), (b) and (c) when compared with (d) and (e); strain RN4220 (—); strain BP108 (···)

strain RN4220 and BP108 under the same conditions of RH achieved by the five various humectants used in the study (Fig. 5). This approach has been described by Peleg and Cole (1998). These differences in the curves can be treated as a reflection of the population's spectrum of sensitivities, or resistances, to osmotic stress caused by various humectants with different characteristics. As shown in Fig. 5, sucrose-fructose (higher  $T_g$ ; panel A) vs glucose-fructose (panel B) at the same 84% RH showed an increased efficacy of osmotic stress in inactivating *S. aureus* RN4220. This is manifested in a lower mode as well as narrower overall distribution in panel A vs panel B. This indicates that not only were the cells more sensitive to the osmotic stress caused by sucrose-fructose, but that there was also a more narrow range of osmotic stress resistances in the population. The glycerol, with its lower  $T_g$ , free membrane permeability, and use as a compatible solute, leads to minimal plasmolysis of the cell, and results in a population with a broad range of sensitivities to the osmotic stress caused by this humectant. The maximum frequency of death comes at a much later time than for that of any other humectants studied. This also correlates to a large number of survivors that are still viable after a considerable time, perhaps suggesting that some portion of the population may be reaching homeostasis under these conditions. The shapes of the distributions are markedly different for those populations of *S. aureus* exposed to NaCl or LiCl (also at 84% RH). The frequency distributions both have a sharp peak at 1 h, with LiCl showing a higher maximum frequency of death at 1 h. These distributions show that a majority of *S. aureus* cells in these populations are inactivated within a short time after being exposed to this stress. A comparatively small number of survivors are still viable after a considerable period of time, particularly in the NaCl conditions. Comparison of these five plots suggests that although the RH of the system was 84% in all five experiments, the sensitivities of the populations were quite different depending on the solute used. This suggests that the mechanism of inactivation may be different for glass-forming solutes when compared with ionic solutes.

The osmoregulatory mutant strain BP108 exhibited similar frequency distribution plots compared with RN4220 (Fig. 5) except that the time to maximum frequency of death was shortened and the distributions were considerably narrower in most cases, particularly for cells exposed to glycerol, glucose-fructose and sucrose-fructose. The frequency plots for BP108 in sucrose-fructose when compared with glucose-fructose were quite similar with the maximum frequency of death only slightly higher and occurring only 2 h later for cells exposed to sucrose-fructose when compared with those exposed to glucose-fructose. The frequency plot for LiCl was virtually identical to that for RN4220, indicating that the effects of

the high concentration of LiCl on both strains were as a result of toxic effects rather than osmotic stress. It was unexpected that the results for the two strains examined would be so similar. These results indicate that the mechanism of cell inactivation when under osmotic stress is not the same for all humectants used to achieve the same RH in the system, because if the mechanism were the same regardless of humectant type, the distributions would be identical for this osmoregulatory mutant. These results also indicate that the two remaining high affinity transport systems for proline (PPI) and glycine betaine (BPI) play a role in osmoregulation of *S. aureus* when under extreme osmotic stress.

## DISCUSSION

The results of this study show that the higher the  $T_g$  of the membrane impermeable solute and therefore the slower the molecular mobility of the system, the longer the lag time before death occurred (e.g. sucrose contributing a higher  $T_g$  than glucose in their respective blends with fructose). Once the cells started to die rapidly, the frequency of death was higher and occurred more quickly in the sucrose-fructose broth than in the glucose-fructose broth. When NaCl was used to adjust RH, the initial inactivation rates were relatively fast when compared with the systems where glass-forming solutes were used, perhaps because NaCl does not affect the mobility of the system. After the initial reduction, the cells survived over a longer period of time, perhaps because *S. aureus* has adapted to survive in conditions of relatively high NaCl concentrations. As glycerol is membrane permeable and causes minimal plasmolysis, cells may not experience as much osmotic stress as with solutes that are not membrane permeable. These effects would be expressed as prolonged survival times, which were observed in this study. One would expect that LiCl as a glass-former would allow for long lag times. However, the high concentrations of ions in the system necessary to reduce the RH may have had a toxic effect on the cells leading to the rapid inactivation observed. Lithium is a chaotrope in the Hofmeister series, with high water attracting potential, and therefore may have entered the cells causing protein precipitation as was observed during preparation of full strength BHI broth with LiCl (Voet and Voet 1990; Wood 1999).

BP108 should be much more sensitive to osmotic stress when compared with the parent strain as BP108 lacks the osmotically sensitive, low affinity glycine betaine/proline transport system (BPII) which transports osmoprotectants into the cytoplasm when the cell is under osmotic stress. Studies with BP108 characterized the high affinity system as being only slightly activated by osmotic pressure (under osmotic stress levels that still allowed cell growth) with

glycine betaine being accumulated independently of osmotic stress (Pourkomialian and Booth 1994). It should not, therefore, be able to stabilize itself successfully as osmotic stress increases. If the cause of cell stress (as expressed in increased lag time) were the same, regardless of the humectant in the system, then the only expected stress would be osmotic. Therefore, this osmoregulatory mutant would be expected to have the same lag time and death rate at the same RH across all systems studied. In this study, BP108 showed different survival rates and underlying distributions of resistances to osmotic stress, depending on the humectant used in the system. Therefore, we propose that it is not only osmotic stress, but also solute-specific characteristics (i.e. molecular mobility and ionic stress) of the humectants that lead to the variation in cell behaviour observed in this study.

Our results are in agreement with Houssin *et al.* (1991) who reported that *E. coli* osmotically stressed by NaCl or glycerol had no change in ATP levels after osmotic shock. These authors concluded that inhibition of growth under osmotically stressed conditions was not caused by energy limitations. However, inhibition of respiration was observed when osmotic stress was caused by NaCl, but did not occur when cells were exposed to glycerol. Additionally, it was demonstrated that both active and facilitated carbohydrate transport systems were affected when cells were under osmotic stress, but glycerol had only a minor effect when compared with NaCl or sucrose. The authors suggested that as there was no evidence of a decrease in ATP levels, conformational changes in the carriers might have caused the inhibition of the transport systems studied as a result of plasmolysis of the cells which cannot be completely reversed even in the presence of compatible solutes.

The results from this study indicate that ATP is not the limiting factor for cell survival under excessive osmotic stress, and therefore cell inactivation was not because of lack of ATP being available for metabolic processes. While the results from this study did not elucidate the mechanism(s) of cell inactivation when *S. aureus* was placed under osmotic stress, they do contribute to the evidence which has shown that mechanisms of inactivation depend on the solute and suggest that the glass transition and molecular mobility of the system may be important means to explain many diffusion-limiting properties of food (Fennema 1996).

The growth of bacterial cells, as defined as balanced increase in the mass of the major polymers (DNA, RNA, proteins and lipids) until the time that the cell achieves critical mass and divides, requires the control of the flow of water (O'Byrne and Booth 2002). Osmoregulation, which typically involves the transport of compatible solutes into the cytoplasm, is all about the control over the influx and efflux of solutes from the cell, with water movement being essentially passive (O'Byrne and Booth 2002). Osmoregula-

tion requires not only the modulation of the activity of transport and enzyme systems, but also involves complex patterns of regulation of gene expression. The major compatible solutes utilized by food-borne micro-organisms include quaternary amines (i.e. betaine and carnitine), amino acids (i.e. proline), amino acid derivatives (i.e. proline betaine), sugars (i.e. trehalose, mannitol), and peptides (i.e. prolyl-hydroxyproline). It is quite clear that food constitutes a rich source of compatible solutes and their precursors, thus higher osmolarities may be required to achieve inactivation of pathogenic and spoilage micro-organisms than would be necessary if compatible solutes were absent from foods (Gutierrez *et al.* 1995; Booth 1998; O'Byrne and Booth 2002). As removing compatible solutes from foods would be virtually impossible, alternative strategies could include preventing the micro-organisms from accumulating compatible solutes by the addition of inhibitors of transport systems for compatible solutes. This also is a difficult strategy to implement. Therefore, an understanding about osmoregulation, including the effects of solute specific characteristics of various humectants used to depress the RH of foods, information on the regulation of the synthesis and activity of the various transport systems, information on the patterns of regulation of gene expression involved in osmoregulation and the understanding of the mechanisms of cell death caused by osmotic stress may generate ideas for optimally utilizing osmotic stress as a food preservation method.

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