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Bacterial maximum non-inhibitory and minimum inhibitory concentrations of different water activity depressing solutes

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ABSTRACT

The NaCl MNICs (maximum non-inhibitory concentrations) and MICs (minimum inhibitory concentrations) for growth of various strains of six bacterial species were determined and then compared with those obtained for seven other solutes. The influence of prior growth conditions on the MNICs and MICs was also evaluated. No significant changes on the MNICs and MICs were found among the strains studied within each species. Among all factors investigated, only growth phase –for Gram-negatives– and growth at high NaCl concentrations led to a change in the NaCl MNICs.

Species could be classified depending on its NaCl MNICs and MICs (in decreasing order) as follows: *Staphylococcus aureus*, *Listeria monocytogenes*, *Cronobacter sakazakii*, *Enterococcus faecium*, *Escherichia coli* and *Salmonella* Typhimurium. Similar results were obtained for KCl, LiCl, and sodium acetate, but not for the remaining solutes investigated (sucrose, glycerol, $MgCl_2$ and $CaCl_2$). Results obtained indicate that, in general, Gram-negatives showed lower MNICs and MICs than Gram-positives for all the solutes, *S. aureus* being the most solute tolerant microorganism. When compared on a molar basis, glycerol showed the highest MNICs and MICs for all the microorganisms –except for *S. aureus*- and LiCl the lowest ones. NaCl MNICs and MICs were not significantly different from those of KCl when compared on a molar basis. Therefore, the inhibitory action of NaCl could not be linked to the specific action of Na^+. Results also showed that the Na^+ tolerance of some species was Cl^- dependent whereas for others it was not, and that factors others than a_w-decrease contribute to the inhibitory action of LiCl, $CaCl_2$ and $MgCl_2$.

1. Introduction

Throughout history, water activity (a_w) reduction, alone or in combination with other agents, has been the basis of many food preservation methods. Thus, food products have been preserved through the addition of solutes such as salt or sugars (Tapia et al., 2007).

The water activity of food can be reduced by directly removing the water, such as in evaporation, drying or freeze-drying processes, or by adding solutes (humectants) that make water less available for microorganisms, enzymes and chemical reactions in general. Given the widespread use of these water activity depressing solutes and their generally acknowledged efficacy as preserving agents, many works have been carried out to determine the concentration of each particular solute required to inhibit bacterial growth (compiled in ICMSF, 2002; Tapia et al., 2007). For comparison purposes, the so-called minimum inhibitory concentration (MIC) is the parameter most frequently used. MIC can be defined as the minimum concentration of a particular solute required to prevent growth in a broth relative to a control without solute (Carson et al., 1995). However, despite the well-known fact that solute concentrations below those leading to complete growth inhibition can also lead to increased lag times and decreased growth rates, in most investigations growth information below the MIC concentration is normally not reported (Lambert and Pearson, 2000). These authors proposed the use of a second and complementary parameter, the NIC (non-inhibitory concentration) which corresponds to the concentration below which the solute has no effect on growth relative to a control. This last parameter is also sometimes called MNIC (maximum non-inhibitory concentration; Cebrián et al., 2009) and not only provides very interesting information about microbial physiology but it might be useful from a practical point of view, since it determines the threshold concentration above which the solute begins to develop its inhibitory activity. Thus, MNIC might be helpful when designing combined processes in which growth inhibition or bacterial inactivation is achieved by the combination of various agents acting at concentrations or intensities below those required if they were used alone. Besides, other applications of MNIC may include the design of selective plating media aimed to determine/quantify the presence of sublethally injured cells (Mackey, 2000).

A critical aspect when evaluating the inhibitory activity of a solute is the influence of the experimental conditions. It has been already demonstrated that the concentration of a particular solute capable of

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inhibiting growth is affected by the organism studied, incubation temperature, pH and atmosphere, and inoculum size (Lambert and Pearson, 2000; Mcmeekin et al., 2000; Stewart et al., 2002; Koutsoumanis et al., 2004), among others. Nevertheless, there are some factors that have only been scarcely studied, such as the magnitude of the intra-specific differences in solute tolerance or if this solute tolerance is influenced by the previous growth conditions. Finally, although a huge number of studies dealing with bacterial osmoregulation systems have been published (reviewed in O'Byrne and Booth, 2002 and in Wood, 2010), the mechanism of action of many of these solutes, e.g. sodium chloride, still remains to be fully elucidated (Bidlas and Lambert, 2008).

The objective of this work was to determine the MNICs and MICs of sodium chloride and seven other specifically chosen solutes (sucrose, glycerol, KCl, LiCl, sodium acetate, $MgCl_2$, and $CaCl_2$) for six bacterial species of relevance for food safety and technology growing in solid media. The influence of a number of environmental factors acting before plating, including growth phase, growth temperature, growth medium pH and NaCl concentration, prior exposure to different sublethal shocks, and the possible intra-specific variations were also evaluated. The mechanisms underlying the changes in the MNICs and MICs were explored as well.

2. Materials and methods

2.1. Strains

For this study four strains of each of the following species: *Cronobacter sakazaki*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus* and two strains of *Enterococcus faeicum* were used. The strains of *C. sakazakii* CECT 858, *E. faecium* CECT 410, *E. coli* CECT 405, CECT 471, CECT 4201, *L. monocytogenes* CECT 932, CECT 4031, CECT 4032, and CECT 5672, *S.* Typhimurium CECT 443, CECT 722, CECT 878, and CECT 4594 and *S. aureus* CECT 976, CECT 4459, CECT 4465, and CECT 4466 were supplied by the Spanish Type Culture Collection (CECT). The strains of *C. sakazakii* NCTC 8155, NCTC 9238, and NCTC 9529 were provided by the National Collection of Type Cultures (NCTC). The *E. faecium* strain ATCC 49624 and *E. coli* strain W3110 (ATCC 27325) were obtained from the American Type Culture Collection (ATCC).

2.2. Growth conditions

Bacterial cultures were maintained frozen at −80 °C in cryovials. Pre-cultures were prepared by inoculating 10 mL of Tryptone Soya Broth (Biolife, Milan, Italy) supplemented with 0.6% (w/v) Yeast extract (Biolife) (TSBYE) with a loopful of growth from a Tryptone Soya Agar supplemented with 0.6% (w/v) yeast extract (Biolife) (TSAYE) plate. This pre-culture was incubated at 37 °C for 12 h in a shaking incubator. 50 μL of this pre-culture were inoculated into 50 mL of different growth media (see below) and incubated until cultures reached the stationary-phase of growth. Exponential-phase cultures were obtained as described for stationary-phase cells but growth was stopped when cultures reached a concentration of approximately 10^8 cells/mL. Unless specifically indicated, exponential and stationary phase cells were obtained after growth in TSBYE at 37 °C.

The influence of the following environmental factors on bacterial MNIC was studied: a) growth temperature, b) growth pH, c) NaCl concentration on the growth medium and, d) exposure to sublethal shocks. For this purpose cells were cultured under the following conditions: a) in TSBYE at 20 °C and TSBYE at 37 °C; b) in TSBYE (pH 7.3) at 37 °C and TSBYE acidified to pH 5.0 with HCl (Panreac S. A., Barcelona, Spain) at 37 °C c) in TSBYE at 37 °C with different concentrations of NaCl (Panreac) added ranging from 0 to 15% w/v and, d) in TSBYE at 37 °C and then exposed for 1 h to acid (pH 4.5), alkaline (pH 9.5 with NaOH, Panreac), cold (4 °C), heat (45 °C) and NaCl (varying concentrations between 1 and 15% w/v) shocks, as described in Cebrián et al. (2012).

2.3. Incubation of samples and survival counting

The recovery medium used was TSAYE with or without different concentrations of NaCl, KCl, LiCl, $MgCl_2$, $CaCl_2$, sodium acetate, sucrose, or glycerol added. After adequately diluting in TSBYE, 0.1 mL samples were pour-plated and then incubated for 24 h at 37 °C, unless a solute was added to the agar so that incubation was extended for up to 21 days. After incubation, colony forming units (CFUs) were counted. Experiments were performed at least in triplicate on independent working days.

2.4. Calculation of MNICs and MICs

Maximum non-inhibitory concentration (MNIC) of each solute was determined as the highest concentration (moles/L) leading to <20% decrease in the number of CFUs recovered as compared to the number of CFUs recovered in TSAYE (no solute added). Minimum inhibitory concentration (MIC) was determined as the lowest concentration (moles/L) of a solute completely inhibiting growth of bacteria in TSAYE, i.e. absence of colony growth after 21 days of incubation at 37 °C.

2.5. Recovery curves and Weibull curve fitting

Recovery curves were obtained by plotting the logarithm of the number of CFUs recovered in the medium with solute added divided by the number of CFUs recovered in the medium without solute added versus the solute concentration.

In order to determine the underlying distribution of tolerances to NaCl of cells grown under different conditions, curves were fitted to the experimental data using a mathematical model based on the Weibull distribution (Stewart et al., 2002) with the GraphPad PRISM® software (GraphPad Software, Inc., USA). Statistical analyses were carried out with the same software.

2.6. Measurement of water activity

Water activity (a_w) of TSAYE plates with different solute concentrations was measured by the dew point method (model CX-1, Decagon Devices, Inc., Pullman, Washington, USA). Measurements were performed by triplicate on independent working days.

3. Results

3.1. Intraspecific variation and influence of prior growth conditions on bacterial MNICs and MICs of sodium chloride

Fig. 1 shows the influence of sodium chloride concentration in the number of CFU recovered in TSAYE (recovery curves) of six bacterial strains each one representative of one of the following bacterial species, three Gram-negative, *C. sakazakii*, *E. coli* and *S.* Typhimurium and three Gram-positive, *E. faecium*, *L. monocytogenes* and *S. aureus*. From these data MNIC and MIC values were calculated as described in Materials and methods. MNIC values ranged between 0.656 M and 2.277 M, and MIC values between 1.198 M and 2.850 M. Species could be classified as a function of their NaCl MNICs and MICs (in decreasing order) as follows: *S. aureus*, *L. monocytogenes*, *C. sakazakii*, *E. faecium*, *E. coli* and *S.* Typhimurium.

Among all factors studied, only the growth phase for Gram-negative species and the growth with NaCl concentrations between the MNIC and the MIC provoked a change in the MNICs of NaCl. Conversely, none of the factors studied resulted in a change in the MICs of NaCl (data not shown). Similarly, no significant differences were found among the MNIC or the MIC values (Fig. 1B) among the different strains tested of each species, indicating that the tolerance of *C. sakazakii*, *E. coli*, *S.* Typhimurium, *E. faecium*, *L. monocytogenes* and *S. aureus* to sodium chloride would be much less strain-dependent than their tolerance to