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Osmotolerance provided by the alternative sigma factors σ^B and rpoS to *Staphylococcus aureus* and *Escherichia coli* is solute dependent and does not result in an increased growth fitness in NaCl containing media

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ABSTRACT

The aim of this work was to examine the role of the alternative general stress sigma factors σ^B and rpoS on the ability of *Staphylococcus aureus* and *Escherichia coli*, respectively, to grow in liquid and solid media of different osmolarity. For this purpose, *S. aureus* strain Newman and its isogenic Δ*sigB* mutant IK84 and *E. coli* strain BJ4 and its isogenic Δ*rpoS* mutant BJ4L1 were grown in media (TSBYE) with different concentrations of NaCl. Growth parameters (lag phase duration, growth rate and maximum number of microorganisms) and limiting growth concentrations (Maximum Non-Inhibitory Concentration – MNIC – and Minimum Inhibitory Concentration – MIC–) were determined. The mechanisms underlying the differences observed between parental and mutant strains were also explored. The absence of the sigma factors σ^B and rpoS led to a decrease in the MNICs and MICs calculated for *S. aureus* and *E. coli*, respectively. Conversely, neither σ^B nor rpoS provided with increased growth fitness to *S. aureus* and *E. coli* cells at NaCl concentrations up to 1.36 M and 1 M, respectively. The decreased osmotolerance of the σ^B and rpoS deficient strains, as compared to their parental strains, was compensated by the addition of glycine-betaine (1 mM) to the growth medium. It was also observed that the decreased tolerance to NaCl of the mutant strains was coincident with a decreased tolerance to sucrose, KCl, and LiCl but not to glycerol, $MgCl_2$, and $CaCl_2$. Results obtained also demonstrate that the increased osmotolerance of stationary growth phase *E. coli* cells, as compared to exponential growth phase ones, would be due to the activation of both rpoS-independent and rpoS-dependent mechanisms. This work will help to understand the mechanisms of bacterial resistance to osmotic stress and the role of the alternative sigma factors σ^B and rpoS in this process.

1. Introduction

Among the different strategies that bacterial cells are capable of developing when exposed to adverse conditions, the use of alternative sigma factors is perhaps the most important one (Abee and Wouters, 1999). Sigma factors are the recognition units of the RNA polymerase and thus, the sigma factor bound to the core enzyme determines the set of genes that is expressed. General stress sigma factors include sigma S (σ^S also known as rpoS) in some Gram-negative, and sigma B (σ^B) in some Gram-positive bacteria, which are considered as functionally homologous by many researchers (Gertz et al., 2000). Both rpoS and σ^B have in common that their activity increases after exposing cells to a number of different stresses, and that they control large regulons that confer resistance against a variety of environmental stresses including heat, high osmolarity, low pH, and oxidizing agents, among others (Hengge, 2010; Price, 2010). Their regulation and specific roles vary depending on the species studied.

At present, there is a lot of information available about their genetic regulation and their role on stress adaptation and survival, but studies aiming to clarify how their activity influences the ability of bacterial cells to grow (growth rate, final number of microorganisms, and growth limits) under different conditions are very scarce. From the data available, it can be concluded that *Escherichia coli* mutants with a decreased rpoS activity display an increased growth rate as compared with their parental strains when grown in limited nutrient media as well as in Luria Bertani (LB) broth at alkaline pH, but that this growth advantage is reduced in LB at neutral pH and disappears at acid pH (Farrell and Finkel, 2003). Similarly, King et al. (2006) observed that the growth advantage of *rpoS* null *E. coli* mutants in minimal media was lower when cultivated under anaerobic conditions, when growth temperature was raised to 44 °C and when chloramphenicol or sucrose (5%) was added to the medium. On the other hand, in a Gram-positive microorganism such as *Listeria monocytogenes*, deletion of *sigB* resulted in little or no change in the growth rate in Defined Growth Media (DGM) but in an increase in the growth rate if 0.5 M NaCl was added to the media

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(Abram et al., 2008a; Heavin et al., 2009). Conversely, deletion of *sigB* resulted in a decreased growth rate in DGM with sorbic or benzoic acid added (Heavin et al., 2009), thus indicating that σ^B expression, at least for *L. monocytogenes*, does not always impose a fitness cost to cells.

High osmolarity is a condition that microbial cells may encounter frequently in their habitats, for example in food environments. Water activity reduction is a food preservation method that has been used since ancient times and thus, microorganisms are very likely to be exposed to this type of stress when present in a number of different food products. It is generally acknowledged that when bacteria are exposed to osmotic stress they respond by rapidly accumulating compatible solutes and by initiating a program of gene expression leading to the synthesis of specific proteins to cope with it (O'Byrne and Booth, 2002; Vijaranakul et al., 1997). RpoS and σ^B activities are known to be increased upon exposure to hyperosmotic conditions and also it is well known that some of the proteins they control are involved in bacterial osmoregulation (Abram et al., 2008a, 2008b; Hengge, 2010; Pané-Farré et al., 2006; Price, 2010). Nevertheless, to the best of our knowledge, how rpoS and σ^B activities influence bacterial growth in media of different water activities has not been studied in detail yet. From the few data available it can be hypothesized that the fitness growth advantage of deleting *rpoS* would be lower in media of reduced water activity since King et al. (2006) observed that in minimal media with a sucrose concentration of 5% the glucose transport rate difference between *rpoS* null mutants and will type cells was much lower than in media without sucrose added. Furthermore, although most of the works concerning the role of these sigma factors on bacterial osmotolerance have been carried out using NaCl as the stressor, preservation of food through water activity reduction can be achieved by the addition of different solutes such as salts, sugars, and polyols. It is well-known that each one of these solutes has its "specific solute effects" besides the bacteriostatic or bactericidal effect they cause through the decrease in water activity (Tapia et al., 2007), but up to date there is little information available on the role of rpoS and σ^B on bacterial osmotolerance to solutes other than NaCl.

The objective of this work was to determine the role of the *Staphylococcus aureus* sigma factor σ^B and the *E. coli* sigma factor rpoS on: 1) the NaCl limiting concentrations for growth, for *S. aureus* and *E. coli* cells and 2) the growth parameters in media with different concentrations of NaCl added. Results obtained using NaCl were compared to those obtained with KCl, LiCl, $MgCl_2$, $CaCl_2$, sucrose and glycerol. The mechanisms underlying the differences in osmotolerance observed between the mutant (Δ*sigB* and Δ*rpoS*) and the corresponding parental strains were explored.

2. Materials and methods

2.1. Strains and growth conditions

E. coli strains W3110 (ATCC 27325) and BJ4 and its isogenic Δ*rpoS* derivative, *E. coli* BJ4L1 (Krogfelt et al., 2000), were kindly provided by Dr. B.M. Mackey from the University of Reading. *S. aureus* strain Newman and its isogenic Δ*sigB* derivative, *S. aureus* IK184 (Kullik et al., 1998) were kindly provided by Brigitte Berger-Bächi from the Institute of Medical Microbiology, University of Zurich.

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 (TSAYE) plate. This pre-culture was incubated at 37 °C for 12 h, in a shaking incubator. 50 mL of this pre-culture was inoculated into 50 mL of TSBYE and incubated under agitation for 24 h at 37 °C, resulting in stationary-phase phase cultures. Exponential-phase cultures were obtained as described for stationary-phase cells but growth was stopped when cultures reached a concentration of 10^8 cells/mL approximately. This point was determined spectrophotometrically (Mañas and Mackey, 2004; Cebrián et al., 2009) and corresponded to 2.5–3.0 h of incubation under our experimental conditions ($Abs_{600} = 0.9$ for *S. aureus* cells and $Abs_{680} = 0.3$ for *E. coli* cells, approximately).

2.2. Calculation of MNIC and MICs in solid media

The medium used for determining the MNICs in solid media was TSAYE with or without different concentrations of NaCl, KCl, LiCl, $MgCl_2$, $CaCl_2$, sucrose, or glycerol added. All the compounds were added to the growth medium before autoclaving. After adequately diluting the bacterial culture (to aprox. 2.5×10^5 CFU/mL), 0.1 mL samples were pour-plated in the different solid media. Plates were incubated for 24 h at 37 °C, unless any of the solutes had been added to the agar, in such case incubation was extended for up to 21 days. It was checked that further incubation times, up to 42 days, did not change colony counts. After incubation, CFUs were counted with an improved image analyzer automatic colony counter, as previously described (Condón et al., 1996). For some experiments glycine-betaine (Sigma-Aldrich) was added to the media to a final concentration of 1 mM.

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

2.3. Calculation of growth parameters and MNICs/MICs in liquid media

Growth kinetics was studied for cells growing in TSBYE with or without the addition of the different solutes evaluated. An appropriate dilution of the bacterial culture containing aprox. 10^5 CFU/mL was inoculated into 180 μL of TBS-YE with different concentrations of solute (final volume 200 μL) in the wells of a microtiter plate to adjust the initial concentration to 10^4 CFU/mL. Microtiter plates were incubated at 30 °C under agitation, and at preset time intervals samples were taken, adequately diluted, pour-plated in TSAYE and incubated at 37 °C. After 24 h incubation, CFUs were counted and growth curves constructed. Growth curves were fitted to the model of Baranyi and Roberts (1994), using the software Microsoft Office Excel 2002 (Microsoft Co., Redmond, Washington, USA) and DMFit (http://www.ifr.ac.uk/safety/DMFit, IFR, Norwich, UK). This model estimates the growth rate (k, 1/h), the duration of the lag phase (lag, hours) and the maximum bacterial concentration (Y_{end}, log CFU). The same software was used to calculate determination coefficients (r^2 values) and standard deviations.

MNIC was determined as the highest concentration leading to less than a 20% decrease in the Y_{end} as compared to that obtained in TSBYE with no solute added, and MIC as the lowest concentration at which growth was not detected. For growth experiments with glycine-betaine, the different growth media were supplemented with 1 mM glycine-betaine.

2.4. Resistance to lethal concentrations of NaCl

TSBYE with NaCl added to a final concentration of 5.1 M (for *S. aureus*) or 3.4 M (for *E. coli*) was inoculated with the bacterial suspension to a cell concentration of 10^7 CFU/mL. Samples were collected after different treatment times ranging from 0.5 to 72 h. After the desired contact time, 0.1 mL samples were removed and appropriate serial