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Survival of *Escherichia coli* during drying and storage in the presence of compatible solutes

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Abstract Five different compatible solutes, sucrose, trehalose, hydroxyectoine, ectoine, and glycine betaine, were investigated for their protective effect on *Escherichia coli* K12 and *E. coli* NISSLE 1917 during drying and subsequent storage. Two different drying techniques, freeze-drying and air-drying, were compared. The highest survival rate was observed when the non-reducing disaccharides sucrose (for *E. coli* K12) and trehalose (for *E. coli* NISSLE 1917) were added. The two tetrahydropyrimidines, hydroxyectoine and ectoine, gave protection to freeze-dried *E. coli* NISSLE 1917 whereas *E. coli* K12 was protected only by hydroxyectoine. Glycine betaine seemed to be harmful for both strains of *E. coli* with both drying techniques. Air-drying gave much better survival rates than freeze-drying. The two strains of *E. coli* differed in their ability to take up compatible solutes.

Introduction

In order to maintain osmotic equilibrium in saline environments, most halophilic and halotolerant micro-organisms are able to synthesize or accumulate small, highly water-soluble organic molecules, the so-called compatible solutes (Brown 1976). Compatible solutes belong to different biochemical classes such as polyols, sugars, amino acids, betaines and tetrahydropyrimidines.

Besides their osmotic function, compatible solutes have been reported to protect enzymes and membranes (Schwab and Gaff 1990; Lippert and Galinski 1992; Jolivet et al. 1982; Rudolph et al. 1986). Several studies on the effect of organic solutes on whole cells during drying and storage have been published (Marshall et al. 1974; Redway and Lapage 1974; Bushby and Marshall 1977; Heckly and Dimmick 1978; Caesar and Burr 1991). These investigations focused primarily on the effects of sugars and polyols. Tetrahydropyrimidines have so far not been examined.

Apart from protection by solutes, the survival of whole cells seems to be influenced by several other factors, for example the drying process (Antheunisse and Arkesteijn-Dijksman 1979), the storage conditions (Mary et al. 1985), and the rehydration procedure (Leach and Scott 1959; Kosanke et al. 1992). The preservation of dried micro-organisms has potential application in pharmacy (shelf-life of pharmaceuticals containing viable cells), agriculture (legume inoculation with rhizobacteria), and research (preservation of strains in culture collections).

The aim of this study was to assess the protective effect of different compatible solutes on two strains of *Escherichia coli* during drying (freeze-drying and air-drying) and subsequent storage. In addition, the accumulation of solutes by the cells was examined.

Materials and methods

Bacterial strains and culture conditions

E. coli K12 (DSM 498) and *E. coli* NISSLE 1917 (industrial strain, used in the pharmaceutical Mutaflor, supplied by Ardeypharm, Herdecke, Germany) were grown in minimal medium 63 (Larsen et al. 1987) containing 100 mM KH_2PO_4, 75 mM KOH, 15 mM $(NH_4)_2SO_4$, 3.9 μM $FeSO_4$, and 22 mM D-glucose. For the cultivation of *E. coli* NISSLE 1917, vitamin solution and trace element solution (Imhoff and Trüper 1977) (1 ml l^{-1}, respectively) were added. Cells were grown aerobically in a 5-l laboratory fermentor (Biostat V, Braun Melsungen, Germany). A volume of 4 l was aerated at 13 l min^{-1} and stirred at 500 rpm. The pH was adjusted to 7.2 with 1 M NaOH and the cultivation temperature was 37° C.

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Drying experiments

Cells were harvested in the stationary growth phase by centrifugation and resuspended with one of the following solutes (1 g bacterial wet weight in 2 ml solution): ectoine, hydroxyectoine, sucrose, trehalose or glycine betaine (625 mM in 100 mM phosphate buffer, pH 7.2). Sucrose, trehalose and glycine betaine were purchased from Sigma (Munich, Germany). Ectoine and hydroxyectoine were isolated from the halophilic producer strains *Halomonas elongata* and strain M52 (unidentified). Phosphate buffer without solute served as a control. Taking into account the extracellular water in the cell pellet, the final solute concentration was approximate 500 mM. Samples of 200 µl cell suspension were placed in multiple well plates (Corning, N.Y., USA) with 24 flat-bottomed wells.

Air-drying was performed in desiccators in the dark at 25° C for 60 h using silica gel (0% relative humidity) or a saturated $CaCl_2$ solution (30% relative humidity), respectively. Freeze-drying was performed for 15 h in the dark after all suspensions had been frozen at –40° C for 1 h. All samples were sealed under nitrogen and stored over periods of 2, 6, 12, and 26 weeks.

Air-dried cells were rehydrated slowly prior to resuspension by placing them in a desiccator with distilled water (100% relative humidity) for 1 h. Freeze-dried cells were processed without prior rehydration. All samples were resuspended in 100 mM phosphate buffer to a total volume of 2 ml. Serial dilutions were also performed in phosphate buffer. For colony counts aliquots of diluted cell suspension were mixed with 4 ml of fluid (40° C) trypcase-soy soft agar (half concentrated), poured onto trypcase-soy agar plates (Biomerieux, France) and subsequently incubated at 37° C. Colonies were counted after 1 day of incubation and controlled after 3 days. To assess the initial viable cell number, viable cell counts of cell suspensions were also made on trypcase-soy agar prior to drying.

Determination of intracellular solute uptake using HPLC.

Samples of cell suspension in solute solutions (approx. 500 mM) or NaCl (approx. 250 mM) were centrifuged after 0–4 h incubation (the preparation of the cell suspension lasted 15 min). Cells were washed with iso-osmotic NaCl-phosphate buffer, pH 7.2, and subsequently freeze-dried. Dry material was extracted with chloroform/water (1:1), using a modified technique of Bligh and Dyer (1959). The water soluble fraction was analysed by 9-fluorenylmethyl chloroformate-HPLC (FMOC-HPLC) for N-reactive compounds as described by Kunte et al. (1993). For the detection of compatible solutes insensitive to the FMOC technique, the water-soluble fraction was desalted and analysed on a NH_2–phase column (Lichrospher 100, Merck) using acetonitrile/water (76:24, v/v) as a solvent. Chromatography was carried out at a flow rate of 1 ml min^{-1} using an LDC/Milton Roy HPLC unit with a refractive index detector.

Results

Influence of drying and rehydration conditions

We obtained similar results for air-drying at both 0% and 30% relative humidity. Therefore only results for 0% relative humidity are presented in the following sections.

A slow rehydration procedure prior to resuspension increased the survival rate of air-dried cells. The same treatment, however, proved to be harmful for freeze-dried cells. As this investigation aimed at optimizing survival rates, freeze-dried cells were processed without prior rehydration.

The structure of dried cell material displayed marked differences between freeze-dried and air-dried cells. Whereas freeze-dried samples appeared fluffy with a rough surface, air-dried cells were tightly packed and had a firm top layer.

Drying and storage of *E. coli* K12

Viable cell counts prior to drying were 11.44 log colony-forming units (cfu) ml^{-1} (standard deviation 0.05) equivalent to 2.75×10^{11} cfu ml^{-1}. Immediately after drying, freeze-dried cells were well protected by the compatible solutes tested (20–60% survival as compared to 4% with phosphate buffer only), with sucrose giving the best results (Fig. 1A, zero storage time). The influence of compatible solutes on air-dried cells, however, proved to be only minor. Some protection was observed with sucrose, trehalose, and hydroxyectoine, whereas ectoine and glycine betaine seemed to be harmful (Fig. 1B, zero storage time).

Samples were stored at 4° C in the dark and after 2, 6, 12, and 26 weeks viable counts were taken (Fig. 1). Freeze-dried samples of *E. coli* K12 showed a large decrease in viable cells during the first 6 weeks of storage.

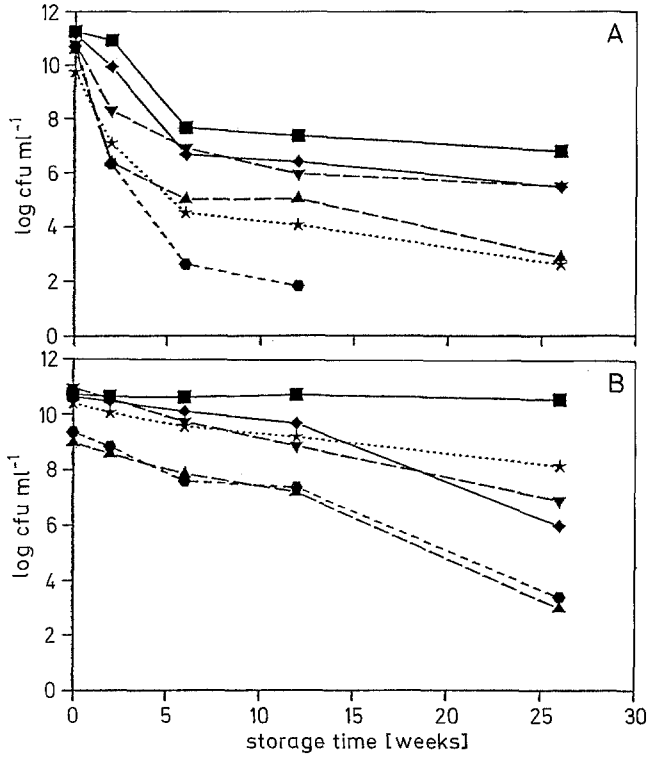

Fig. 1 Effect of compatible solutes on freeze-dried (**A**) and air-dried (**B**) cells of *Escherichia coli* K12 during storage at 4° C. Counts were performed on four parallel samples with standard deviations between 0.03 and 0.7 log colony forming units (cfu) ml^{-1}: ■ sucrose, ◆ trehalose, ▼ hydroectoine, ★ control, ▲ ectoine, ● glycine betaine

A protective effect was observed using sucrose, trehalose, and hydroxyectoine. Ectoine seemed to have no significant influence, whereas glycine betaine proved to be harmful to the cells.

Generally, air-dried cells showed much higher survival rates than freeze-dried cells. Unprotected air-dried samples (control in phosphate buffer), that had been stored for 26 weeks gave viability counts of about 2.5×10^8 cfu ml^{-1} compared to about 3×10^2 cfu ml^{-1} for freeze-dried samples. Therefore, protective effects of compatible solutes were much less pronounced in air-dried samples. A remarkable protection was, however, obtained with sucrose. Except for an initial drop in viable cell counts during drying, there was no decrease in the survival rate in the presence of sucrose over a period of 26 weeks, so that optimal stabilization of the cells was achieved. The other solutes tested failed to improve the shelf-life of air-dried cells.

Drying and storage of *E. coli* NISSLE 1917

Viable cell counts prior to drying were 11.20 log cfu ml^{-1} (standard deviation 0.06) equivalent to 1.58×10^{11} cfu ml^{-1}. Immediately after drying, similar results were obtained as for *E. coli* K12. All compatible solutes increased the survival after freeze-drying, while no obvious protection was seen after air-drying (not shown). In contrast to *E. coli* K12, trehalose gave better results than sucrose for both treatments. All samples were stored for 6 weeks (Fig. 2). As before, unprotected freeze-dried cells of *E. coli* NISSLE 1917 rapidly lost viability. With the exception of glycine betaine, all tested compatible solutes protected the cells, with trehalose giving the best results. Again, air-dried cells of *E. coli* NISSLE 1917 showed a higher survival than freeze-dried cells over a period of 6 weeks (air-dried: approx. 10^9 cfu ml^{-1}, freeze-dried: approx. 10^3 cfu ml^{-1}). A slight protection of air-dried cells was observed with trehalose, while the other solutes had no or slightly negative effects.

Uptake of compatible solutes

Using isocratic and FMOC-HPLC gradient techniques we investigated whether the species-specific differences in compatible solute protection were due to intracellular accumulation of solutes. Following the suspension of cells in protecting solutions (which provided osmotic stress for the cells) ectoine, hydroxyectoine, sucrose, and glycine betaine were immediately taken up by both strains of *E. coli* to an intracellular concentration of about 0.4 μmol mg^{-1} dry weight. Using an estimated cell volume of approximate 1 μl mg^{-1} dry weight, the intracellular solute concentration reached about 400 mM, which agrees fairly well with an extracellular concentration of approximate 500 mM. However, differences were observed with respect to the accumulation of trehalose. Trehalose could not be detected inside the cells of *E. coli* K12 over a period of 4 h, but instead glucose, alanine, and γ-aminobutyric acid were found, while the glutamate pool was low compared to cells resuspended in sucrose (Fig. 3). Thus, with the intruding solute sucrose the relative proportions of glutamate, γ-aminobutyric acid and alanine remained fairly unchanged as compared to buffer, whereas the nonpermeable trehalose caused marked changes, namely an increase in γ-aminobutyric acid and alanine and a decrease in glutamate. Glucose probably originated from extracellularly degraded trehalose, as both intra- and extracellular glucose levels increased, while that of

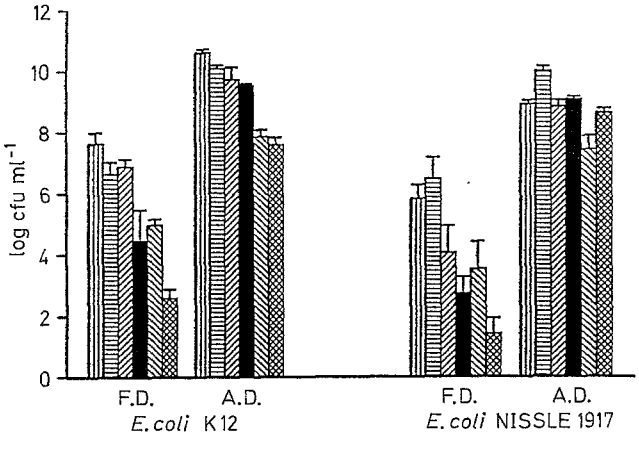

Fig. 2 Comparison of the protective effect of compatible solutes on two strains of *E. coli* during freeze- and air-drying and subsequent storage for 6 weeks at 4° C. Counts were performed on four parallel samples: (*F.D.* freeze-drying, *A.D.* air-drying) ▥ sucrose, ▤ trehalose, ▨ hydroxyectoine, ■ control, ▩ ectoine, ▦ glycine betaine

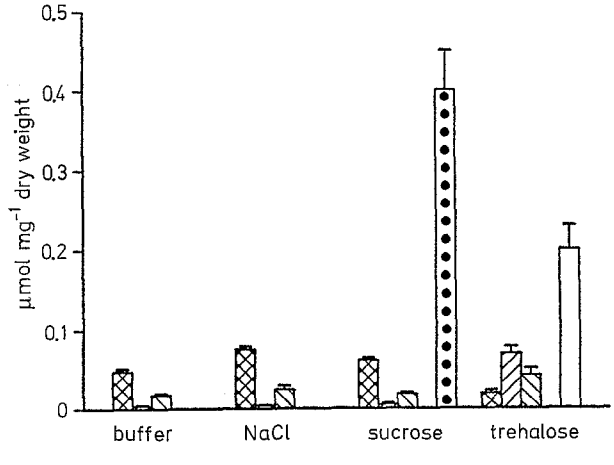

Fig. 3 Intracellular amounts of glutamate, γ-aminobutyric acid, alanine, sucrose and glucose in cells of *E. coli* K12 after resuspension in phosphate buffer alone or phosphate buffer with 312.5 mM NaCl, 625 mM sucrose or 625 mM trehalose. Means and standard deviation of three independent experiments are shown: ▦ glutamate, ▨ γ-aminobutyric acid, ▩ alanine, ⊡ sucrose, □ glucose

external trehalose decreased. The cytoplasmic concentration of glucose (0.2 μmol mg^{-1} dry weight, however, was not sufficient to balance the extracellular trehalose concentration. The application of NaCl (another non-intruding solute) did not have a similar effect on the amino acid composition of the cells (Fig. 3).

In contrast to strain K12, *E. coli* NISSLE 1917 accumulated trehalose intracellularly. Only minor amounts of glucose were found inside the cells, and in addition, concentrations of glutamate, alanine, and γ-aminobutyric acid were lower than in *E. coli* K12 (not shown).

Discussion

We were able to demonstrate that the addition of compatible solutes prior to freeze-drying may markedly increase the survival rate of the two *E. coli* strains tested. With the exception of trehalose, which did not penetrate *E. coli* K12, all solutes were detected within the cells at a concentration near equilibrium.

A comparison of freeze-drying and air-drying showed that freeze-dried cells were much more labile than air-dried cells. The survival rate decreased within hours after drying, a phenomenon also observed by Cox and Heckly (1973) with *Serratia marcescens* and by Nei et al. (1966) with *E. coli*. Air-drying seems to be the method of choice for long-term storage, as the cells proved to remain very stable. An active adaptation of the cells to the new conditions cannot be excluded, since the drying process is rather slow. Furthermore, the tight packing and a firm top layer obtained with air-dried cells may provide better protection against damage caused by oxygen.

Our study also revealed that the influence of some solutes on the survival of *E. coli* differed for drying and storage. For example, glycine betaine protected cells during freeze-drying but caused damage during storage. Marshall et al. (1974) and Redway and Lapage (1974) described a number of substances that were protective during freeze-drying of different bacteria but had a negative influence during storage. Crowe et al. (1990) postulated a relatively non-specific mechanism of stabilization by protective agents during freezing of proteins and membranes [the preferential exclusion model of Arakawa and Timasheff (1985)], whereas in the dry state a more specific interaction would take place. This could explain the different protective effects of some solutes immediately after drying as compared to storage.

The finding that the disaccharides served as the best protecting agents is in accordance with the results of other workers (Marshall et al. 1974; Redway and Lapage 1974; Bushby and Marshall 1977; Heckly and Dimmick 1978; Van Laere 1989). On the basis of the "water replacement hypothesis" of Clegg et al. (1982) this is probably explained by the presence of OH groups in the protecting molecules. The observation that hydroxyectoine is a better protectant than ectoine seems to support this concept. However, we believe that a range of effects has to be considered. If, for example, the generation of free radicals influences cell survival, some solutes could also play a role as scavengers. Smirnoff and Cumbes (1989) studied the capacity of compatible solutes as hydroxyl-radical scavengers. They found that sucrose was the best scavenger whereas glycine betaine was ineffective. However, the study of Smirnoff and Cumbes (1989) did not include tetrahydropyrimidines.

The different effects of the disaccharides (sucrose is the best protectant for *E. coli* K12 and trehalose for *E. coli* NISSLE 1917) may be due to different uptake properties with respect to trehalose. Whereas *E. coli* NISSLE 1917 was able to use trehalose as an intracellular protectant, *E. coli* K12 was not. Boos et al. (1990) investigated transport and metabolism of trehalose in a number of *E. coli* strains (not including K12 or NISSLE 1917). They proposed the following sequence of events: at low osmolarity trehalose is taken up and degraded inside the cells, whereas at high osmolarity it is cleaved to glucose in the periplasm. Glucose is subsequently taken up, resynthesized to trehalose and used as an osmoprotectant. In our study, we found trehalose and minor amounts of glucose inside the cells of *E. coli* NISSLE 1917, which agrees with the results of Boos et al. (1990). *E. coli* K12, however, accumulated glucose from extracellularly degraded trehalose, but seemed to be unable to resynthesize trehalose. In addition, the cytoplasmatic glutamate concentration dropped while that of γ-aminobutyric acid and alanine rose. Hence, in the absence of other solutes, glutamate is possibly converted into γ-aminobutyric acid by decarboxylation. This would result in the conversion of an anion into a zwitterion, which may be more compatible with cell functions.

The production of γ-aminobutyric acid from amino acids and amines by marine bacteria has been shown before (Mountfort and Pybus 1991, 1992). If these changes were triggered by osmotic stress exerted by trehalose, then other non-intruding solutes such as NaCl should have a similar effect. This was not the case (Fig. 3). However, it still remains to be shown whether NaCl is effectively excluded by resting cells or transiently accumulated, thus releaving osmotic gradients. The cytoplasmic changes induced by trehalose may be partly responsible for the cell protection still observed with this solute in *E. coli* K12. However, we cannot exclude the possibility that the stabilization is caused solely by external trehalose at the membrane level.

We conclude that non-reducing disaccharides such as sucrose and trehalose as well as tetrahydropyrimidines are promising candidates for further protection studies on whole organisms. These studies should pursue the optimization of solute concentrations and combinations of solutes and further investigate the partial protective effects of solutes against freezing, drying and storage.

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