APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1996, p. 259–261 Vol. 62, No. 1
0099-2240/96/$04.00+0

Effect of Compatible Solutes on Survival of Lactic Acid Bacteria Subjected to Drying

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Received 25 July 1995/Accepted 30 October 1995

Four strains of lactic acid bacteria were investigated to determine if a relationship exists between accumulation of compatible solutes and the ability of cells to survive drying. Betaine was the major solute found in these lactic acid bacteria subjected to salt stress. Survival of cultures subjected to drying was considerably enhanced when this solute was accumulated by cells.

Lactic acid bacteria are often preserved by freezing and/or drying for use as starter cultures in the food and feed industries. Dried starter cultures compare favorably to frozen starter cultures because of lower transport and storage costs. However, considerable inactivation of cells occurs during the drying process. Therefore, it is necessary to understand the physiological response of the organisms to drying (10).

Like many other organisms, lactic acid bacteria confronted with a decreased water activity (a_w) over a long period respond by accumulation of compatible solutes such as betaine and carnitine (3, 5).

These compounds are thought to be beneficial for lactic acid bacteria, not only during osmotic stress but also during drying. The organisms are probably not able to accumulate compatible solutes during the short drying process, and therefore they should be accumulated prior to the drying process (4).

Presumably, salt-tolerant strains can accumulate compatible solutes more efficiently than salt-sensitive strains and hence should be better protected against drying. In the present study, four strains of osmotically stressed lactic acid bacteria were studied to determine if a direct relation exists between their ability to accumulate compatible solutes and their survival after drying.

Organisms and growth medium. The organisms used were *Enterococcus faecium* URL-EF1 (Unilever Research Laboratory, Vlaardingen, The Netherlands) and *Lactobacillus halotolerans* ATCC 35410, which are both osmotolerant strains; the osmosensitive strain *Lactobacillus bulgaricus* URL-LB1 (Unilever Research Laboratory); and *Lactobacillus plantarum* P743 (Netherlands Institute for Dairy Research, Ede, The Netherlands), which was previously studied by Kets and de Bont (4). Experiments were performed with either MRS medium as described by de Man et al. (2) or defined medium (DM) as described by Kets et al. (5).

Salt tolerance. Cells were subjected to salt stress by including NaCl in the medium to obtain initial data about the salt tolerance of the strains tested in this investigation. The maximum NaCl concentrations at which growth was still possible in MRS medium were only 0.5 M for *L. bulgaricus* and 1.5, 2.5, and 2.3 M for *L. plantarum*, *L. halotolerans*, and *E. faecium*, respectively. In DM, the maximum NaCl concentration for *L. bulgaricus* was 0.3 M, while those for *L. plantarum*, *L. halotolerans*, and *E. faecium* were 1.3, 1.0, and 1.5 M, respectively. In these batch experiments, 15 mM lactose was replaced by 30 mM glucose as a carbohydrate source for *E. faecium* and *L. halotolerans* cultivated in DM. In triplicate, serum bottles containing 49.5 ml of DM or MRS medium were flushed with N_2 and inoculated with 0.5 ml of a culture of cells grown in either DM or MRS medium. *E. faecium* and *L. bulgaricus* were cultured at 37°C. *L. plantarum* and *L. halotolerans* were cultured at 30°C. Growth was monitored until 72 h by optical density determinations at 660 nm. *L. bulgaricus* was the most salt-sensitive strain tested in both media. The rich, complex MRS medium sustained growth at higher NaCl concentrations than DM did. Remarkably, *L. halotolerans* did not grow when no NaCl was included in DM (not shown) yet was more salt sensitive in DM than *L. plantarum* and *E. faecium*.

Accumulation of compatible solutes in complex medium. Lactic acid bacteria require a complex group of compounds for growth (2). Therefore, accumulation of compatible solutes in complex diluted MRS medium (DMRS medium) containing 2.75 g of MRS medium (Difco) per liter was determined. Cells were grown in a fermentor with a 1-liter working volume at a D of 0.02 h^{-1} (4). The growth temperatures for *L. bulgaricus*, *L. plantarum*, *L. halotolerans*, and *E. faecium* were 37, 30, 34, and 37°C, respectively. The medium was supplemented with various amounts of NaCl. Compatible solutes were analyzed by high-performance liquid chromatography as described by Kets et al. (5), and amino acid concentrations were determined by the method of Kunte et al. (7) with a Chromspher 5 C_{18} column (Chrompack, Bergen op Zoom, The Netherlands).

Betaine and carnitine are both present in DMRS medium, which contains yeast extract and beef extract (5). Although both compounds are important compatible solutes for bacteria, no growth conditions under which *L. bulgaricus* accumulated either of the two solutes were found (Table 1). Similarly, Hutkins et al. (3) showed that *L. bulgaricus* ATCC 8144 transported no betaine at elevated salt levels. *L. plantarum* subjected to salt stress accumulated both betaine and carnitine when the NaCl concentration added was 1 or 1.3 M. Measures (8) found accumulation of glutamate and proline in *L. plantarum*. However, ^{13}C nuclear magnetic resonance analysis (not shown) of cell preparations revealed no proline accumulation in this strain during growth in DMRS medium. Strikingly, *L. halotolerans* cultured in DMRS medium accumulated betaine but not carnitine when the medium was supplemented with 0.4

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TABLE 1. Accumulation of solutes by chemostat-grown lactic acid bacteria cultivated in DMRS medium and subjected to salt stress

Organism	NaCl addition (M)	Amt of accumulated solute (μmol g [dry wt] of cells^{-1})[a]		
		Betaine	Carnitine	Total amino acids
Lactobacillus bulgaricus	0	—	—	257
	0.1	—	—	371
	0.3	—	—	252
Lactobacillus plantarum	0	—	—	227
	1	255	159	349
	1.3	282	258	342
Lactobacillus halotolerans	0	31	—	737
	0.4	106	—	127
Enterococcus faecium	0	5	104	215
	1.0	123	85	68

[a] Values are means of duplicate determinations. —, not detected at concentrations over 1 μmol g (dry weight) of cells^{-1}.

M NaCl. Cells of *E. faecium*, either stressed or unstressed, contained a high intracellular level of carnitine, while the betaine concentration strongly increased, reaching 123 μmol g (dry weight) of cells^{-1}, under osmotic stress. Similar observations have been made for *Listeria monocytogenes* subjected to increasing salt stress (6).

Accumulation of betaine in DM. As mentioned above, complex DMRS medium contains betaine and carnitine, thus preventing clear observations of the effects of the former compatible solute on the physiology of cells. Consequently, a betaine-free medium was used.

Growth of *L. bulgaricus* at elevated NaCl concentrations did not result in accumulation of betaine. Again, as observed for cells grown in DMRS medium, intracellular amino acid levels responded to a reduction in the a_w (Table 2). These levels were due to higher concentrations of intracellular aspartate, glutamate, and alanine (not shown).

Growth of *L. plantarum* in DM changed the balance of accumulated solutes remarkably in comparison with growth in DMRS medium. Compared with DM without NaCl, DM containing 1 M NaCl reduced the amount of accumulated amino acids. Addition of 2 mM betaine to the medium during osmotic stress did not affect the amino acid composition in cells, but it did decrease the amount of amino acids when added to DM not containing NaCl.

L. halotolerans accumulated substantial amounts of betaine, and in its absence the organism was able to grow by accumulating amino acids. In particular, proline accumulated (131 μmol g [dry weight] of cells^{-1} in the absence of betaine versus 57 μmol g [dry weight] of cells^{-1} in the presence of betaine). Similar results were obtained for *Lactococcus lactis*; in this case, proline was replaced by betaine when the latter was included in the medium (9). Also, addition of only carnitine to DM, as in a study by Beumer et al. (1), enhanced growth of cells subjected to osmotic stress (not shown).

Amino acid levels in *E. faecium* decreased in the presence of NaCl. This unexpected observation may be explained by the ability of the organism to accumulate unknown compounds, as was found for ^{13}C nuclear magnetic resonance spectra of extracts of cells cultured in complex medium (not shown). Analysis of these compounds in DM awaits further elucidation.

Effect of betaine on survival of cells after drying. The effect of betaine on survival after drying was tested with DM in a chemostat as described above. Cells cultured were harvested by centrifugation (16,000 × *g*) under steady-state conditions and washed in 0.02 M potassium phosphate containing the concentrations of salt included in the growth medium. Resuspended cells (2 ml) were dried in petri dishes by exposure to air (approximately 35% relative humidity, 30°C, 2.5 h). The petri dishes were subsequently kept at 5°C in a desiccator containing a saturated solution of LiCl (a_w = 0.12) for 72 h. Dried samples were resuspended, and serial dilutions were plated on MRS agar plates. The resulting colonies from samples taken before and after drying were counted, and the survival percentage was calculated (4).

Betaine included in osmotically stressed medium clearly protected *L. plantarum*, *L. halotolerans*, and *E. faecium* against drying (Table 2). *L. bulgaricus* was not able to accumulate this solute, and adding betaine to the culture medium indeed did not protect the organism when the samples were dried (not shown). In media which were not salt stressed, addition of betaine did not improve survival after drying. In the cases of *L. halotolerans* and *E. faecium*, increased survival in the presence of only NaCl may be attributable to proline or accumulated compounds found in ^{13}C nuclear magnetic resonance spectra, respectively. From the results presented in Table 2, we concluded that there is a direct relationship between the presence of compatible solutes in lactic acid bacteria and their ability to survive drying.

TABLE 2. Accumulation of solutes and survival after drying of chemostat-grown lactic acid bacteria cultivated in DM and subjected to salt stress in the presence or absence of betaine

Organism	Additive		Amt of accumulated solute (μmol g [dry wt] of cells^{-1})[a]		Survival (%)[b]
	NaCl (M)	Betaine (2 mM)	Betaine	Total amino acids	
Lactobacillus bulgaricus	0	−	—	227	0.05 ± 0.03
	0.15	−	—	383	0.4 ± 0.2
Lactobacillus plantarum	0	−	—	660	4.3 ± 1.8
	1	−	—	402	11.7 ± 1.0
	0	+	42	119	1.9 ± 0.3
	1	+	192	444	26.0 ± 2.3
Lactobacillus halotolerans	1	−	—	575	37.1 ± 1.8
	1	+	534	400	55.0 ± 6.5
Enterococcus faecium	0	−	—	192	17.1 ± 5.3
	1	−	—	77	40.6 ± 2.8
	0	+	47	239	38.7 ± 5.5
	1	+	391	49	66.1 ± 14.4

[a] Values are means of duplicate determinations. —, not detected at concentrations over 1 μmol g (dry weight) of cells^{-1}.

[b] Ratio of number of viable cells after drying relative to number of viable cells before drying, expressed as a percentage (mean ± standard deviation [n = 3]).

The financial support of the Unilever Research Laboratory, Vlaardingen, The Netherlands, is gratefully acknowledged.

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