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Effect of Sodium Chloride on the Intracellular Solute Pools of *Listeria monocytogenes*

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The concentrations of intracellular solutes in *Listeria monocytogenes* were examined in cells grown at various concentrations of NaCl. At 5% NaCl, cells contained elevated concentrations of potassium and glycine betaine compared with concentrations in cells grown without NaCl. At 7.5% NaCl, cells contained increased concentrations of K^+, glycine betaine, glycine, alanine, and proline. Only glycine betaine, choline, or glycine promoted growth on a solidified defined medium containing 4% NaCl; there was no growth at higher concentrations of NaCl in the defined medium.

Listeria monocytogenes is a gram-positive, rod-shaped bacterium of widespread distribution in the environment. It is able to live as an intracellular pathogen in humans and animals (6, 7, 10, 14). It has been isolated from a range of food samples, and there have been major outbreaks of listeriosis associated with contaminated foods. The organism is of concern to the food industry, particularly as it can survive and grow at refrigeration temperatures and high salt concentrations, e.g., up to 10% (18). This tolerance to osmotic stress suggests an adaptation of the organism to the osmotic environment.

The adaptation of bacteria to a high-salt environment by the intracellular accumulation of compatible solutes has been extensively studied in gram-negative bacteria. Solutes commonly accumulated include potassium ions, amino acids, imino acids, quaternary ammonium compounds, and carbohydrates. The genetics and control of the uptake mechanism have been well characterized in *Escherichia coli* and *Salmonella typhimurium*, showing an integrated response involving accumulation of K^+, glutamate, betaine, and trehalose, dependent on the nature of the environment and the degree of upshock (5). The physiological response of gram-positive bacteria to external osmotic potential has received considerably less attention. *Streptomyces* species accumulate proline, glutamine, and alanine because of increased synthesis when grown at enhanced salt concentrations (11). *Staphylococcus aureus* accumulates glutamine, proline, and betaine (1, 13, 19) in saline conditions. Proline accumulation has also been observed in *Bacillus subtilis*, but when betaine was present, this was accumulated in preference to proline (2, 21, 24). This paper describes the intracellular solutes accumulated in *L. monocytogenes* in response to increased salinity and osmotic stress.

MATERIALS AND METHODS

Growth of bacteria. *L. monocytogenes* was grown in coryneform broth containing (liter^{-1}) 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 5 g of glucose (pH 7.2) or a defined medium containing (liter^{-1}) 8.0 g of K_2HPO_4, 3.1 g of $NaH_2PO_4 \cdot 2H_2O$, 1.0 g of NH_4Cl, and 0.4 g of $MgSO_4$ (pH 6.8). The defined medium base was supplemented with (liter^{-1}) 0.1 g of L-leucine, 0.1 g of L-norleucine, 0.2 g of DL-valine, 0.6 g of L-glutamine, 0.1 g of L-methionine, 0.2 g of L-histidine, 0.2 g of L-arginine, 0.1 g of L-tryptophan (23), 1 ml of vitamin solution (22), 1 ml of trace element solution (8), and glucose (0.5%, wt/vol). The media were solidified by addition of 1.5% (wt/vol) agar (Difco no. 3). Media were supplemented with sodium chloride as required. Cells were inoculated into 500 ml of medium in 3-liter baffled flasks as a 1/50 dilution from an overnight culture in identical medium, grown at 30°C with shaking (200 rpm) to mid-exponential growth, and harvested by centrifugation (5,000 × *g* for 10 min).

Measurement of cell volume. Cytoplasmic and cell volumes were determined by the differential penetration of [^{3}H]water, [^{14}C]mannitol, and [^{14}C]carboxymethyl inulin (20). Harvested cells were resuspended to an optical density at 600 nm (OD_{600}) of ca. 20 in the defined medium base with the appropriate salt concentration. The cell suspension (4 ml) was incubated at 30°C with mixing for 1 min with [^{3}H]water (40 μCi) and ^{14}C-mannitol (1.6 μCi) or [^{3}H]water (40 μCi) and [^{14}C]hydroxymethyl inulin (1.6 μCi) for the determination of cytoplasmic volume and cell volume, respectively. Aliquots (0.5 ml) were centrifuged rapidly (12,000 × *g*, 30 s), and the supernatant fluid was removed. The cell pellet was resuspended in 0.5 ml of defined medium base. Aliquots of cell suspension and the supernatant fluid were counted in Instagel scintillation cocktail (Packard Instrument Co.) with a Tri-Carb liquid scintillation counter (Packard) with dual label counting by full-spectrum analysis.

Estimation of intracellular osmotic potential. The internal osmolality of *L. monocytogenes* grown at different salt concentrations was estimated from the change in cell volume over a range of external solute concentrations (25). Exponentially growing cells of *L. monocytogenes* were harvested by centrifugation (6,000 × *g*, 10 min) and resuspended in a minimal volume of medium at 5°C. Aliquots (50 μl) of cell suspension were diluted into 950 μl of buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.0) containing various concentrations of NaCl at room temperature. The OD_{600} was measured after 1 min with a SP8-150 spectrophotometer (Pye Unicam). The effect of the refractive index on optical density was corrected for by measuring the optical density of 50 μl of bacterial suspension in 950 μl of standard Ficoll solutions in 50 mM HEPES, pH

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7.0. The refractive index of Ficoll solutions was determined with a PR1 digital refractometer (Atago Co. Ltd., Tokyo, Japan). The reciprocal of this corrected value is proportional to the surface area and enables the determination of cell volume in arbitrary units as the 3/2 power of the surface area (12, 25). Boyle-van't Hoff plots were prepared by plotting cell volume as a percentage of the original against the reciprocal of the external osmolality.

Measurement of intracellular solutes. (i) Potassium. An aliquot (10 ml) of mid-exponential-phase culture was rapidly filtered through a prewashed GF/F glass fiber filter (Whatman) under vacuum and immediately washed with 5 ml of 5 mM HEPES–1 mM $MgSO_4$, pH 7.0, containing NaCl at the concentration in the growth medium. Filters were placed in 4 ml of distilled water and incubated in a boiling water bath for 3 min. Potassium was measured by flame photometry after appropriate dilution of the sample. Background potassium was corrected for by a control sample consisting of bacterium-free culture supernatant filtered and processed in an identical manner. Potassium content was related to cell protein by centrifuging 1.5 ml of the bacterial culture and quantitating cell protein in the pellet by a modified Lowry method (9).

(ii) Amino acids. Mid-exponential-phase cells were harvested by centrifugation (5,000 × *g* for 10 min), washed in 5°C 5 mM HEPES, pH 7.0, containing sodium chloride at a concentration equal to that in the growth medium, and resuspended in water to an OD_{600} of ca. 20. Aliquots were taken for protein estimation, and a sample was diluted with an equal volume of (5°C) trichloroacetic acid (20%, wt/vol) and stored on ice for 30 min. The precipitated cell debris was removed by centrifugation (5,000 × *g* for 10 min), and trichloroacetic acid was removed by three extractions with equal volumes of diethyl ether. Residual ether was removed under a stream of N_2. The extract was freeze-dried and redissolved in Li-citrate sample buffer (Beckman Instruments, Inc.), and amino acids were analyzed on a model 6300 amino acid analyzer (Beckman Instruments Inc.).

(iii) Betaine. Mid-exponential-phase cells were harvested by centrifugation, washed with 5°C water containing sodium chloride at a concentration equal to that in the growth medium, and resuspended in water to an OD_{600} of ca. 20. Cell material was precipitated with an equal volume of (5°C) 1.2 M perchloric acid and incubated at 0°C for 30 min, and the precipitate was removed by centrifugation (5,000 × *g* for 10 min). The supernatant was neutralized with KOH and incubated at 0°C for 1 h. The precipitate was removed by centrifugation (5,000 × *g* for 10 min). Supernatants were freeze-dried, redissolved in D_2O (Sigma, Poole, Dorset, United Kingdom), and filtered through a 0.2-μm-pore-size filter, and nuclear magnetic resonance spectra were obtained on a JEOL GX-400MHZ spectrometer. Betaine peaks were identified by comparison with betaine standards and quantitated by reference to an acetone internal standard.

(iv) Carbohydrate. Perchloric acid extracts of mid-exponential-phase cells were analyzed for total carbohydrate by the anthrone method after removal of sugar phosphates and reducing sugars by boiling with 0.2 M HCl and then with 0.2 M NaOH (16, 17). Standards containing trehalose were treated similarly.

RESULTS

Measurement of cell volume of *L. monocytogenes* grown under various salt concentrations showed no change in cytoplasmic volume over the range of 0 to 7.5% NaCl, with a water-permeable, mannitol-impermeable space of 2.73 to 3.99 μl/mg of protein. Total cell volume showed no change between 0 and 5% NaCl, with a water-permeable, inulin-impermeable space of 4.35 to 4.75 μl/mg of protein. At 7.5% NaCl the total cell volume decreased to 3.94 μl/mg of protein (Table 1). Since the cytoplasmic volume had decreased only slightly, this was presumably due to a decrease in the thickness of the cell wall. Standard cell suspensions (OD_{600} = 20) showed no significant variation in protein concentration dependent on salt concentration during growth, and therefore these measured volume/protein ratios reflected volume changes rather than changes in protein content of the bacteria. This maintenance of cytoplasmic volume over a wide range of salinity of 0 to 7.5% NaCl (equivalent to 0 to 1.28 M) indicated a maintenance of turgor pressure in the cell. This could be achieved either by maintaining a higher internal osmotic potential under all conditions or by increasing the internal osmotic potential by uptake of solutes to maintain a difference in the osmotic potentials of the internal and external environments.

TABLE 1. Cell volumes and potassium and betaine concentrations in *L. monocytogenes* grown at various salt concentrations in complex media

NaCl (% [wt/vol])	Cytoplasmic vol [μl/mg of protein (*n*)]	Cell vol [μl/mg of protein (*n*)]	K^+ [M (*n*)]	Betaine (M)
0	2.73 ± 0.66 (9)	4.75 ± 0.90 (9)	0.163 ± 0.044 (3)	0.065
2.5	3.99 ± 0.23 (6)	4.35 ± 0.23 (6)	0.226 ± 0.010 (3)	0.076
5	3.13 ± 0.52 (9)	4.70 ± 0.23 (6)	0.196 ± 0.047 (3)	0.358
7.5	3.10 ± 0.49 (6)	3.94 ± 0.09 (6)	0.319 ± 0.100 (3)	0.588

Internal osmotic potential. The internal osmolality of *L. monocytogenes* was estimated from the effect of high salt concentrations on the cell volume as measured by the OD_{600}. Boyle-van't Hoff plots showed that below a certain value of the reciprocal of external osmolality, *L. monocytogenes* behaved as an ideal osmometer, with a linear relationship between cell volume and the reciprocal of external osmolality (Fig. 1). At high external osmolalities this relationship broke down, with a large decrease in cell volume occurring with relatively small changes in osmolality. Extrapolation of the linear portion of the graph enabled the derivation of a value for the nonosmotic volume at infinite osmolality to be obtained. Taking Fig. 1 as an example, the nonosmotic volume is 34% of the original volume. From the cell volume measurements obtained by the use of permeant probes, with a total cell volume of 4.75 μl/mg of protein this gave a nonosmotic volume of 1.615 μl/mg of protein and therefore an original osmotic volume of 3.135 μl/mg of protein. When acting as an ideal osmometer in the linear portion of the graph, the turgor pressure is zero, enabling the application of the Boyle-van't Hoff relationship to calculate the internal osmolality in the original medium (25). Results from these experiments (Table 2) indicate an internal osmolality of approximately 0.55 osmol/kg in *L. monocytogenes* grown in the absence of sodium chloride, with an apparent increase to approximately 0.73 to 0.83 osmol/kg at 7.5 and 5% NaCl, suggesting the accumulation of an internal solute in response to the external salt concentration.

Intracellular solutes. Measurement of the steady-state intracellular potassium concentration in exponentially growing cells of *L. monocytogenes* showed a potassium concentration of 0.163 M in cells growing in the absence of external sodium chloride. There was no significant increase in the

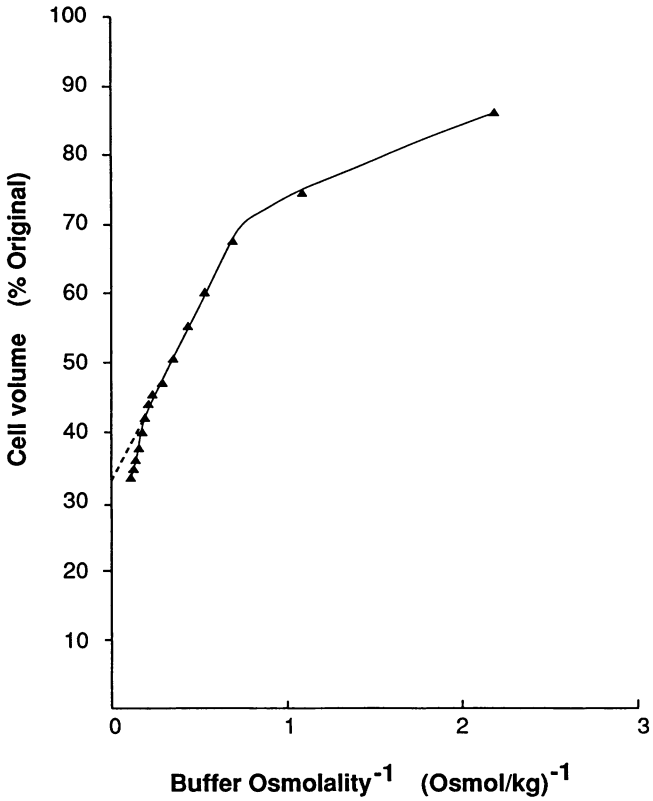

FIG. 1. Boyle-van't Hoff plot of *L. monocytogenes*. Cell volume in arbitrary units was calculated as the 3/2 power of the surface area, where surface area is proportional to optical density of the bacterial suspension after correction for effects of refractive index changes (see Materials and Methods). Values were plotted as the percentage of the value obtained in the absence of NaCl.

internal K^+ concentration with up to 5% NaCl in the growth medium. At an external sodium chloride concentration of 7.5% (wt/vol) the internal K^+ concentration doubled to 0.319 M, suggesting the accumulation of K^+ as an osmoprotectant at high salt concentrations (Table 1).

The concentration of intracellular betaine showed no increase with up to 2.5% salt but showed a ninefold increase in concentration, up to 0.588 M, at 7.5% (wt/vol) sodium chloride (Table 1). In contrast, there was no change in the majority of the amino acids during growth with up to 5% NaCl (Table 3). At 7.5% NaCl there was a general increase in the concentration of many of the amino acids; however, the principal contributions to higher cellular concentrations were glutamate (52 to 280 mM), glycine (7 to 52 mM), alanine (10 to 110 mM), and proline (9 to 42 mM). The total concentration of amino acids increased substantially from 166 mM with no salt to 716 mM at 7.5% NaCl. No difference in the concentration of intracellular nonreducing sugar was detected over this range of growth and NaCl conditions (data not shown).

TABLE 2. *L. monocytogenes* growth rates and internal osmolality calculated from Boyle-van't Hoff plots

Growth medium[a]	Doubling time (h)	Internal osmolality [osmol/kg (*n*)]
CB	1	0.55 ± 0.14 (4)
CB + 5% NaCl	1.4	0.83 ± 0.20 (3)
CB + 7.5% NaCl	2.6	0.73 ± 0.20 (3)

[a] CB, coryneform broth.

TABLE 3. Intracellular concentrations of free amino acids in *L. monocytogenes* grown at different NaCl concentrations in complex media

Amino acid	Amino acid concn (mM) (*n* = 3) after growth with:		
	No NaCl	5% NaCl	7.5% NaCl
Asp	13.56 ± 4.91	11.38 ± 1.41	45.01 ± 18.03
Thr	6.15 ± 2.38	4.43 ± 1.30	37.83 ± 21.85
Ser	6.36 ± 4.07	5.12 ± 1.56	24.71 ± 14.74
Asn	1.64 ± 2.24	2.23 ± 0.71	0.00 ± 0.00
Glu	52.01 ± 18.90	37.62 ± 6.60	280.85 ± 55.01
Gln	2.09 ± 2.13	3.10 ± 2.10	7.28 ± 2.03
Gly	7.16 ± 3.35	8.60 ± 0.95	52.65 ± 17.09
Ala	9.47 ± 9.06	23.29 ± 4.67	109.95 ± 19.15
Val	5.33 ± 6.35	7.01 ± 1.95	20.09 ± 11.65
Met	2.96 ± 1.87	2.74 ± 0.62	10.80 ± 4.70
Cys	0.03 ± 0.07	0.00 ± 0.00	0.49 ± 0.03
Ile	2.77 ± 2.97	2.70 ± 1.38	10.48 ± 7.00
Leu	5.42 ± 5.88	9.44 ± 3.26	38.58 ± 21.18
Tyr	1.75 ± 0.98	1.25 ± 0.41	4.07 ± 2.64
Phe	5.03 ± 3.65	5.27 ± 1.71	8.58 ± 3.42
gaba[a]	2.27 ± 2.79	1.99 ± 0.67	0.06 ± 0.10
Trp	3.65 ± 4.06	1.48 ± 0.73	0.00 ± 0.00
Orn	0.83 ± 0.43	0.44 ± 0.11	1.03 ± 0.09
Lys	16.60 ± 8.48	9.31 ± 3.37	12.78 ± 7.33
His	4.11 ± 0.67	1.53 ± 0.57	3.54 ± 1.54
Arg	8.16 ± 3.64	5.06 ± 1.21	5.52 ± 2.75
Pro	9.33 ± 3.26	3.65 ± 0.72	42.03 ± 24.68
Sum	166.69	147.63	716.33

[a] gaba, gamma aminobutyric acid.

Osmoprotectants. *L. monocytogenes* grew well on a solid, defined medium in the absence of sodium chloride. The presence of 4% (wt/vol) NaCl prevented growth at 30°C, but this inhibition was relieved by 1 mM betaine (colonies formed at 3 days). Growth was also promoted by the presence of 1 mM choline and 1 mM glycine, although these were substantially less effective than betaine (with the latter, colonies were present at 6 days but not at 3 days, and with the former, pinprick-size colonies were present at 6 days). Neither 1 mM alanine nor 1 mM proline promoted growth in the presence of 4% NaCl.

DISCUSSION

L. monocytogenes can grow over a wide range of salt concentrations, and as in other bacteria this adaptability appears to be achieved by the ability of the organism to accumulate intracellular solutes.

Use of optical density measurements to determine cell shrinkage and hence intracellular osmolality is a crude method involving large corrections for refractive index effects (25). The estimates obtained, however, indicated that *L. monocytogenes* maintains a relatively high internal osmolality of approximately 0.55 osmol/kg when grown at low salt concentrations, a value similar to that found in *B. subtilis* (25). There was evidence of a slight increase in the internal osmolality when the cells were grown in 7.5% sodium chloride; however, the difference was slight in comparison with the increase in the external salt concentration. The

values obtained are likely to be underestimates due to the loss from the cells of rapidly mobile solutes such as K^+.

At a salt concentration of 2.5% (wt/vol), there were no increase in the accumulation of any of the measured solutes and no change in the generation time of *L. monocytogenes*. At 5% NaCl, the cells contained increased concentrations of betaine only, giving an overall increase of solutes (K^+, betaine, and amino acids) from 0.395 to 0.698 M. At 7.5% NaCl, increased concentrations of K^+, betaine, and amino acids gave an overall concentration of 1.623 M. The increased intracellular solute concentration (change in concentration of 1.228 M) observed between growth at 2.5% NaCl and growth at 7.5% NaCl (change in NaCl concentration of 0.855 M) is probably insufficient to provide complete compensation for the increased salinity, as this change in salt concentration represents an increase in osmolality of 1.74 osmol/kg. There is therefore the possibility of further, as yet unidentified osmoprotectants in the cell, especially as the activity coefficient of the cytoplasmic solutes is probably not unity because of interaction with cellular components. Attempts to measure intracellular Na^+ concentrations were unsuccessful because of high background levels of sodium present in the media (data not shown).

Proline was not a major osmoprotectant under the growth conditions studied. Betaine present in the complex media may have been accumulated preferentially and inhibited proline uptake, as has been reported for *B. subtilis* (24). Proline did not alleviate inhibition of growth by sodium chloride in a defined medium lacking betaine, suggesting that proline may not be accumulated as an osmoprotectant in this organism. Furthermore, the proline analogs L-azetidine-2-carboxylic acid and 3,4-dehydroproline failed to inhibit the growth of *L. monocytogenes* in either the presence or absence of NaCl (data not shown). This may indicate that the organism lacks a proline uptake system and furthermore suggests that the betaine uptake system cannot take up proline, unlike the betaine uptake system in *S. typhimurium* (3, 4). Alternatively, this organism may not be susceptible to intracellular L-azetidine-2-carboxylic acid or 3,4-dehydroproline. The ability of choline to promote growth on a defined medium containing NaCl may indicate a role as a precursor for betaine, as reported for *E. coli* (15). The ability of glycine to promote growth on a defined medium is of interest. This effect was obtained only on solid media; similar stimulation of growth in a liquid medium of defined composition with 4% NaCl was not observed (results not shown). The reason for this discrepancy is unclear. During growth on defined media, in the absence of NaCl, glycine was not an essential nutrient and is thus presumably synthesized by anabolic metabolism. This anabolic route appears to be incapable, however, of supplying sufficient glycine to enable growth at 4% NaCl on defined medium. Alanine did not promote growth on a defined medium with NaCl despite its accumulation during growth in coryneform broth with 7.5% NaCl. The true role of such amino acids as osmoprotectants in these conditions remains unclear. Alteration of their intracellular concentration may be a consequence of altered growth rate during batch culture rather than a protective response to osmotic stress.

The ability of *L. monocytogenes* to grow in high concentrations of salt may be an important factor enabling the organism to survive and grow in foods, e.g., soft cheeses. An understanding of the organism response could indicate mechanisms for controlling its growth in foods.

This work shows that the response of *L. monocytogenes* to osmotic stress is similar to that of many other bacteria: the organism accumulates K^+, betaine, and glutamate in response to osmotic stress; however, there was no evidence for proline acting as an osmoprotectant. The possible role of glycine in osmoregulation awaits further elucidation.

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