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Glutamate uptake in *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 208 and its enhancement by a combination of Mn^{2+} and Mg^{2+}

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Aims: To demonstrate the mechanism of glutamate uptake in the dairy strain *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 208, and to characterize key aspects of the system.
Methods and Results: Glutamate uptake proceeded via an active transport system requiring an exogenous source of energy. The system also transported aspartate and glutamine. It was unique, with a K_t of 2·8 μmol l^{-1} and a V_{max} of 900 μmol s^{-1} (g dry weight)$^{-1}$. The activity was optimal at pH 7·3 and 50°C, was independent of the glutamate charge, and was enhanced by Mn^{2+} + Mg^{2+} in combination. Inhibition of the activity by uncouplers and ionophores showed that transport was driven by an ATP-dependent mechanism involving the proton-motive force. This inhibition was partially abolished in the presence of both Mn^{2+} and Mg^{2+}.
Conclusions: We demonstrated for the first time that an active transport system governs the uptake of the essential amino acid glutamate in *Lact. delbrueckii* subsp. *bulgaricus* CNRZ 208, the activity of which is enhanced by a combination of Mn^{2+} and Mg^{2+}.
Significance and Impact of the Study: The potential of the findings is discussed with reference to the growth of *Lact. delbrueckii* subsp. *bulgaricus* in mixed-strain cultures for the dairy industry.

INTRODUCTION

Lactic acid bacteria are commercially important microorganisms used as mixed-strain cultures in the manufacture of fermented products. They show an absolute requirement for essential nutrients, including amino acids (Desmazeaud 1983). Frequently, their transport occurs across the cell membrane against concentration gradients using an active system consisting of coupling of the carrier to the metabolic machinery. General models have been proposed, which involve chemiosmotic coupling to ion gradients or direct chemical coupling of the transport to ATP or intermediates of oxidative phosphorylation (Konings *et al*. 1994).

In contrast to vertebrates (Slotboom *et al*. 1999), requirement for glutamate (Glu) remains poorly characterized in bacteria (Ledesma *et al*. 1977; Morishita *et al*. 1981; Benateya *et al*. 1986; Konings *et al*. 1994). Despite amino acid transport now being well described in some lactic acid bacteria (Konings *et al*. 1994), only a few papers have been devoted to amino acid uptake in the genus *Lactobacillus* (Strobel *et al*. 1989; de Giori and de Valdez 1994; Nakajima *et al*. 1998).

In the dairy industry, *Lact. delbrueckii* subsp. *bulgaricus* (*Lact. bulgaricus*) is used on a large-scale in yoghurt manufacturing as a mixed-strain culture with *Streptococcus thermophilus*. Glutamate, which results from casein hydrolysis, is recognized as an important factor for significant growth of both organisms, probably owing to multiple roles including transamination processes. Thus, in this study, we present evidence for and characterize the uptake of Glu in *Lact. bulgaricus* with reference to growth in milk.

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MATERIALS AND METHODS

Organism and culture conditions

Lact. bulgaricus CNRZ 208 (ATCC 11842) was obtained from the INRA-CNRZ collection, Jouy-en-Josas, France. Stock cultures were propagated in 10% (w/v) skimmed milk

containing 0·1% (w/v) bromocresol purple and stored at –20°C, and utilized to inoculate MRS (de Man *et al*. 1960), incubated overnight statically at 37°C.

The influence of growth conditions on Glu uptake was tested in a chemically defined medium (Ledesma *et al*. 1977) in which Glu was present as a free amino acid (1 mmol l^{-1}) or glutamate-containing dipeptide at the same concentration.

Preparation of cells for uptake experiments

For uptake experiments, 1% (v/v) of a late exponential-phase cell culture were inoculated again in MRS and grown under the same conditions (unless otherwise stated). Exponential-phase cells were then harvested by centrifugation (12 500 *g*, 10 min, 4°C). The pellet was washed twice with 50 mmol l^{-1} N-(2-hydroxyethyl piperazine)-N′-3-propane sulphonic acid equilibrated with KOH (HEPPS/KOH) to pH 7·3, and suspended in the same buffer (unless otherwise stated). The optical density of cell suspensions at 650 nm (not used elsewhere) was adjusted to 2 (equivalent to 1·2 g dry weight l^{-1}), as measurements indicated a linear correlation between the Glu uptake and the cell concentration of up to 1·8 g dry weight l^{-1}.

Transport assays

Cells were depleted by starvation for 2 h at 37°C in 50 mM HEPPS/KOH pH 7·3, under shaking (120 strokes min^{-1}) and then energized for 10 min with 20 mmol l^{-1} glucose (unless otherwise stated). Reactions were initiated by adding 100 μmol l^{-1} of L-[1–4-^{14}C]Glu to the cell suspension. At given time intervals 0·2 ml of the reaction mixture was removed and filtered through a 450-nm filter (HA-type, Millipore Corp. Bedford, MA). Filters were washed twice with 4 ml of 50 mM HEPPS/KOH pH 7·3 prewarmed to the assay temperature, dried under an infrared lamp and placed in scintillation vials containing 5 ml of the cocktail (Ready-Solv. Hp/b, Beckman Instrument Inc., Fullerton, CA). Radioactivity was determined with a liquid scintillation spectrophotometer (LS 2800 type, Beckman) with a counting efficiency of more than 95% for ^{14}C. The initial rate of uptake was determined from the initial slopes of transport kinetic curves as nmoles of Glu taken up per second and per g of dry weight.

The pH dependency of transport was determined in the following buffers (all 50 mmol l^{-1}): 4-morpholineethane sulphonic acid (MES) buffer, pH 4–6; potassium phosphate, pH 6–7·5; HEPPS/KOH, pH 6–8·5; and Tris/HCl, pH 7·5–9·5.

Miscellaneous

[1–4-^{14}C]L-Glu (specific radioactivity 9·3 TBq mol^{-1}) was purchased from the Commissariat à l'Énergie Atomique (Saclay, France). All chemicals were of reagent grade and were obtained from commercial sources. All amino acids were L- unless otherwise stated. Mean values of transport data with standard deviation were calculated on the basis of three independent experiments, and the results varied by less than 10%.

RESULTS

Evidence for an active uptake system

In contrast to negligible uptake in de-energized cells, significant uptake of Glu was observed in *Lact. bulgaricus* CNRZ 208 in the presence of glucose or lactose as the energy source. Maximum uptake activity [125 ± 10 nmol s^{-1} (g dry weight)$^{-1}$] was measured after 10 min energization with 20 mmol l^{-1} glucose.

Table 1 Effect of amino acids, glutamate-related compounds and various peptides on glutamate uptake in *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 208

Unlabelled amino acid	Uptake inhibition (%)	Analogue or peptide	Uptake inhibition (%)
Gln, Asp	83	Carbobenzoxy-Glu	98
Val	62	Glu-Val	91
Ala, Trp	35	Glu-α-anilide	87
Phe, Lys	29	D-Glu	55
Met, Ser	23	γ-Methyl ester-Glu	45
Leu, Arg	13	Glu-Thr-Tyr, Val-Gly-Ser-Glu	30
Gly	8	Acetyl-Glu	15
His	0	Val-Glu, γ-Glu-Ala	10

Unlabelled 1 mmol l^{-1} amino acid, analogue or peptide and 100 μmol l^{-1} [^{14}C]glutamate were added simultaneously to the energized-cells reaction mixture.

Specificity of glutamate uptake

The specificity of Glu uptake was first investigated by performing uptake experiments in the presence of unlabelled amino acids, peptides or structural analogues (Table 1). Glu uptake was not inhibited by histidine, glycine, arginine, leucine, Glu containing peptides or acetyl L-Glu. In contrast, analogues with modified α-amino or α-carbonyl functions were inhibitors, in particular L-Glu α-anilide and carbobenzoxy L-Glu. Aspartate, glutamine and valine were also powerful inhibitors (K_i of the same magnitude, 5·3 μmol l^{-1}). The other amino acids had little if any effect.

The specificity of the Glu uptake was further characterized with chase experiments. Unlabelled Glu was added to cells that were accumulating ^{14}C-Glu, the concentration of which being 10-fold lower than that of the unlabelled compound. The incorporation of radioactivity decreased rapidly. Similarly, the radioactivity retained by the cells was also displaced but to a lower extent by unlabelled glutamine (1 mmol l^{-1}).

Transport kinetics

The initial rate of Glu uptake was studied over a wide range of concentrations (50 μmol l^{-1}–2 mmol l^{-1}). A single phasic Michaelis-Menten-type saturation kinetics was observed, indicating the presence of a single uptake system. At pH 7·3, the affinity constant (K_t) value was 2·8 μmol l^{-1} and the V_{max} value 900 μmol s^{-1} (g dry weight)$^{-1}$, reflecting a high level of activity.

Expression of Glu uptake

The initial rate of Glu uptake occurred unchanged when cells were cultivated in a complex medium (MRS) or in the defined medium of Ledesma *et al*. (1977) in which Glu was present as a free amino acid or as a Glu-containing dipeptide. Thus, the Glu transport system might be constitutive.

Effect of temperature and pH

The initial rate of glutamate uptake was optimal at 50°C and was reduced to 70% at the optimal temperature of growth (42°C). It was sensitive to pH and buffer type with an optimum pH of 7·3 for all buffers. However, the uptake in potassium-phosphate or in Tris/HCl buffers was lower than in HEPPS/KOH (60% and 20%, respectively).

Effect of metal ions

Some monovalent and divalent cations stimulated Glu uptake, in particular Mg^{2+} and Mn^{2+} (Table 2). The stimulation was maximal when both cations were present, each 2·5 mmol l^{-1}. The V_{max} values in the presence of both Mg^{2+} and Mn^{2+}, or Mn^{2+} or Mg^{2+}, were 27-, 11- and seven-fold higher, respectively, than that in the absence of cations. The effect of Cu^{2+} (10 mmol l^{-1}) provoked a 50% reduction in activity after 5 min with a nonlinear kinetics. As expected for activators, all the cations that enhanced the rate of uptake had no effect on the affinity constant.

Table 2 Effect of cations on glutamate uptake in *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 208

Cations (mmol l^{-1}, as chlorides)	Initial rate of glutamate uptake (as percentage of control)
Control (no cations)	100
Mg^{2+} 1 + Mn^{2+} 4	1250
Mg^{2+} 2·5 + Mn^{2+} 2·5	1700
Mg^{2+} 4 + Mn^{2+} 1	560
Mg^{2+} 5	870
10	770
Mn^{2+} 5	580
10	350
Ca^{2+} 5	280
10	45
Zn^{2+} 10	27
Cs^{+} 10	415
Rb^{+} 10	405
Li^{+} 10	360
NH_4^{+} 10	45
Na^{+} 10	28

Effect of metabolic inhibitors

To determine the energy source used in the process, uptake was measured in the presence of various metabolic inhibitors (Table 3). Transport in the optimal conditions (in the presence of both Mg^{2+} and Mn^{2+}) was not affected by KF, whereas residual uptake (RU) was 39% in the presence of NEM and 15% with IAA. The strongest inhibitory compounds for uptake of Glu were the uncoupler CCCP (RU 27%) and the inhibitors of ATPase, chlorhexidine (RU 1%) and DCCD (RU 2%). In addition, the residual uptake in the presence of the inhibitor was often (21 out of 33 combinations) higher with the cation(s) than without, indicating a counter action of both the ions and the inhibitors.

DISCUSSION

The uptake of Glu in *Lact. bulgaricus* depended on the availability of metabolic energy as described for *L. lactis* (Konings *et al*. 1994) and *Lact. helveticus* (de Giori and de Valdez 1994; Nakajima *et al*. 1998) but not for *Enterococcus faecalis* (Reid *et al*. 1970).

Table 3 Influence of Mg^{2+} and Mn^{2+} cations on the effect of metabolic inhibitors on glutamate and glucose uptake in *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 208

	Initial rate of uptake (% of the control) of Glutamate			Glucose	
		In the presence of cations			
Inhibitor (mmol l^{-1})	In the absence of cations	(Mn^{2+} 5 mmol l^{-1})	(Mg^{2+} 5 mmol l^{-1})	(Mn^{2+} + Mg^{2+}:	each 2·5 mmol l^{-1})
Control (no inhibitor)	100	275 *(100)*	315 *(100)*	595 *(100)*	100
Na Azide (10)	32	195 *(71)*	315 *(100)*	162 *(27)*	50
Carbonyl cyanide-m-chloro-phenylhydrazone (0·05)	100	200 (*73)*	214 *(68)*	162 *(27)*	ND
2,4-Dinitrophenol (1)	86	14 *(5)*	188 *(60)*	126 *(21)*	63
Gramicidin (0·03)	25	23 *(8)*	72 *(23)*	50 *(9)*	ND
Nigericin (0·01)	6	10 *(4)*	7 *(2)*	15 *(2)*	ND
K Cyanide (10)	19	18 *(7)*	13 *(4)*	5 *(1)*	73
Iodo acetic acid (10)	16	10 *(4)*	3 *(1)*	86 *(15)*	90
K Fluoride (10)	100	193 *(70)*	157 *(50)*	595 *(100)*	ND
K Arsenate (10)	92	20 *(7)*	43 *(14)*	454 *(76)*	ND
N-Ethylmaleimide (1)	100	221 *(80)*	207 *(66)*	233 *(39)*	ND
Dicyclohexylcarbodiimide (0·1)	ND	ND	ND	15 *(2)*	50
Chlorhexidine digluconate (0·25)	ND	ND	ND	6 *(1)*	55

Values in italic given in brackets are relative values to the control for each column.
ND, not determined.

The best transport observed in HEPPS/KOH compared with that in Tris/HCl could result from a difference in the exchange of the buffer cation with the internal K^+ ions leading to a valinomycin-like effect. Transport activity declined rapidly when the pH was lower than the optimal value (7·3) but not as abruptly as reported for *Lact. casei* (Strobel *et al.* 1989). This influence of pH could not be explained by the proportion of uncharged and charged forms of Glu as described for *L. lactis* (Konings *et al.* 1994) and *Lact. casei* (Strobel *et al.* 1989), where only the noncharged acid form enters the cells. As for *Ent. faecalis*, the transport system of *Lact. bulgaricus* has no preference for either the acidic or the anionic form of Glu (Poolman *et al.* 1987). At the pH of milk (i.e. 6·7), uptake was 38% of that determined at the optimal pH (7·3) although the proportion of uncharged Glu remains more than 99%. Moreover, the optimal pH is higher than the pH range for growth (6·7–5·5). In contrast both pH were the same for *Lact. casei* (pH 6; Strobel *et al.* 1989).

The optimal temperature for uptake by *Lact. bulgaricus* differs from that for growth (42°C). Similar trends were observed with three strains of *Lact. lactis* (Hemme, D and Solliec, C., unpublished data), but not for *Lact. casei*, where both optima were the same (Strobel *et al.* 1989). Although uptake activity was reduced at 42°C, *Lact. bulgaricus* could initiate growth in the initial conditions of yoghurt manufacture (pH 6·7 and 42°C). Hopefully, this nonmaximal transport of Glu did not concern all amino acids during the growth of *Lact. bulgaricus,* e.g. the optimal pH for valine is 5·8 (de Giori, S and Hemme, D., unpublished data).

Unlike the specific system of *Lact. casei* (Strobel *et al.* 1989), the high-affinity Glu uptake system we have described was shared by aspartate and glutamine as observed in *Ent. faecalis* (Reid *et al.* 1970) and *L. lactis* (Konings *et al.* 1994). The inhibition of Glu uptake by Glu-Val, carbobenzoxy L-Glu and glutamine shows that the amino group of α carbon is essential for binding, the chain length and the conformational state (produced by charge distribution) playing probably some additional role in the specificity of transport.

The inhibitory effect of arsenate and IAA on Glu uptake in *Lact. bulgaricus* clearly indicated the requirement of ATP for transport. No other energy-rich phosphorylated intermediate (e.g. phosphoenol pyruvate) might be involved, as uptake was not affected by KF, an inhibitor of enolase. The total inhibition of Glu uptake and the only partial inhibition of glucose entry by DCCD suggested the involvement of the membrane-bound H^+-ATPase in coupling glycolysis to the transport activity as observed for *Lact. casei* (Strobel *et al.* 1989).

The combined stimulatory effect of Mg^{2+} and Mn^{2+} on uptake activity has never been described. The V_{max} we reported is higher in the presence of Mg^{2+} and Mn^{2+} than in the absence of these cations, without change in the K_t values. This may reflect a direct effect of these cations on the uptake system or an indirect effect through the stimulation

of the membrane-bound ATPase complex by Mg^{2+}. This cation is commonly added to the reaction mixture in uptake experiments with *L. lactis* and *Leuconostoc* sp. (Foucaud *et al*. 2001) and *Lact. casei* (Strobel *et al*. 1989), although the optimal concentrations were not determined.

Optimal concentrations of Mg^{2+} and Mn^{2+} required for maximum uptake were 2·5 mmol l^{-1} each. In milk, the Mg^{2+} concentration (5–8 mmol l^{-1}) is close to the optimum. In contrast, it is too low (5 μmol l^{-1}) for Mn^{2+} especially because 95% of Mn^{2+} is bound to caseins. In *Lactobacillus*, Mn^{2+} plays an important role (Weinberg 1997; Elli *et al*. 2000). A 200-μmol l^{-1} Mn^{2+} concentration is required to obtain the 30-mmol l^{-1} functional intracellular concentration by *Lact. plantarum* (Archibald and Minh-Ngoc 1984). Although the milk and cheese Ca^{2+} content is high (25 mmol l^{-1} and up to 250 mmol l^{-1}, respectively), the free form of Ca^{2+} is rare, so this ion may not influence the uptake activity of *Lact. bulgaricus* in dairy processes.

Altogether, our results have provided evidence that in *Lact. bulgaricus*, all forms of Glu and also glutamine and aspartate are transported by an ATP-dependent mechanism and that the activity of this system is enhanced by the combination of Mg^{2+} and Mn^{2+}.

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