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Glutamine Synthesis Is Essential for Growth of *Streptococcus thermophilus* in Milk and Is Linked to Urea Catabolism

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Growth of a glutamine synthetase-deficient mutant of *Streptococcus thermophilus* was compared to that of the parent strain in milk that was not supplemented or was supplemented with ammonium chloride, glutamine, or the urease inhibitor flurofamide. It was concluded that one of the functions of urease is to supply ammonia for the synthesis of glutamine.

In contrast to the other lactic acid bacteria present in starter cultures, *Streptococcus thermophilus* possesses a urease, which converts urea into ammonia and carbon dioxide (5, 10). Even if this enzyme is not required for the growth of *S. thermophilus* in milk (8), we observed recently that its inhibition by flurofamide decreased the growth of most of the strains tested (9). One hypothesis to explain this result is that ammonia and carbon dioxide produced from urea could be used in several biosynthesis routes, such as amino acid production. In the present study, we investigated the importance of glutamine synthesis for the growth of *S. thermophilus* in milk and its relation with the catabolism of urea.

Inactivation of the gene encoding a glutamine synthetase. The nucleotide sequence of the putative glutamine synthetase gene, named *glnA*, was obtained from the *S. thermophilus* LMD-9 genome sequence, produced by the U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). The *glnA* gene was PCR amplified from DNA of *S. thermophilus* CNRZ385 (Table 1) using the oligonucleotides olgln01 (5′ AGCAATACAATGTTCGG 3′) and olgln02 (5′ GTAAAGA TACCACCTCTGG 3′). The resulting 2.6-kb PCR fragment was then cloned into the TOPO XL vector (Invitrogen, Groningen, The Netherlands) and transferred into *Escherichia coli* TOP10. The resulting plasmid was named pGS21. A 1.1-kb deletion within the *glnA* gene was performed by digestion of pGS21 with BglII (Eurogentec, Seraing, Belgium), which has two cutting sites in *glnA*. After ligation (fast-link DNA ligation kit; Epicentre Technologies, Madison, Wis.), the resulting plasmid, named pGS39, was digested with HindIII and PstI to release a 1.6-kb fragment containing the deleted *glnA* gene. This fragment was inserted in the plasmid pG$^+$host9::ISS1 (6), previously digested with the same restriction enzymes (this also eliminated the ISS1 element from the vector). The resulting plasmid, named pGS45, was obtained in *E. coli* TIL206 and then introduced into *S. thermophilus* CNRZ385 using a previously described protocol (4). The procedure for gene replacement described by Biswas and coworkers (2) was then applied to the *glnA* gene, except that all culture media were supplemented with 0.25 g liter^{-1} glutamine. The resulting glutamine synthetase-negative mutant was named GS200. Presence of the deletion and absence of the vector in GS200 was checked by PCR.

Glutamine synthetase activity. After growth of the parent and mutant strains at 37°C, cells were harvested by centrifugation, washed with 0.9% NaCl, and resuspended in imidazole-HCl buffer (100 mM, pH 7.15). Glutamine synthetase activity was then measured using the "forward reaction assay" described by Bender and coworkers (1). One unit of glutamine synthetase activity was defined as the amount of enzyme producing 1 μmol of glutamyl hydroxamate per min at 37°C. The parent strain displayed a similar glutamine synthetase activity, whether the medium was supplemented with glutamine or not (Table 2). No activity could be detected when measurements were done in the presence of 15 mM of the glutamine synthetase inhibitor methionine sulfoximine. As expected, no glutamine synthetase activity could be detected in strain GS200.

Growth of the parent strain in milk. After growth in M17 broth at 37°C, cells were harvested by centrifugation, washed twice with 0.9% NaCl, and inoculated, at a concentration equivalent to 0.007 absorbance unit (575 nm), in reconstituted skim milk (100 g liter^{-1}; EPI ingrédients, Ancenis, France) that had been heated for 10 min at 80°C. Sterile 8-ml tubes were filled with 7.8 ml of inoculated milk and hermetically sealed. They were then incubated at 37°C and sampled regularly during 12 h. Growth in milk was measured after clarification of the medium (3). Some cultures were also done in the presence of 10 mM ammonium chloride, 0.25 g liter^{-1} L-glutamine, or 10 μM of flurofamide. Flurofamide is a potent inhibitor of urease, and growth of *S. thermophilus* strains in the presence of this compound is equivalent to that of mutant strains deficient in urease (7). Figure 1A shows that growth of strain CNRZ385 in milk is lower when urease is inhibited.

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source
Strain		
E. coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80 *lacZ* ΔM15 Δ*lacX74 recA1 deoR araD139*(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
TIL206	TG1 (*supE hsd* Δ5*thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15])*repA*$^+$ for construction with pG$^+$host9	UBSP[a]
TIL401	TIL206 with pG$^+$host9::ISS1	UBSP
GS21	TOP10 with pGS21	This study
GS39	TOP10 with pGS39	This study
GS45	TIL206 with pGS45	This study
S. thermophilus		
CNRZ385	Natural isolate	URLGA[b]
GS55	CNRZ385 with pGS45	This study
GS200	CNRZ385 with deleted *glnA* gene	This study
Plasmid		
Topo XL	PCR cloning plasmid	Invitrogen
pG$^+$host9::ISS1		UBSP
pGS21	Topo XL with PCR fragment from CNRZ385 containing *glnA* (2.6 kb)	This study
pGS39	pGS21 with a 1.1-kb deletion in *glnA*	This study
pGS45	pG$^+$host9::ISS1 with the deleted *glnA* gene at sites PstI/HindIII	This study

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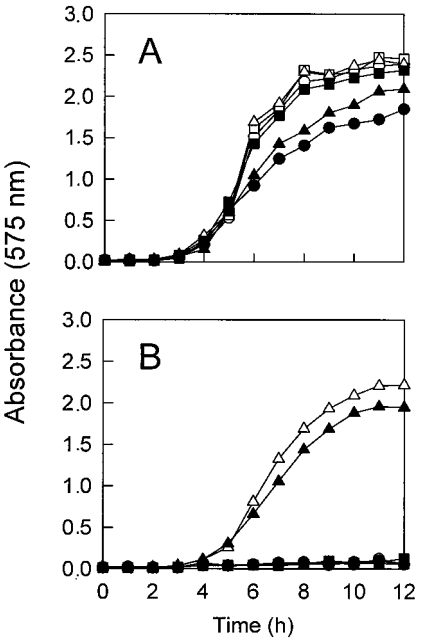

FIG. 1. Effect of ammonia and glutamine on the growth of *S. thermophilus* CNRZ385 (A) or the glutamine synthetase-deficient mutant GS200 (B) in milk and in milk supplemented with flurofamide (representative graphs selected from three repeated experiments). Cultures in milk (○), milk plus ammonium chloride (□), milk plus glutamine (△), milk plus flurofamide (●), milk plus flurofamide plus ammonium chloride (■), and milk plus flurofamide plus glutamine (▲).

Indeed, final biomass concentration is approximately 23% lower in the presence of flurofamide. The same result was obtained when the cultures were repeated (mean value of 2.43 ± 0.11 in the absence of flurofamide and 1.77 ± 0.12 in the presence of flurofamide). In the presence of flurofamide, i.e., when urease was inhibited, glutamine and ammonia stimulated the growth of the strain. Indeed, after 12 h of growth, the mean biomass concentration was 1.77 ± 0.12, 2.03 ± 0.14, and 2.30 ± 0.16 for the cultures in the presence of flurofamide, of flurofamide plus glutamine, and of flurofamide plus ammonium chloride, respectively. The stimulating effect of ammonium chloride and glutamine was not observed in the absence of the urease inhibitor. It may thus be proposed that the stimulating effect of the metabolism of urea could be due, at least partly, to the production of ammonia, which may be used in biosynthetic reactions, such as glutamine synthesis.

Concentrations of ammonia and urea were assayed using an enzymatic kit (r-biopharm, Saint Didier Au Mont d′Or, France). As expected, no consumption of urea occurred in the cultures supplemented with flurofamide (Table 3). The amount of nitrogen present in urea and ammonia ($N_{urea+ammonia}$) was calculated using the following relation: $N_{urea+ammonia} = 2 \times [urea] + 1 \times [ammonia]$. It is noteworthy that when CNRZ385 was cultivated in nonsupplemented milk, there was a significant decrease of $N_{urea+ammonia}$, compared to the amount present initially in milk (6.78 mM versus 8.41 mM). During its growth in milk, *S. thermophilus* was thus able to assimilate nitrogen present in milk ammonia and urea. No decrease of $N_{urea+ammonia}$ could be highlighted (Student test) in cultures supplemented with ammonium chloride, flurofamide, or glutamine. This does not necessarily mean that ammonia was not assimilated in these culture media. Indeed, this result may be explained by the fact that the relative amount of ammonia used by *S. thermophilus* was too low compared to the level of precision of the assays of ammonia and urea.

TABLE 2. Glutamine synthetase activity of *S. thermophilus* CNRZ385 and GS200

Strain	Growth medium	Glutamine synthetase activity (U mg protein^{-1})[a]	
		Measurement in the absence of methionine sulfoximine	Measurement in the presence of methionine sulfoximine
CNRZ385	M17	0.0190 ± 0.0018	<0.0005
	M17 + 0.25 g liter^{-1} glutamine	0.0240 ± 0.0026	<0.0005
GS200	M17	ND	ND
	M17 + 0.25 g liter^{-1} glutamine	<0.0005	<0.0005

[a] Means of three measurements ± standard deviations. ND, not determined.

TABLE 3. Ammonia and urea concentration in milk and in cultures of *S. thermophilus* CNRZ385 after 12 h of growth[a]

Samples	Presence of:			Concentration (mM)		
	Flurofamide	Ammonium chloride	Glutamine	Urea	Ammonia	$N_{urea+ammonia}$[b]
Noninoculated milk[c]	−	−	−	3.81	0.79	8.41
	−	+	−	3.75	10.29	17.79
Cultures of *S. thermophilus*	−	−	−	<0.1*	6.78*	6.78*
	−	+	−	<0.1*	17.01*	17.01
	−	−	+	<0.1*	7.90*	7.90
	+	−	−	3.69	0.59	7.97
	+	+	−	3.77	9.52	17.06
	+	−	+	3.67	1.01	8.35

[a] The values are means of three experiments.
[b] Amount of nitrogen present in urea and ammonia.
[c] Addition of flurofamide or glutamine did not change ammonia and urea concentration of milk. *, Values after 12 h of growth differed from those of the corresponding noninoculated medium ($P < 0.05$).

Growth of the glutamine synthetase-deficient mutant in milk. Strain GS200 had only a very limited growth in milk (Fig. 1B), indicating that glutamine synthesis is essential for the growth of *S. thermophilus*. The addition of glutamine, but not of ammonium chloride, stimulated the mutant. It is noteworthy that the growth of GS200 in the presence of glutamine was slightly lower when milk was also supplemented with flurofamide ($P < 0.05$).

In milk, glutamine is present in peptides and caseins and is also present as a free amino acid. In the present study, we showed that these sources of glutamine do not fulfill the requirements of *S. thermophilus* and that some glutamine has to be produced via the glutamine synthetase. Furthermore, as glutamine addition stimulated the wild-type strain only when the catabolism of urea was inhibited by the addition of flurofamide, it is likely that ammonia production from urea is essential for the glutamine synthetase to have a sufficient in vivo activity. These results support the idea that one of the physiological functions of urease in *S. thermophilus* is to supply ammonia for the synthesis of glutamine. To our knowledge, this is the first study showing the importance of ammonia assimilation for the growth of lactic acid bacteria in milk.

Nucleotide sequence accession number. The nucleotide sequence of the *S. thermophilus* CNRZ385 *glnA* gene has been deposited in GenBank under the accession number AY764257.

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