Relative Rates of Amino Acid Import via the ABC Transporter GlnPQ Determine the Growth Performance of *Lactococcus lactis*

Faizah Fulyani, Gea K. Schuurman-Wolters, Dirk-Jan Slotboom, Bert Poolman

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute & Zernike Institute for Advanced Materials, University of Groningen, Groningen, The Netherlands

ABSTRACT

The GlnPQ transporter from *Lactococcus lactis* has the remarkable feature of having two substrate-binding domains (SBDs) fused to the N terminus of the transmembrane domain (TMD), and thus four SBDs are present in the homodimeric complex. Although X-ray structures and ligand binding data are available for both SBDs, little is known of how different amino acids compete with each other for transport via GlnPQ. Here we show GlnPQ has a broader substrate specificity than previously thought, with the ability to take up asparagine, glutamine, and glutamic acid, albeit via different routes and with different affinities. Asparagine and glutamine compete with each other at the level of binding to SBD1 and SBD2 (with differences in dissociation constant), but at the same time SBD1 and SBD2 compete with each other at the level of interaction with the translocator domain (with differences in affinity constant and rate of transport). Although glutamine transport via SBD1 is outcompeted by physiological concentrations of asparagine, SBD2 ensures high rates of import of the essential amino acid glutamine. Taken together, this study demonstrates that even in the presence of competing asparagine concentrations, GlnPQ has a high capacity to transport glutamine, which matches the high needs of the cell for glutamine and glutamate.

IMPORTANCE

GlnPQ is an ATP-binding cassette (ABC) transporter for glutamine, glutamic acid, and asparagine. The system is essential in various Gram-positive bacteria, including *L. lactis* and several pathogens. Here we show how the amino acids compete with each other for binding to the multiple SBDs of GlnPQ and how these SBDs compete with each other for substrate delivery to the transporter. Overall, our results show that GlnPQ has evolved to transport diverse substrates via different paths and to optimally acquire the abundant and essential amino acid glutamine.

The ATP-binding cassette (ABC) superfamily is one of the largest and the best-studied protein families, which is present in most if not all members of the three kingdoms of life (1). ABC transporters translocate solutes across the cell or organellar membrane at the expense of ATP hydrolysis (1, 2). The basic unit of ABC transporters consists of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs), together referred to as the translocator (1). While the sequence and structure of the TMDs vary, the NBD of the proteins is highly conserved and is the hallmark of the ABC superfamily (3). The domains of the translocator can be present as separate subunits or fused to each other in many possible combinations. In addition, prokaryotic ABC transport systems involved in solute uptake employ accessory proteins that capture the ligands and deliver them to the translocator. These so-called substrate-binding proteins (SBPs) are present in the periplasm of Gram-negative bacteria, or they can be tethered to the membrane via a lipid or protein anchor (in Gram-positive bacteria and archaea). In a subset of ABC transporters the SBP is fused to the TMD, resulting in most cases in more than one SBD per functional complex (4).

GlnPQ is an ABC importer that belongs to the PAO (polar amino acids and opines) family (5). Numerous studies have reported the important roles of this transporter in pathogenic and nonpathogenic bacteria (6–8). In *Lactococcus lactis*, a nonpathogenic Gram-positive bacterium, GlnPQ provides the main route for fast import of glutamine, glutamate and asparagine (9). Moreover, it serves as the only transport system for glutamine and deletion of the *glnPQ* gene is lethal for the cell if glutamine or glutamate is not supplied in the form of peptides (10).

Two distinct substrate-binding domains (SBDs) are present in GlnPQ from *L. lactis*, namely, SBD1 and SBD2 (10, 11). These SBDs are arranged in tandem, resulting in four SBDs per translocation unit. In GlnPQ, SBD2 is proximal to the translocator, while SBD1 is distal. Structures of SBD1 and SBD2 were resolved in different states by X-ray crystallography: a closed conformation of SBD1 (1.7-Å resolution) with asparagine bound (unpublished results), an open conformation of SBD1 (1.4-Å resolution), a closed conformation of SBD2 (0.95-Å resolution) with glutamine bound, and an open unliganded form of SBD2 (1.5-Å resolution) (11). Although the two SBDs have a similar fold and similar binding pockets, the ligand affinities and specificities of SBD1 and SBD2 differ enormously. SBD1 has relatively high affinity for asparagine (K_D [equilibrium dissociation constant] = 0.2 μ M) and

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Address correspondence to Bert Poolman, b.poolman@rug.nl.

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low affinity for glutamine ($K_D = 92 \mu\text{M}$), while SBD2 binds glutamine with high affinity ($K_D = 0.9 \mu\text{M}$).

On the basis of uptake experiments, we now show that glutamate is transported by both SBD1 and SBD2, and, in line with previous studies (10, 12), the pH dependence suggests that glutamic acid is the translocated species. Differential scanning calorimetry (DSC) shows that 5 mM glutamate shifts the temperature of unfolding of SBD2 by 2.6°C ; such an effect was not observed for SBD1, yet glutamate uptake is detected.

The conformational dynamics of the SBDs in GlnPQ has been probed by single-molecule spectroscopy (9). It was found that *apo* SBDs could be in open and closed conformations. In the presence of ligand, the SBDs are in the closed state more frequently, but the lifetime of the closed liganded state is similar to that of the closed unliganded state. Thus, the binding mechanism is a special type of induced fit that includes rare open-to-closed transitions in the absence of ligand. The transport rate correlates reciprocally with the lifetime of the closed state, at least qualitatively (9).

Although functional and structural information is available for GlnPQ, little is known of how different amino acids compete with each other for transport in a system with multiple SBDs. We now have determined the growth of *L. lactis* as a function of glutamine, glutamate, and asparagine availability in the medium and have performed transport assays in parallel. We employed *L. lactis* strain GKW9000, which has the chromosomal *glnPQ* genes deleted (9). The strain was complemented in *trans* using plasmids containing *glnPQ* or mutant derivatives, in which either SBD1 or SBD2 was deleted (GlnPQ-SBD2 and GlnPQ-SBD1, respectively) (9). The cell growth and transport analyses reveal how amino acids compete with each other for uptake via GlnPQ and show the advantage of having two SBDs with different specificities.

MATERIALS AND METHODS

Strains and growth conditions. *Lactococcus lactis* NZ9000 (13) and GKW9000 (9) were used as hosts to express the genes for GlnPQ, GlnPQ-SBD2, or GlnPQ-SBD1. *L. lactis* NZ9000 is a derivative of *L. lactis* MG1363 containing the *nisRK* genes in the *pepN* locus of the genome (13). *L. lactis* GKW9000 was constructed by deleting the *glnPQ* genes by homologous recombination, using *L. lactis* NZ9000 as the parent (for details, see reference 9). In addition, mutants of full-length transporters in which one of the SBDs was inactivated were also tested, namely, GlnPQ(E184F) and GlnPQ(D417F); the numbers refer to the amino acid position predicted from the full-length gene (including the signal sequence). Details about gene constructs have been described by Gouridis et al. (9). *L. lactis* cells were cultivated semianaerobically at 30°C in a special chemically defined medium (CDM*) containing free amino acids, 1% (wt/vol) glucose, and 5 $\mu\text{g/ml}$ chloramphenicol. The CDM composition is described in Table 1. Semianaerobic refers to the conditions in closed tubes or sealed 96-well plates, but no precautions were taken to prevent oxygen from entering the medium during inoculation.

Growth assay and analysis. A single colony was picked and grown overnight in CDM* plus 2.7 mM asparagine and 3 mM dipeptide alanyl-glutamine (Sigma). The next morning, cells were inoculated to an optical density at 600 nm (OD_{600}) of ~ 0.05 in 5 ml fresh medium and allowed to grow to an OD_{600} of ~ 0.5 . The cells were harvested and washed twice with 30 ml of 50 mM cold and sterile K_2HPO_4 - KH_2PO_4 (pH 6.5) and resuspended in this buffer to an OD_{600} of ~ 0.5 . Thirty microliters of preculture was used to inoculate a total volume of 300 μl of CDM* supplemented with 0.01% *L. lactis* NZ9700 culture medium (containing the inducer nisin A [14]), 5 $\mu\text{g/ml}$ chloramphenicol, and 1% (wt/vol) glucose. Various combinations of asparagine, glutamine, glutamate, and dipeptide were added to the CDM* as indicated in the figure legend for each exper-

TABLE 1 Composition of chemically defined medium^a

Component	Concn, g/liter
Amino acid mix, 20 $\times$ solution (adjusted to pH 7.0, filter sterilized)	
L-Alanine	4.75
L-Glutamine	7.8
L-Asparagine	7
L-Arginine	2.5
L-Lysine	8.75
L-Isoleucine	4.25
L-Methionine	2.5
L-Phenylalanine	5.5
L-Serine	6.75
L-Threonine	4.5
L-Tryptophan	1
L-Valine	6.5
Glycine	3.5
L-Histidine	3
L-Leucine	9.5
L-Proline	13.5
Metal mix, 400 $\times$ solutions, filter sterilized (in H_2O)	
A	
MgCl ₂	160
CaCl ₂	20
ZnSO ₄ · 7H ₂ O	2
B	
CuSO ₄ · 5H ₂ O	1.2
CoCl ₂	0.08
C	
FeCl ₂ · 4H ₂ O	2
D	
MnSO ₄	14
Basic medium, 2.7 $\times$ solution (set to pH 6.4 with HCl, autoclaved)	
Distilled H ₂ O	750 ml
Tyrosine	0.58
KH ₂ PO ₄	27.2
K ₂ HPO ₄	50.2
(NH ₄) ₃ -citrate	1.2
Na-acetate · 3H ₂ O	2
Vitamin mix, 100 $\times$ solution (adjusted to pH 7.0, filter sterilized)	
Nicotinic acid (niacin amide)	0.2
Thiamine hydrochloride	0.1
Riboflavin	0.1
Ca-pantothenate	0.1
4-Aminobenzoic acid	1
Biotin	1
Folic acid	0.1
Vitamin B ₁₂ (cyanocobalamin)	0.1
Orotic acid	0.5
2-Deoxythymidine (thymidine)	0.5
Inosine	0.5
DL-6,8-Thioctic acid	0.25
Pyridoxamine dihydrochloride	0.5
Pyridoxal-HCl	0.2
Base mix, 100 $\times$ solution (in 0.2 M NaOH, filter sterilized)	
Adenine	1
Uracil	1
Xanthine	1
Guanine	1

^a All the stock solutions were stored at -20°C , except basic medium, which was kept in the dark at room temperature. One liter of CDM (2 $\times$ solution) contains the following: basic medium, 750 ml; vitamin mix, 20 ml; amino acid mix, 100 ml; metal mix A, 5 ml; metal mix B, 5 ml; metal mix C, 5 ml; metal mix D, 5 ml; and base mix, 20 ml. CDM* is CDM without glutamine and asparagine.

iment. Growth was monitored at 600 nm in transparent 96-well microtiter plates (Greiner BioOne), using an automated plate reader (Biotek). Growth was followed for at least 24 h with either 15-min or 30-min intervals between OD measurements. The growth rate (μ) was determined by fitting the curves to a modified logistic model as described in reference 15. Model fitting was performed using Mathematica software. The growth experiments were done in at least two replicates of duplicates (full data sets used for this paper), but in total the number of replicates is much higher.

DSC. The SBDs were isolated and purified as described previously (11). The purified SBDs without a His tag were dialyzed overnight against 50 mM potassium phosphate, 1 mM EDTA, and 1 mM NaN_3 at pH 5.0, 6.0, or 7.0. Differential scanning calorimetry (DSC) experiments were performed using the VP-DSC, MicroCal with protein concentrations of 4 μM for SBD12 and 6 μM for SBD1 or SBD2; dialysis buffer was used as the reference. The apparent melting temperature (T_m) of SBDs in the absence or in the presence of 5 mM substrate (glutamine or glutamate) was determined against the reference, using a heating rate of 60°C/hour. The measured unfolding curve was irreversible, implying that the protein precipitated during/after unfolding. As a result, the T_m can be compared only under the same experimental conditions, and quantitative thermodynamic information cannot be extracted.

Amino acid transport assay. *L. lactis* strains GKW9000 harboring the expression constructs or the empty vector pNZ8048 (13) were grown in M17 medium supplemented with 1.0% (wt/vol) glucose (here referred to as GM17 medium) to an OD_{600} of ~ 0.4 and induced for 1 h with 0.01% culture supernatant of the nisin A-producing strain NZ9700 (containing ~ 10 $\mu\text{g/liter}$ of nisin A) (14). Cells were harvested, washed, resuspended to an OD_{600} of ~ 50 in ice-cold 10 mM piperazine- N,N' -bis(2-ethanesulfonic acid) (PIPES)-KOH plus 80 mM KCl (pH 6.0), and kept on ice until use. Uptake experiments were performed at final protein concentrations of 2.5 to 250 $\mu\text{g/ml}$ in 30 mM PIPES-KOH, 30 mM MES-KOH, and 30 mM HEPES-KOH, pH 6.0. Next, the cells were equilibrated at 30°C, and 10 mM glucose and 5 mM MgCl_2 were added. After 3 min, the uptake reaction was started by addition of either [^{14}C]glutamine or [^3H]asparagine; the specific radioactivity was adjusted in the different experiments to have the disintegrations per minute (dpm) at least 10-fold above the background, and the final amino acid concentrations are indicated in the figures or figure legends. For studies of (competitive) inhibition by amino acids, unlabeled glutamine or asparagine was mixed with the labeled substrate. At given time intervals, samples were taken and diluted in 2 ml ice-cold 100 mM LiCl to stop the uptake reaction. The samples were rapidly filtered through a 0.45- μm -pore-size nitrocellulose filter (BA85; Whatman/GE Healthcare, Buckinghamshire, United Kingdom). The filter was washed once with 2 ml 100 mM LiCl and submerged in Emulsifier Scintillator Plus scintillation fluid (Packard Bioscience). The radioactivity on the filters was determined by liquid scintillation counting. Initial rates of transport were estimated from the linear part of uptake over time.

RESULTS AND DISCUSSION

Amino acid dependence of growth. Based on genome sequencing, *L. lactis* IL1403 and MG1363 have all the genes necessary to synthesize the 20 standard amino acid (16, 17); however, some of the genes are not functional due to, mutations or small deletions, as reported for *leuA* and *leuB*, which are involved in branched-chain amino acid (BCAA) biosynthesis (18). Furthermore, regulation of amino acid metabolism involves control of the enzymes' activity and their synthesis in response to the presence of certain metabolites (19). In *L. lactis*, regulation of amino acid biosynthesis is globally controlled by CodY (19). Excess isoleucine has been shown to block several CodY-dependent amino acid biosynthesis pathways, thus inhibiting growth (19–21). Moreover, an excess of one branched-chain amino acid inhibits growth by competing out others that are taken up via the same transport system (22). The

FIG 1 Growth dependence of *L. lactis* on asparagine, glutamine, and glutamate. Growth of *L. lactis* NZ9000, GKW9000(pNZ8048) (empty plasmid), and GKW9000 expressing wild-type *glnPQ* in *trans* is shown. Cells were grown in 96-well plates in CDM* supplemented with either 3 mM glutamine, 3 mM glutamate, 2.7 mM asparagine, or a combination of these amino acids as indicated.

amino acid requirement for optimal *L. lactis* growth varies from one strain to another. Generally, synthetic medium for *L. lactis* should contain at least six amino acids, i.e., isoleucine, leucine, valine, histidine, methionine, and glutamate (or glutamine) (23–25). Several other amino acids, such as asparagine, alanine, arginine, and threonine, are important as growth-stimulating factors (22, 24, 25). The composition and concentration of amino acids in the growth medium give rise to variation in the growth rate of *L. lactis*.

Glutamine (or glutamate) is known to be essential for *L. lactis*, but the organism is able to synthesize asparagine itself (17, 18). *L. lactis* converts intracellular glutamine into glutamate and vice

FIG 2 Apparent melting temperatures ($T_{m,s}$) of SBD1 (A), SBD2 (B), and SBD12 (C) determined by DSC. Measurements were done in 50 mM KP_i (pH 6.0) plus 1 mM EDTA and 1 mM NaN_3 . The DSC profiles of SBD1, SBD2, and SBD12 are depicted without substrate (solid lines) and in the presence of 5 mM sodium glutamate (dotted lines) or 5 mM glutamine (dashed lines). See Table 2 for T_m measurements at different pHs. Cp, heat capacity.

versa, using glutaminase and glutamine synthetase, respectively (26, 27). Figure 1 shows that glutamine (or glutamate) and asparagine are required for growth of *L. lactis* NZ9000 (Fig. 1M and P, respectively; Fig. 1D, G, and J show where the medium lacks either glutamine, glutamate, or asparagine). Apparently, under our experimental conditions, the synthesis of asparagine is insufficient to support growth (Fig. 1G and J), even though the amino acid is not considered to be essential for *L. lactis*.

To bypass the import of glutamine or glutamate via GlnPQ, we added a glutamine- or glutamate-containing peptide to the medium, which is taken up via the di/tripeptide transport systems DtpT and Dpp (28). Deletion of the *glnPQ* gene results in the inability of *L. lactis* (GKW9000) to grow on medium containing glutamine and asparagine (Fig. 1N), but the strain can grow in the presence of alanyl-glutamine (Fig. 1T) or if the strain is complemented in *trans* with the *glnPQ* genes (Fig. 1O). We also found that GlnPQ is not the only transporter for glutamate or asparagine, as slow but significant growth of *L. lactis* GKW9000 was

detected when these amino acids were added to the medium (Fig. 1Q and T, respectively). Glutamate may be taken up via the secondary transporter AcaP (29) or GltPQ (16), a protein that has been annotated as a putative glutamate ABC transporter. Taken together, the growth data indicate that GlnPQ is the only transport system for glutamine but not for glutamate and asparagine.

Glutamate binding probed by DSC. We have previously shown by isothermal titration calorimetry (ITC) that SBD1 binds glutamine and asparagine, whereas SBD2 binds glutamine (11). However, we never detected binding of glutamate by either SBD1 or SBD2, which is surprising because GlnPQ transports glutamate in the form of glutamic acid (10). We now used differential scanning calorimetry (DSC) to determine the unfolding temperatures (T_m s) of SBD1 and SBD2 in the absence and presence of glutamine and glutamate to assess qualitatively whether or not the SBDs bind glutamate. We found that glutamine shifts the T_m s of SBD1 and SBD2 by 3.4°C and 14.3°C, respectively (Fig. 2A and B, dashed lines). Glutamate, in contrast, causes a shift in T_m of SBD2 by 2.6°C but gives no shift with SBD1 (Fig. 2B and A, respectively, dotted lines); the shift in the T_m of SBD2 is larger with decreasing pH (Table 2), which is consistent with a mechanism whereby only glutamic acid causes the closing of the substrate-binding protein. Since the pK_a of the glutamate side chain is relatively low (~4.2 in aqueous media), millimolar concentrations of glutamate are required to saturate SBD2. The binding of glutamate to the tandem SBD gives slightly larger shifts in T_m than when the separate SBD1 and SBD2 are analyzed (Fig. 2C and Table 2). Since the unfolding was irreversible, absolute values cannot be given. The differences in T_m are too small to conclude that mutual stabilization of the SBDs in the tandem SBD occurs.

Glutamate transport by GlnPQ. In order to determine the contributions of SBD1 and SBD2 to the transport of glutamate via GlnPQ, we performed *in vivo* uptake assays, using *L. lactis* GKW9000 complemented with either GlnPQ-SBD1 or GlnPQ-SBD2. We found that glutamate was taken up at high rate via SBD2 (Fig. 3A). The transport via SBD1 was low but significantly above background and saturated with an apparent K_m of about 500 μ M (Fig. 3B). If we take a pK_a of 4.2 for glutamate $\rightleftharpoons$ glutamic acid and a medium pH of 6, then the data correspond to a K_m of ~7 μ M for glutamic acid. Taken together, the DSC and transport data show that glutamate (glutamic acid) can be taken up via either SBD1 or SBD2 but that SBD2 is by far the preferred path.

Glutamate dependence of growth and roles of SBD1 and SBD2. To determine the roles of the individual SBDs in the growth of *L. lactis*, we expressed GlnPQ-SBD1 or GlnPQ-SBD2 in *L. lactis* GKW9000. The cells were grown in CDM* supplemented with glutamate. As stated above, GlnPQ is not the only transporter for

TABLE 2 Apparent melting temperatures of SBDs determined by DSC

Protein	T_m (°C) ^a at pH:								
	5.0			6.0			7.0		
	Control	+Gln	+Glu	Control	+Gln	+Glu	Control	+Gln	+Glu
SBD1	63.4	66.8	63.7	60.8	64.2	60.8	57.2	58.5	57.4
SBD2	63.5	78	70	61.9	76.2	64.5	57.2	71	58.2
SBD12	62.4	66, >76	63.2, >67	59.8	63.1, >76	61.2, 67.9	55.7	57.5, 67.3	56.1

^a The T_m s of SBD1, SBD2, and SBD12 of GlnPQ were measured at different pHs in either the absence or presence of 5 mM glutamine (Gln) or glutamate (Glu) as indicated. Where two values are given, the first and second correspond to the T_m s of SBD1 and SBD2 in the tandem SBD12, respectively.

FIG 3 (A) Glutamate transport in *L. lactis* GKW9000 carrying pNZ8048 (□) or expressing GlnPQ (Δ), GlnPQ-SBD1 (○), or GlnPQ-SBD2 (▽). The uptake of 250 μ M [14 C]glutamate is shown as a function of time. (B) The glutamate uptake rates of cells expressing GlnPQ-SBD1 (○) or carrying the empty plasmid pNZ8048 (□) were determined as a function of [14 C]glutamate concentration.

glutamate but is needed for maximal cell growth (Fig. 4A). With glutamate instead of glutamine as a source of glutamine and glutamate, *L. lactis* expressing wild-type GlnPQ (Fig. 4B) or GlnPQ-SBD2 (Fig. 4D) grew well with concentrations as low as 1 mM (red lines), but GlnPQ-SBD1 required a higher concentration of glutamate (Fig. 4C). The difference in glutamate requirement can be understood when the amino acid affinities and specificities of the SBDs are taken into account. Asparagine binds with high affinity to SBD1 (11), but binding to SBD2 could not be detected. Given that asparagine can be transported via SBD2, it presumably binds the amino acid with very low affinity and thus has limited effect on the growth in the presence of glutamate. In cells expressing GlnPQ-SBD1, the ~ 3 mM asparagine present in the CDM* outcompetes the glutamate when present at 1 mM concentration (red trace in Fig. 4C), which is not the case in wild-type GlnPQ or GlnPQ-SBD2 (red traces in Fig. 4B and D, respectively). When the glutamate concentration was raised to an equimolar concentration of asparagine, the cell growth was restored (green trace in Fig. 4C). In wild-type GlnPQ (Fig. 4B), glutamate can be taken up via SBD1 and SBD2, and the competition by asparagine is less severe.

In our experimental setup, the high concentration of potas-

sium phosphate (~ 100 mM) in CDM supports a higher cell yield than in standard CDM, which contains ~ 20 mM. We determined the growth of cells expressing GlnPQ, GlnPQ-SBD1, GlnPQ-SBD2, and empty plasmid in standard CDM (Fig. 4F, G, H, and E, respectively) and found that the outcome was similar to that with high-phosphate CDM. In addition, we tested full-length GlnPQ with either SBD1 or SBD2 inactivated via E184F and D417F mutations, respectively (see Fig. S1 in the supplemental material); we noted that SBD2(D417F) no longer bound glutamine, whereas SBD1(E184F) had some residual glutamine and asparagine uptake activity. The results were (qualitatively) similar to those for the corresponding SBD deletion mutants. In summary, GlnPQ supports growth of *L. lactis* through transport of glutamate (glutamic acid) via SBD1 and SBD2, but the transport via SBD1 is more severely inhibited by asparagine.

Glutamine dependence of growth and role of SBD1 and SBD2. GlnPQ is the sole importer for glutamine in *L. lactis*, whereas multiple transporters for glutamate are present. As shown in Fig. 5A, cells expressing empty plasmid pNZ8048 show no growth when cells are dependent on glutamine as the source of glutamate and glutamine. The glutamine dependence of growth of *L. lactis* expressing GlnPQ-SBD2 (Fig. 5E) or the E184F mutant (Fig. 5F) was comparable to that of wild-type GlnPQ (Fig. 5B), whereas cells expressing GlnPQ-SBD1 required an order of magnitude more glutamine than the wild-type GlnPQ (Fig. 5C). In line with what was observed for glutamate, the difference in glutamine requirement for cells expressing GlnPQ-SBD1 is due to competition with asparagine, which binds to SBD1 with high affinity (11). In wild-type GlnPQ, glutamine can be taken up via SBD1 and SBD2 of GlnPQ, and thus the competition by asparagine is less severe (Fig. 5B). Cells expressing GlnPQ(D417F) (Fig. 5D) grew less well than those expressing GlnPQ-SBD1 (Fig. 5C), which can be explained by the lower level of expression of transporters with two rather than one SBD. The lower level of expression also applies to GlnPQ(E184F); however, this mutant still shows residual transport via SBD1.

Growth inhibition by asparagine. Next, we determined the growth of *L. lactis* at various concentrations and ratios of glu-

FIG 4 Growth of *L. lactis* as a function of glutamate concentration in CDM* containing a high (upper graphs) or low (lower graphs) concentration of potassium phosphate. *L. lactis* GKW9000 carrying pNZ8048 (A and E) or expressing GlnPQ (B and F), GlnPQ-SBD1 (C and G), or GlnPQ-SBD2 (D and H) was grown in CDM* containing 2.7 mM asparagine and glutamate at a concentration of 0, 1 mM, 3 mM, 10 mM, or 30 mM.

FIG 5 Growth of *L. lactis* as a function of glutamine concentration in CDM* containing high concentrations of potassium phosphate buffer. *L. lactis* GKW9000 carrying pNZ8048 (A) or expressing GlnPQ (B), GlnPQ-SBD1 (C), GlnPQ (D417F) (D), GlnPQ-SBD2 (E), or GlnPQ (E184F) (F) was grown in CDM* containing 2.7 mM asparagine and glutamine at concentration of 0 (black), 3 mM (red), 10 mM (blue), or 30 mM (gray).

tamine and asparagine (Fig. 6). *L. lactis* GKW9000 expressing GlnPQ-SBD1 grew poorly in medium containing 1 mM glutamine plus 0.1 mM asparagine (Fig. 6B). The growth rate improved when asparagine was raised to 0.3 mM, but the lag became rather long. When the glutamine concentration was increased to 3 mM, the lag phase was shortened again. Apparently, the concentration of asparagine needed for growth has a defined optimum: some asparagine is needed, but when the concentration becomes too high, it outcompetes glutamine uptake via SBD1. The latter is evident from the inhibition of growth when the asparagine concentration is raised from 0.3 mM to 2.5 mM at a glutamine concentration of 3 mM.

Presumably a metabolic pathway is induced by asparagine. In fact, asparagine is important for CodY-dependent amino acid biosynthesis, especially in media with excess isoleucine (19). Thus, growth of *L. lactis* is slow when the asparagine concentration is too low to induce most likely CodY-dependent amino acid biosynthesis; it also becomes slow when the asparagine concentration is high and glutamine uptake is competed out. With a second glutamine (and glutamic acid)-specific SBD (SBD2) in wild-type GlnPQ, growth is less severely affected by asparagine (Fig. 6A), although asparagine-bound SBD1 will compete for translocation with glutamine-bound SBD2 (9).

Growth of cells expressing GlnPQ-SBD2 is simpler to explain than that of cells with GlnPQ-SBD1. For cell growth, minimums of 2.5 mM asparagine and 1 mM glutamine are needed (Fig. 6C). Growth of cells expressing GlnPQ-SBD2 is not affected by 2.5 mM asparagine, confirming the high selectivity of SBD2 for glutamine. Additionally, when growth was made dependent on glutamate instead of glutamine, the effects of asparagine competition were less pronounced (see Fig. S2 in the supplemental material), presumably because *L. lactis* then uses one or more alternative transporters for glutamate.

FIG 6 Competition between asparagine and glutamine for transport via GlnPQ affects the growth of *L. lactis* GKW9000. Cells expressed GlnPQ (A), GlnPQ-SBD1 (B), or GlnPQ-SBD2 (C). Cells were grown in CDM* plus asparagine and glutamine in various concentration combinations as indicated.

FIG 7 Substrate transport in *L. lactis* GKW9000 expressing GlnPQ (panels i), GlnPQ(D417F) (panels ii), or GlnPQ-SBD1 (panels iii). (A) Uptake rates at 25 and 250 μM glutamine, reported by [¹⁴C]glutamine, in the presence of a 10- or 100-fold excess of glutamine or asparagine. (B) Uptake rates at 25 and 250 μM asparagine, reported by [³H]asparagine, in the presence of a 10- or 100-fold excess of asparagine or glutamine. Cartoon illustrations of GlnPQ and derivatives are presented above each of the transport data. The blue and the red circles correspond to glutamine and asparagine, respectively.

In vivo uptake of glutamine. To substantiate our findings inferred from the growth studies, we performed *in vivo* transport experiments and monitored the competition between asparagine and glutamine. Figure 7A shows the rate of uptake of [¹⁴C]glutamine in cells expressing GlnPQ (panel i), GlnPQ(D417F) (panel ii), or GlnPQ-SBD1 (panel iii). Radiolabeled glutamine at 25 and 250 μM was used to report the effect of a 10- or 100-fold excess of asparagine on the transport of glutamine. At 25 μM glutamine, SBD1 ($K_D = 92 \mu\text{M}$) is not saturated with substrate, whereas SBD2 ($K_D = 0.9 \mu\text{M}$) is saturated; to approach substrate saturation of SBD1, we used a glutamine concentration of 250 μM. For comparison, we also determined the effects of 10- and 100-fold excesses of glutamine on the uptake of [¹⁴C]glutamine. Figure 7A,

panel i, shows that the excess of glutamine has a larger impact on the uptake rate of [¹⁴C]glutamine than on that of asparagine. However, the effect is reversed if SBD2 is inactivated by mutation (panel ii) or by deletion (panel iii). These data, and taking into account the absolute rates of transport, show that in wild-type GlnPQ the majority of the import of glutamine is via SBD2. The difference in glutamine transport activity of GlnPQ-SBD1 and GlnPQ(D417F) is explained by the higher level of expression of the protein with a single SBD, as reported previously (9).

In vivo uptake of asparagine. We also monitored the uptake of [³H]asparagine in the same cells and under identical conditions as for the transport of [¹⁴C]glutamine (Fig. 7B). What is striking is that the rate of uptake of [³H]asparagine is increased 3-fold when

FIG 8 Asparagine transport in *L. lactis* GKW9000 expressing GlnPQ-SBD2 (○) or carrying the empty plasmid pNZ8048 (□). The uptake rate was determined as a function of asparagine concentration.

SBD2 is inactivated by the D417F mutation (panels i and ii); the expression levels of GlnPQ and GlnPQ(D417F) are comparable. We have previously shown that the D417F mutation prevents closing of the SBD2 (in the absence and presence of glutamine) and that the closed unliganded state of SBD2 inhibits (asparagine) import via SBD1 (9). The failure of SBD2(D417F) to close prevents interaction of the SBD with the membrane domain of the transporter and thus precludes inhibition of transport via SBD1, unlike the situation when wild-type SBD2 is present.

The stronger apparent inhibition of [¹⁴C]glutamine uptake by asparagine than the other way around is explained by the difference in affinity of SBD1 for these amino acids. The competition of asparagine and glutamine is at the level of binding to SBD1 (Fig. 7B, panel i versus ii) but also at the level of competition between SBD1 and SBD2. The latter is indicated by the stronger (relative) inhibition of asparagine uptake via glutamine in GlnPQ(D417F) than in GlnPQ-SBD1 (panel ii versus iii). Finally, although transport of [³H]asparagine by *L. lactis* GKW9000 expressing GlnPQ-SBD2 is negligible at micromolar concentrations, at submillimolar concentrations the signal is well above background. From this we infer an apparent K_m for asparagine transport via SBD2 of 0.5 to 1 mM (Fig. 8).

In summary, we demonstrate the versatility of GlnPQ in the uptake of essential (glutamate or glutamine) and growth-stimulating (asparagine) amino acids. GlnPQ has a higher capacity to transport glutamine than asparagine for several reasons: (i) glutamine is transported via SBD1 and SBD2 much faster than asparagine is, (ii) glutamine is transported with high and low affinity, and (iii) transport via SBD2 is more efficient than that via SBD1. The higher capacity to transport glutamine is consistent with the higher needs of protein synthesis for glutamine and glutamate than for asparagine (22) and the role of these amino acids in cell volume regulation (30). Glutamate is the most abundant amino acid in bacteria, including *L. lactis* (12), and glutamate is readily converted from glutamine when this amino acid is imported. We also show that for optimal growth, the compositions and concentrations of media need to be designed carefully.

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